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Protein separation by monolithic capillary electrochromatography

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Abstract

This work presents the separation of model proteins by capillary electrochromatography involving a monolithic stationary phase with C_4 functionality. The monolith was fabricated in UV-transparent capillaries by employing a slight modification of a recently published photopolymerization procedure. With the number of theoretical plates per column ranging between 11 000 and 33 000, the separation efficiency proved to be lower than capillary zone electrophoresis where plate numbers ranged between 18 000 and 66 000. However, higher resolution was obtained due to the additional chromatographic separation mechanism. Inter- and intra-column reproducibility were evaluated, the latter could be significantly improved when using a rinsing procedure that contained 0.05% sodium dodecylsulfate in the mobile phase. Plate heights became nearly independent of mobile phase velocities higher than 0.5 mm/s indicating that high velocities can be applied without sacrificing efficiency. Furthermore, peak heights showed a dependence on injection times. For proteins, an increase in capacity factors was found when increasing the percentage of organic solvent in the mobile phase.

Keywords: Electrochromatography; Monolithic columns; Capillary columns; Proteins

1. Introduction

Since its implementation in the capillary format [1–3] capillary electrochromatography (CEC) has attracted much attention in the analytical community [4–6]. One reason is the underlying dual separation mechanism that is based on both a thermodynamic (i.e. partitioning) and a kinetic process (i.e. electrokinetic migration) [7–10]. This powerful combination of two distinctly different separation mechanisms is furthermore accompanied by a flat, plug-like flow profile. It is electroosmotically generated and lessens band broadening thereby allowing for higher separation efficiencies when compared to HPLC [1,3,11–14]. Initially, the widespread use of individual, mostly silica-based particles as stationary phase packing materials rendered column manufacture a tedious task. Reasons were the difficulties associated with the need for retaining frits and the frequently observed problem of bubble formation at those frits [15,16]. The recent advent of monolithic, mostly polymeric, columns instead of individual particles has reduced many of these earlier problems of CEC [17–24]. Capillaries are now simply filled with a monomer mixture together with a porogenic

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solvent. Consecutive photo or thermal initiation then yields the porous polymer without the need for a retaining frit. The right choice of monomer and porogenic solvent enables the generation of tailored stationary phases. The possibility of implementing this methodology in microfluidic devices has also been demonstrated [25–29].

Due to very high mass transfer rates, monolithic stationary phases have been shown to possess plate heights that are nearly mobile phase velocity-independent. High flow rates can therefore be applied without a loss in efficiency. This particular advantage over individual particles can be considered as being the major reason for their current popularity in both HPLC and CEC [19,20,23,25,30–35]. In some cases they also allow for improved detection limits due to a dependence of peak height on injection time [36].

A recent article by Ngola et al. describes the use of conduct-as-cast polymer monoliths where, after the polymerization has been completed, no pressure or hydrodynamic flow has to be applied to flush the capillary. All steps can be conveniently carried out using electrokinetic flow [29]. One of the monomers described in their study, based on butyl acrylate, might be well suited for the separation of proteins due to its C_4 group, a functionality widely exploited in protein separations using HPLC [37]. Several authors have investigated the potential of CEC for the separation of proteins. In most cases, reversed-phase or ion-exchange modes were responsible for the overall separation process [9,20,38–42].

This article will describe the separation of model proteins by CEC using a monolith based on butyl acrylate by employing a modified version of the polymerization procedure published by Ngola et al. [29]. The dependence of plate height on mobile phase velocity is examined as well as the dependence of peak height on injection time. Moreover, in order to gain acceptance as a new analytical technique, it is required to establish reproducibility. Hence, inter- and intra-column reproducibilities will be discussed. The intra-column reproducibility can be greatly enhanced when applying an appropriate cleaning procedure in-between runs. Lastly, the dependence of the electroosmotic flow (EOF) and the capacity factors on the percentage organic solvent in the mobile phase will be investigated.

2. Experimental

2.1. Instrumentation

Experiments were conducted using a laboratory-built CEC instrument. All capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). Platinum wire (0.5 mm diameter) for the electrodes was purchased from Goodfellow (Cambridge, UK). Positive potentials were applied to the inlet vial using a Spellman Model CZE 1000R high-voltage power supply (Happauge, NY, USA). The capillary current was measured via a current to voltage converting resistor at 20 Hz using a PCI-1200 (12 bit resolution) data acquisition board (National Instruments, Austin, TX, USA). Injection and separation voltages were controlled through an analog output port of the board using a program written in LabView 5.1 graphical programming language (National Instruments). Detection was provided using either a Unicam 4225 UV detector (Mississauga, Canada) that was operated at 214 nm with a sensitivity of 0.01 absorbance units full scale and a rise time of 0.1 s or an ISCO CV^4 variable-wavelength capillary electrophoresis detector (for Fig. 3b CZE only) operated with the same sensitivity but a rise time of 0.4 s. Absorbance signals were collected at 20 Hz and digitized using the previously described board. Data were presented in graphical form by reading the files into IGOR Pro Version 3.15 (WaveMetrics, Lake Oswego, OR, USA) and analyzed using GRAMS/32 Version 4.01 (Thermo Galactic, Salem, NH, USA). Photopolymerization was carried out using a General Electric H85A3 high-pressure mercury vapor lamp (General Electric, Toronto, Canada). Spectral output was 4.1 mW/cm^2 with the lamp placed 50 cm above the capillary. The spectral output was measured using an Indicator Model 154BT power meter (Laser Instrumentation, Chertsey, UK).

2.2. Materials and reagents

(3-Methacryloyloxypropyl)trimethoxysilane (MTS), butyl acrylate (BAC), 1,3-butanediol diacrylate (BDDA), 2-acrylamido-2-methyl-1-propanesulfonic acid 99% (AMPS), Amberlite IRA-900 ion-exchange resin, myoglobin (horse heart), transferrin (human),

α-lactalbumin (bovine milk), naphthalene, acenaphthene, anthracene, pyrene, fluoranthene, chrysene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, Sigma Ultra grade sodium tetraborate decahydrate, monobasic sodium phosphate, lauryl sulfate (SDS), A.C.S.-grade absolute ethanol and HPLC-grade *N,N*-dimethylformamide were all acquired from Sigma–Aldrich (Oakville, Canada). Benzoin methyl ether 98% (BME) was purchased from Fluka (Sigma–Aldrich, Oakville, Canada), HPLC-grade acetonitrile and HPLC-grade methanol from Fisher (Neapan, Canada). A.C.S.-grade sodium hydroxide was purchased from ICN Biomedicals (Aurora, OH, USA), A.C.S.-grade glacial acetic acid from J.T. Baker (Phillipsburg, NJ, USA). All buffers and aqueous sample solutions were prepared using HPLC-grade water (NANOpure, Barnstead, Dubuque, IA, USA).

Phosphate buffer 5 mM pH 6.8 was prepared as follows: 150 mg monobasic sodium phosphate was dissolved in approximately 150 ml HPLC-grade water and the pH adjusted by dropwise addition of 1 M sodium hydroxide solution. The solution was then further diluted to 250 ml with HPLC-grade water in a volumetric flask. The same procedure applies for the 5 mM pH 7.4 phosphate buffer. Borate buffer 5 mM pH 10.0 was prepared by dissolving 477 mg sodium tetraborate decahydrate in 500 ml HPLC-grade water and the pH adjusted to 10.0. The solution was then further diluted to 1 l with HPLC-grade water. All solutions were filtered with a Millex (Millipore, Bedford, MA, USA) 0.45 μm non-sterile syringe driven filter unit prior use. The solutions were prepared freshly on a monthly basis; phosphate buffers were kept frozen at −20 °C in order to minimize bacterial growth. Mobile phase was prepared on a daily basis by mixing the desired volumetric amounts of acetonitrile and borate buffer. Brown 1.5 ml polypropylene flat top microcentrifuge tubes (Fisher) served as mobile phase and sample reservoirs. They were exchanged each run in order to minimize buffer depletion [43]. Protein stock solutions were also made freshly on a daily basis by dissolving the desired amount in 1 ml of 5 mM pH 7.4 phosphate buffer. Thiourea stock solutions were made monthly using the same buffer. Stock solutions of polyaromatic hydrocarbons were prepared in *N,N*-dimethylformamide on a monthly base as well. All sample solutions were prepared just prior to injection by diluting the stock solutions with the respective mobile phase to yield the desired concentrations.

2.3. Column preparation

The capillary was initially filled with a solution consisting of 20% (v/v) MTS, 30% (v/v) glacial acetic acid and 50% (v/v) water and left overnight with the capillary ends sealed. Then, 1.5 ml monomer (BAC) and 1.5 ml crosslinker (BDDA) solutions were prepared by adding approximately 150 mg of Amberlite ion-exchange resin to each solution. The solutions were then stirred overnight in the dark in order to remove inhibitors. The next day, the solutions were centrifuged for removal of the ion-exchange resin.

The column preparation procedure is a modified version of the one described by Ngola et al. [29] who used 2,2′-azobisisobutyronitrile instead of BME which is used by our group and others [22,32]. Briefly, a casting solution was prepared consisting of 20% ethanol, 60% acetonitrile and 20% 5 mM pH 6.8 phosphate buffer by volume. Then 3.0 mg AMPS and 15.0 mg BME were dissolved in 1 ml of this solution, followed by the addition of 150 μl BDDA, 340 μl of BAC and 1.5 μl of MTS. After ultrasonication for 30 s, the capillary was first rinsed with this solution for 2 min. The capillary ends were then sealed and the capillary was put under the mercury vapor lamp for 25 min. Since only the distance from the inlet to the point of detection was to be polymerized, the remaining parts (detector to outlet end) were masked with black electrical tape. After irradiation, the tape was removed and the polymer inspected for cracks or bubbles. Bubbles were usually found at the inlet and outlet ends and these parts consequently cut off. Unless indicated otherwise resulting capillary lengths were 38 cm total with 18.5 cm monolith (inlet to detector). Unreacted monomer was removed by applying a voltage of 10 kV for 30 min with the outlet end as the cathode using a mobile phase of acetonitrile–5 mM pH 10.0 borate (80:20, v/v). The capillary was then preconditioned for 4 h, again at 10 kV, with mobile phase consisting of acetonitrile–5 mM pH 10.0 borate (50:50, v/v) (this is essentially the time needed to reach absorbance baseline stability). Cur-

rent measurements revealed that the resistance in the polymerized section was 1.9 times as high as in the non-polymerized one. The only limitation to capillary lifetime determined so far was irreversible breakage of the capillary.

3. Results and discussion

3.1. The use of UV-transparent capillaries

Fast completion of the polymerization reaction, avoiding elevated temperatures and thus easier control of the polymerization conditions are the main advantages when employing a photoinitiated reaction compared to other, e.g. thermally initiated reactions [22,32]. BME is a photoinitiator built around the benzoyl chromophor and known to exhibit strong absorption in the UV-range as well as good photochemical reactivity [44]. Scanning electron microscope images of the polymer were used to verify that the use of BME as a photoinitiator did not change the gross structure of the polymer as described by Ngola et al. [29]. Images of a polymer filled capillary are shown in Fig. 1. By visual inspection, particle size is on the order of 0.5–1 μm. Finer structures (i.e. nanometer scales) and pore-size distributions will be the subject of future characterization studies.

The use of UV-transparent capillaries allows photopolymerization to be restricted to the exposed regions of the capillary. To accomplish a similar polymerization with conventional polyimide-coated capillaries, the protective polyimide cladding/coating would have to be removed and this would lead to high fragility. However, as shown in Fig. 2, UV-transparent capillaries have the drawback that they possess wave-guiding properties that can interfere with the absorption measurement. This in turn makes

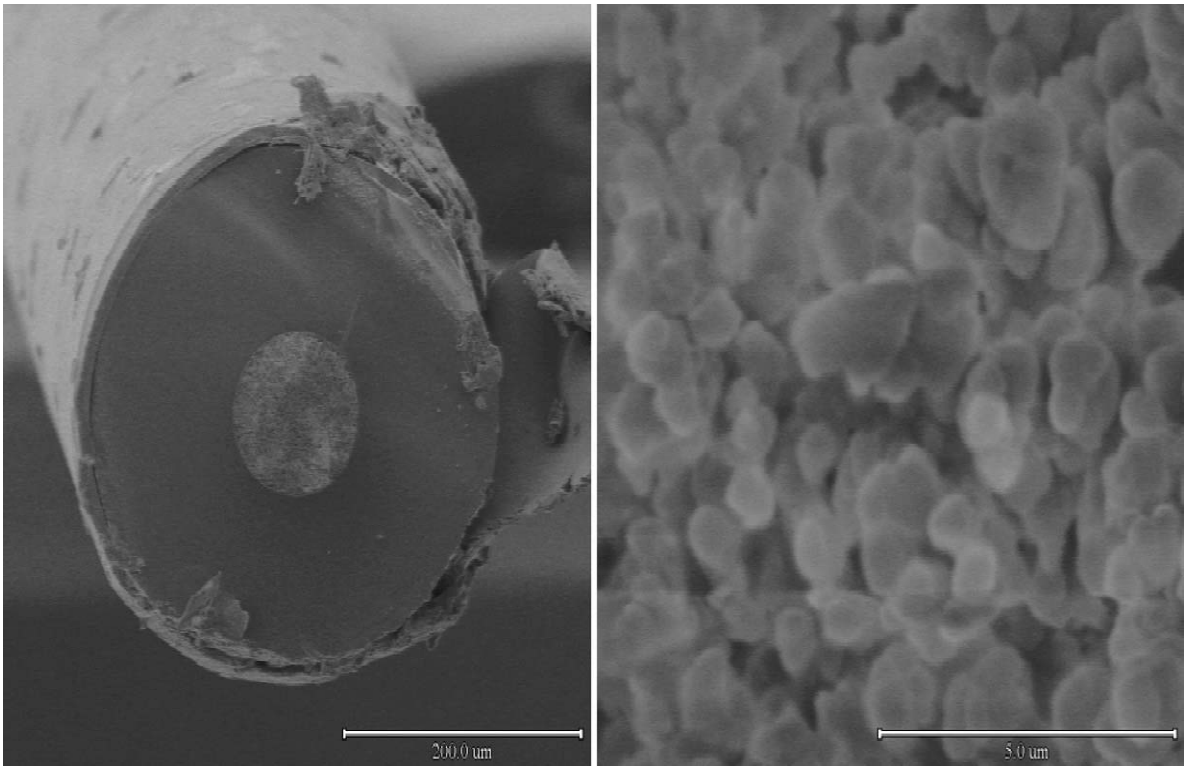

Fig. 1. Scanning electron microscope images of a UV-transparent capillary (100 μm I.D.×360 μm O.D.) with polymer monolith and a detailed view of the polymer. The capillary was coated with vacuum deposited gold to improve conductivity, both images were collected at 7 kV using a Hitachi (S-2300) coupled to a Kevex image analysis system.

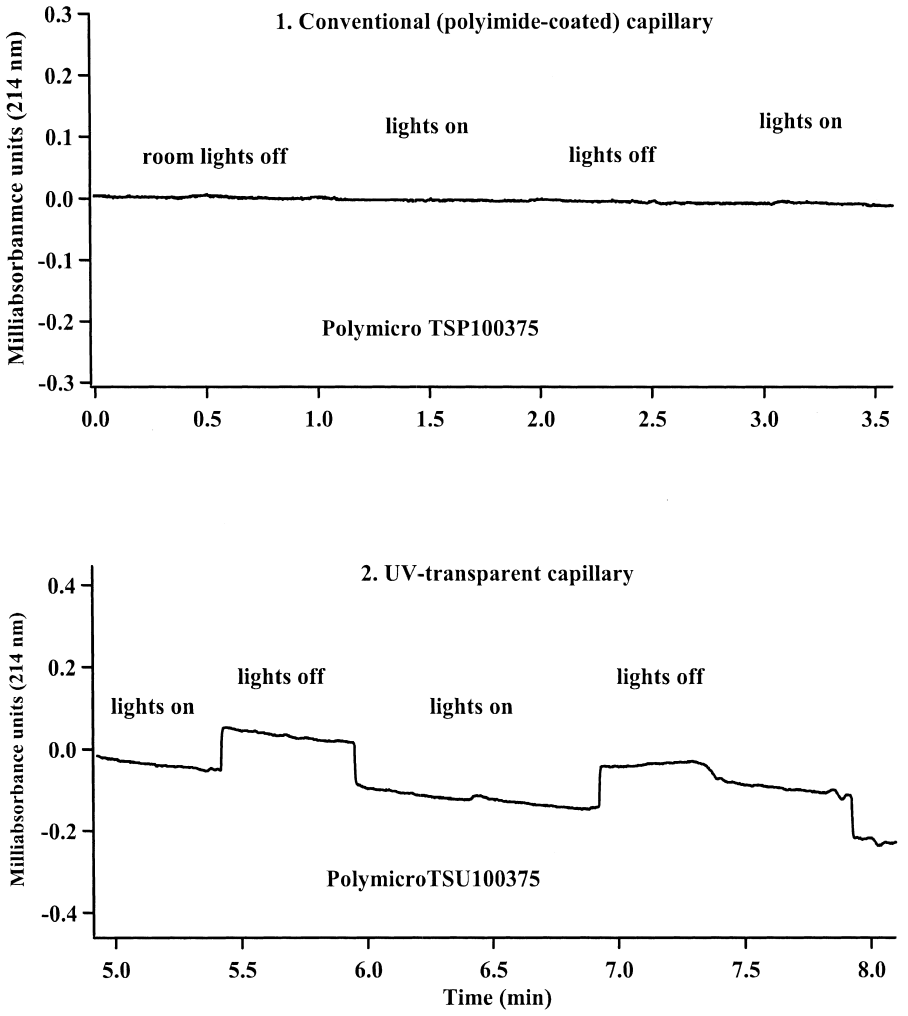

Fig. 2. Influence of room lights on the baseline of a conventional polyimide-coated capillary (100 μm I.D.×386 μm O.D.) and a UV-transparent capillary (100 μm I.D.×360 μm O.D.). Both capillaries were filled with a mobile phase of acetonitrile–5 mM pH 10.0 borate buffer (50:50, v/v). No voltage was applied.

them very sensitive to changing levels of light in the laboratory environment. Light levels should therefore remain constant, and be minimized, to obtain the maximum signal-to-noise ratio and to avoid baseline fluctuations.

3.2. Separation of model proteins and comparison with capillary zone electrophoresis

It was confirmed experimentally by several research groups that the flat flow profile of CEC leads to higher efficiencies than HPLC [3,20,39,40,45–48]. In order to further foster the acceptance of CEC, especially regarding the separation of charged analytes, a comparison with capillary zone electrophoresis (CZE) is also indicated [39,42,49–51]. The CZE method described here uses a relatively standard method that employs only the aqueous component of the mobile phase, i.e. it is carried out with 100% 5 mM pH 10.0 borate buffer. No coating was applied to the capillary walls in the CZE method. The resulting electrochromatograms and electropherograms are presented in Fig. 3a and 3b which shows the different elution profiles obtained by the

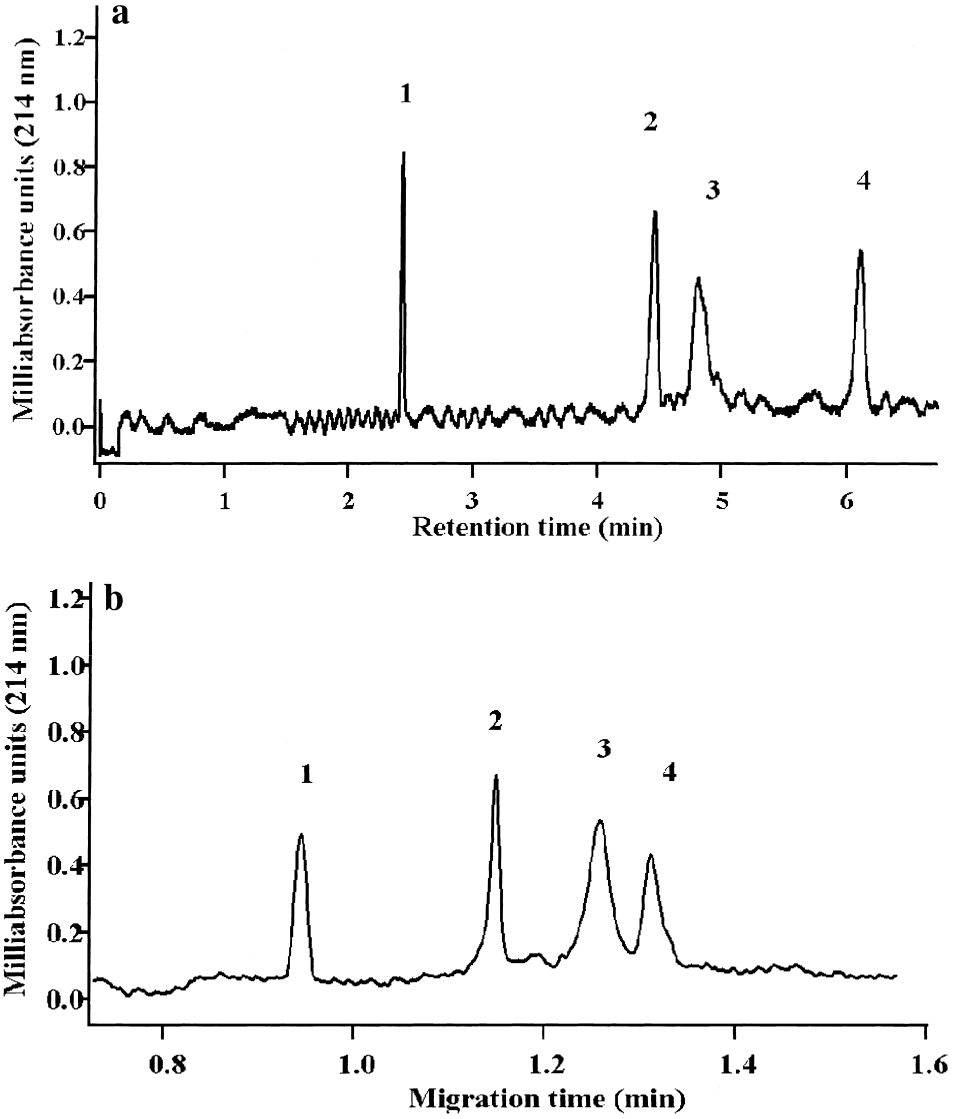

Fig. 3. Comparison of the separation of three model proteins between CEC and CZE. Thiourea is used in both cases as an unretained marker. Both UV-transparent capillaries have the same dimensions, i.e. 37.3 cm, with 21.1 cm to detector (containing the monolith in the case of CEC), 100 μm I.D.×360 μm O.D. Elution order and concentrations (identical in both cases): (1) thiourea 41 μg/ml, (2) myoglobin 366 μg/ml, (3) transferrin 463 μg/ml and (4) α-lactalbumin 339 μg/ml. (a) CEC, mobile phase acetonitrile–5 mM pH 10.0 borate buffer (50:50, v/v), injection 5 s/2 kV, separation 15 kV. (b) CZE, mobile phase 5 mM pH 10.0 borate buffer, injection 5 s/0.5 kV, separation 17.3 kV.

two techniques. The resolution is improved using the CEC method. Table 1 compares migration/retention times, peak widths at half height and number of theoretical plates per column. Here, CZE shows faster analysis times and higher efficiencies. Current measurements revealed that, in the CEC method, the resistance of the polymerized segment, i.e. inlet to detector, is 1.9 times as high as in the non-polymerized one (detector to outlet). When using the same capillary dimensions, i.e. 37.3 cm total length and 21.1 cm inlet to detector, the electric field strength in the CZE method must be adjusted to the same value

Table 1
Comparison of average migration/retention times, average peak widths at half height and average number of theoretical plates ($n=5$) per column between CEC and CZE for the separation of model proteins under the conditions described in Fig. 1

	Thiourea	Myoglobin	Transferrin	α-Lactalbumin
Average retention time (s)				
CEC	150	268	291	368
CZE	57	70	76	79
Average width at half height (s)				
CEC	1.4	3.6	6.4	4.7
CZE	0.9	0.7	1.4	0.9
Average number of theoretical plates per column				
CEC	64 000	29 000	11 000	33 000
CZE	26 000	66 000	18 000	48 000

as in the polymerized segment of the CEC method. Since 15 kV of separation voltage in CEC leads to an electric field strength of 0.463 kV/cm in the polymerized segment, the applied voltage in the CZE method was therefore adjusted to 17.3 kV thus leading to the same field strength.

In a separate experiment the maximum protein concentrations were determined that could be loaded onto the column under the given injection conditions before significant peak overlap and distortion take place. They were found to range between 1900 μg/ml for α-lactalbumin, 2200 μg/ml for myoglobin and 2600 μg/ml for transferrin, respectively (results not shown).

Since proteins possess charged moieties as well as hydrophobic ones their separation in CEC is deemed to be a complex interplay of both chromatographic and electrophoretic mechanisms. In order to prove that the photopolymer is principally capable of separating analytes based on a chromatographic mechanism alone, several neutral analytes were also separated. Fig. 4 illustrates the separation of eight polyaromatic hydrocarbons.

3.3. Inter- and intra-capillary reproducibilities

Inter-capillary reproducibility, i.e. the reproducibility from capillary to capillary relates primarily to the reproducibility of the fabrication process. Although the results are sometimes disenchanting, the use of an internal standard can often compensate for variations in retention times and secure reliable peak identification. Intra-capillary reproducibility, on the other hand, describes the reproducibility of the separation process in one capillary column. Column or reagent instabilities, integration errors, flow rate variations (caused, for example, by temperature fluctuations) as well as variations in the amount of analyte injected are common factors to be considered here [52,53]. For the specific case of protein separations in CZE, irreversible adsorption to the capillary walls has been shown to lessen reproducibility [54–56]. In CEC, irreversible adsorption to the stationary phase should therefore be taken into account. The use of an internal standard for peak area and retention times might improve intra-capillary reproducibility as well since it can compensate for injection and flow rate variations. It should also be assumed that, like in CZE, peak areas in CEC can be expected to vary with retention times since molecules do not pass the detector cell with constant velocity. Using corrected peak areas, i.e. dividing the peak area by the retention time might aid here as well [56,57].

In this study, thiourea was used as an internal standard. Since it is commonly used in CEC as an EOF marker, it should compensate for EOF variations. Unlike proteins, thiourea does not adsorb onto the stationary phase material or the capillary walls and can therefore also compensate for injection variations (problems caused by adsorption could otherwise easily be mistaken as injection errors).

For the inter-capillary reproducibility, 10 capil-

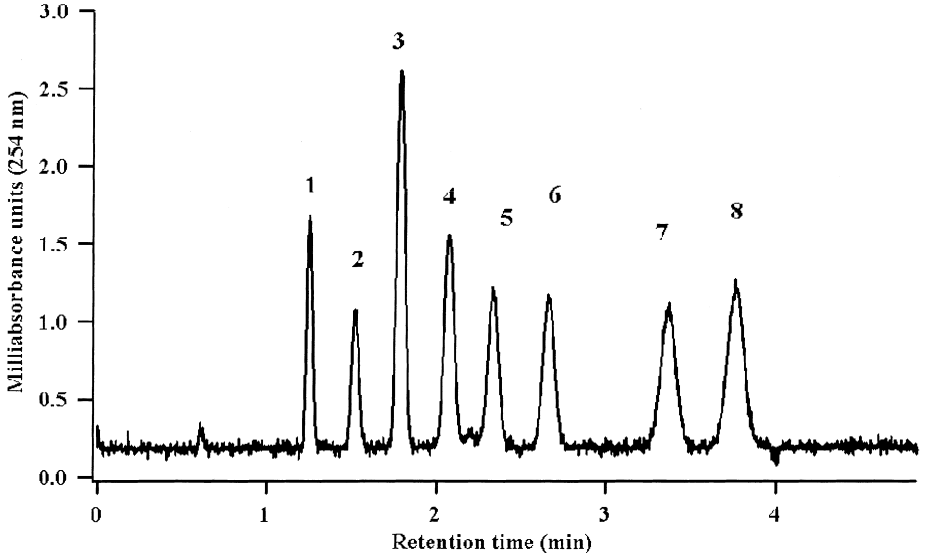

Fig. 4. CEC of polyaromatic hydrocarbons. Capillary dimensions are 10.0 cm inlet to detector and 19.2 cm total length, 100 μm I.D., 360 μm O.D. Mobile phase: acetonitrile–5 mM pH 8.1 Tris buffer (80:20, v/v), injection 5 s/5 kV, separation 15 kV. Elution order: (1) naphthalene 480 μg/ml, (2) acenaphthene 800 μg/ml, (3) anthracene 30 μg/ml, (4) pyrene 190 μg/ml, (5) fluoranthene 240 μg/ml, (6) chrysene 50 μg/ml, (7) benzo[*k*]fluoranthene 111 μg/ml and (8) benzo[*a*]pyrene 100 μg/ml.

laries were manufactured on 10 different days. A standard protein mixture consisting of myoglobin, transferrin and α-lactalbumin was then run on each capillary five consecutive times. For each protein, the mean retention time and mean peak area obtained from each of the 10 capillaries was used to quantify the inter-capillary reproducibility. The RSD of the five runs with each capillary were used for the intra-capillary reproducibility. All manufacturing conditions were kept as constant as possible (see Column preparation section). The results from both inter- and intra-capillary reproducibility can serve as a quality criterion when testing new capillaries.

Table 2 shows the results of the inter-capillary study. When comparing the means of the retention times from each day, a reproducibility of about 15% is observed for the proteins. This value is improved significantly when using relative retention times. The same behaviour is seen for peak areas (with the exception of α-lactalbumin). Comparing the intra-capillary reproducibilities on each day (Table 3) average RSD values for the absolute retention times

Table 2
Inter-capillary reproducibilities of 10 capillaries, with five consecutive runs on each capillary

	Thiourea	Myoglobin	Transferrin	α-Lactalbumin
RSD (%) of absolute means of five runs on each capillary				
Retention time	7.7	13.4	14.4	18.4
Area	8.3	7.1	18.9	7.9
RSD (%) of relative means (using thiourea as internal standard)				
Retention time		5.4	6.9	11.6
Area		6.6	15.7	9.2

See text for further details.

Table 3
Intra-capillary reproducibilities of 10 capillaries, with five consecutive runs on each capillary

	Thiourea	Myoglobin	Transferrin	α-Lactalbumin
Average of RSD (%) of absolute values				
Retention time	2.1	2.5	2.8	3.1
Area	5.6	9.3	10.7	8.5
Average of RSD (%) of relative values				
Retention time		1.4	1.6	1.7
Area		6.9	10.1	6.0

See text for further details.

of around 3% are obtained. They can be improved to about 1.5%, again using the internal standard. The average intra-capillary reproducibility of absolute peak areas is around 9%, it could also be improved to around 7% using the internal standard. The use of corrected peak areas did not bring any improvement (results not shown).

Considering the improvement brought by the internal standard it is expected that differences in the EOF velocity from capillary to capillary can be held responsible for the differences in retention times. Since our laboratory-built instrument is not thermostated, these variations could be caused, for example, by temperature fluctuations. Slight variations in the length of the monoliths also have to be taken into account here. The differences are much smaller within one capillary and are again improved by the internal standard, temperature fluctuations might be once more the cause. The relatively low reproducibility of peak areas (both inter- and intra-capillary) could be caused by variations of the amount of sample injected since the use of thiourea as an internal standard brings improvement here as well. The exact cause of those variations remains the subject for further research. Adsorption of proteins to the wall might also play a role. More efficient rinsing procedures might alleviate this factor.

3.4. Rinsing procedure

As mentioned above, an adequate rinsing procedure in-between runs in order to minimize adsorption of the proteins to the capillary walls should improve the intra-capillary reproducibilities. Several rinsing procedures have been described in the CE literature [58]. Apart from the need for effective cleaning, the rinsing step should not damage the walls or the stationary phase, should not lead to excessive Joule heating and should not be too time consuming. Two methods, each involving 16 consecutive runs, are compared here. The first does not apply a rinsing step at all. The second one rinses for 5 min with mobile phase containing 0.05% SDS (w/v), followed by 8 min reconditioning with pure mobile phase in order to stabilize the current to its initial value. It can be seen in Table 4 that the reproducibility of the relative peak areas can be significantly improved with the rinsing step. The retention time reproducibility remains unaffected (with the exception of α-lactalbumin), indicating that rinsing and reconditioning time are long enough and

Table 4
Comparison of intra-capillary reproducibilities ($n = 16$) with and without a rinsing step in-between runs, refer to text for details

	Myoglobin	Transferrin	α-Lactalbumin
RSD (%) of relative retention times			
Rinsing	1.6	1.3	1.5
Non-rinsing	1.4	1.4	0.5
RSD (%) of relative peak areas			
Rinsing	4.5	3.2	3.2
Non-rinsing	7.9	12.1	5.4

not obscured by potential adsorption of SDS to the stationary phase.

3.5. Relationship between plate height and velocity

As pointed out already in the Introduction, one major reason for the current popularity of monoliths in both CEC and HPLC lies in their ability to allow for rapid mass transfer rates. Fig. 5 shows the Van Deemter plots obtained for thiourea and the three model proteins. At mobile phase velocities above 0.5 mm/s, all three protein plots exhibit nearly velocity-independent plate heights, i.e. the C-term (which describes resistance to mass transfer) in the Van Deemter equation becomes essentially velocity-independent. Consequently, high velocities can be used without sacrificing efficiency. The upper limit could not be determined due to arcing of the electrodes at higher voltages. A steep descent of the graph for thiourea at lower velocities highlights the influence of the B-term in the Van Deemter equation (longitudinal diffusion). This descent is not seen in the plots for the proteins due to their lower diffusion coefficients.

3.6. Relationship between plate height and injection time

Fig. 6 illustrates the relationship between peak height and injection time for thiourea, myoglobin and α-lactalbumin. All three plots demonstrate that an increase in peak height is observed by prolonging the injection time. A similar behavior has been reported earlier by Quirino et al. [36] for a sol–gel monolith. Since mobile phase and sample solvent were the same in our work, i.e. they did not differ in ionic strength or percentage organic solvent, neither preconcentration caused by electrokinetic sample stacking (caused by differences in the ionic strength between sample and mobile phase) nor chromatographic field enhancement (caused by differences in hydrophobicity between sample and mobile phase) should be expected [59]. Considering the dependence of detection limits on peak heights [60], these

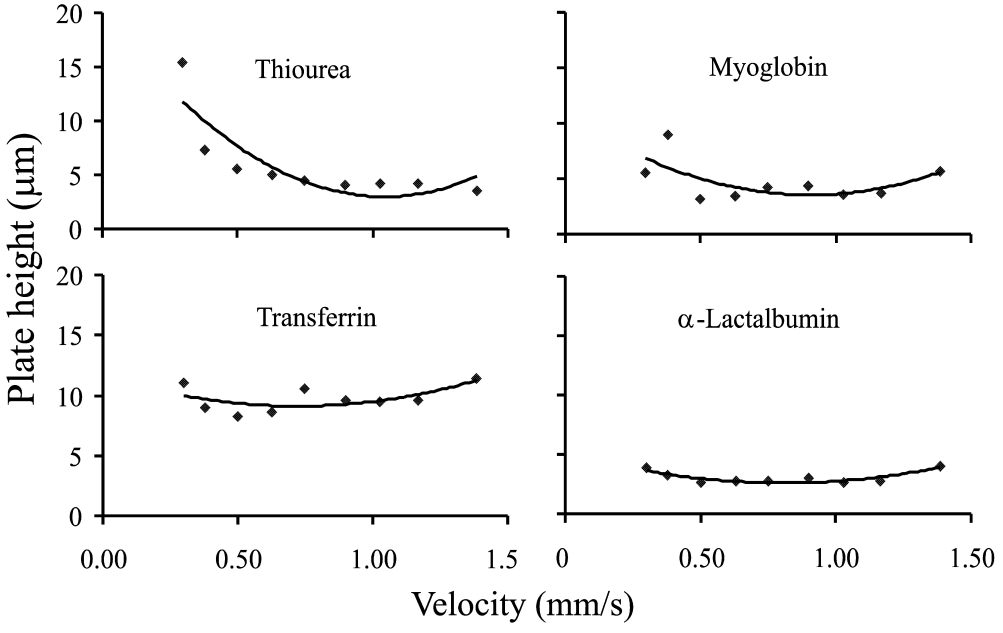

Fig. 5. Relation between plate height and velocity. Analyte concentrations were 30 μg/ml thiourea, 149 μg/ml myoglobin, 150 μg/ml transferrin and 129 μg/ml α-lactalbumin. A second-order polynomial regression line was added to the data for the sole purpose of highlighting the trends. All other conditions as described in the Experimental section.

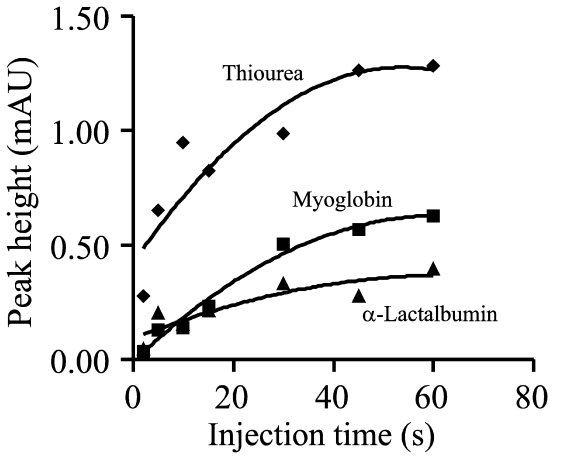

Fig. 6. Relation between plate height and injection time. A second-order polynomial regression line was added to the data for the sole purpose of highlighting the trends. Analyte concentrations were 51 μg/ml thiourea, 218 μg/ml myoglobin and 189 μg/ml α-lactalbumin. All other conditions as described in the Experimental section.

findings might be highly beneficial when detecting dilute quantities of analyte.

3.7. Influence of the percentage acetonitrile on the EOF and capacity factors

The change in EOF as a function of percentage acetonitrile in the mobile phase is illustrated in Fig. 7. A decrease in EOF is observed when increasing the percentage acetonitrile. The sigmoidal profile is similar to profiles obtained by other groups using both CZE and CEC [9,61].

However, increases in capacity factor with increasing percentage acetonitrile were found for the three model proteins (Fig. 7) and may appear to be unexpected. For the CEC method, the capacity factor k' was calculated as is done in LC [37]:

$$k' = \frac{t_r - t_0}{t_0} \quad (1)$$

with t_r being the analyte retention time and t_0 being the retention time of thiourea considered to be an unretained marker. From chromatographic theory, an increase in capacity factor indicates an increase in analyte–stationary phase interaction. In reversed phase chromatography (or electrochromatography), increasing the percentage of organic solvent would be expected to reduce the analyte–stationary phase

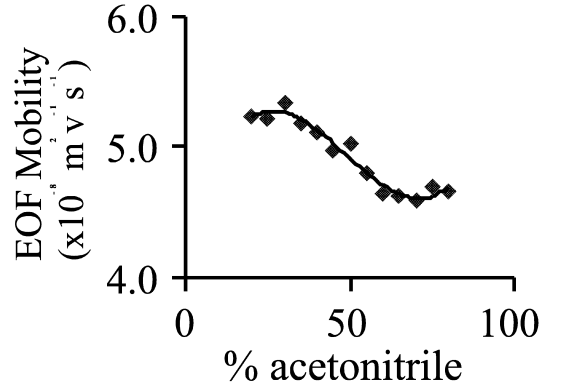

Fig. 7. Velocity of the electroosmotic flow at different percentages acetonitrile in the mobile phase with the aqueous component of the mobile phase being 5 mM pH 10.0 borate buffer. A third-order polynomial regression line was added to the data for the sole purpose of highlighting the trends. All other conditions as described in the Experimental section.

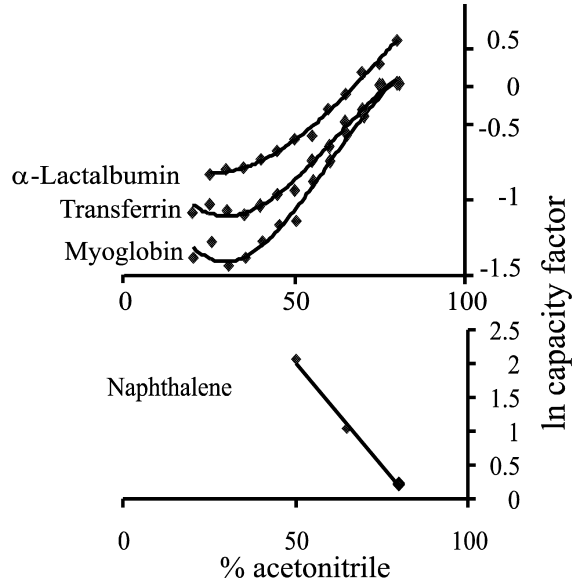

Fig. 8. Plots of ln capacity factor versus different percentages of acetonitrile in the mobile phase. The aqueous component in the mobile phase was always 5 mM pH 10.0 borate buffer. A third-order polynomial regression line was added to the data for the sole purpose of highlighting the trends. All other conditions as described in the Experimental section.

interaction and explains the behavior of the small, neutral analyte naphthalene as illustrated in Fig. 8. In the case of the proteins, there are combined electrophoretic and chromatographic separation mechanisms at work in CEC and an increase in the percentage organic solvent brings an increase in the capacity factor. However, it was pointed out in a recent publication by Rathore and Horváth [62] that using this formula one obtains a number that, unlike its equivalent in HPLC, does not contain any thermodynamic or mechanistic information. Thus, it is inappropriate to draw conclusions about the degree of analyte–stationary phase interaction for proteins, that possess electrophoretic mobilities and may be unfolded to varying degrees, from the calculated capacity factor. The capacity factor in CEC more accurately serves as a mere peak locator useful for the calculation of resolution and selectivity. Still, it can be safely said that in the steep part of the curve, small changes in the percentage of acetonitrile can have a significant impact on the apparent retention of the proteins. This may have a significant impact on the robustness (and reproducibility) of the CEC method. It also provides a mechanism to tune the selectivity and overall resolution of CEC separations.

4. Conclusions

Highly efficient separations for model proteins were obtained using the described photopolymerized monolith. The monolith is easily manufactured using UV-transparent capillaries. Intra- and inter-capillary reproducibilities were evaluated in order to gain insight regarding potential error sources. Both types of reproducibilities could be improved using thiourea as an internal standard. Future work will aim to reduce the error introduced by injection and EOF variations. Protein separations proceed via a combined electrophoretic and chromatographic process that is highly sensitive to mobile phase composition. High electric field strengths can be applied without sacrificing resolution, the upper limit is thus far undetermined due to instrumental shortcomings. The height of the analyte peaks is increased by prolonging the injection time, which should be highly beneficial when analyzing small quantities of proteins.

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