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Capillary isoelectric focusing of proteins and microorganisms in dynamically modified fused silica with UV detection☆

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Abstract

We suggest a method for the reproducible and efficient capillary isoelectric focusing of proteins and microorganisms in the pH gradient 3–10. The method involves the segmental injection of the simple ampholytes, the solution of the selected electrolytes, and the sample mixture of bioanalytes and carrier ampholytes to the fused silica capillaries dynamically modified by poly(ethylene glycol), PEG 4000, which is added to the catholyte, the anolyte and injected solutions. In order to receive the reproducible results, the capillaries were rinsed by the mixture of acetone/ethanol between analyses. For the tracing of the pH gradients the low-molecular-mass p*I* markers were used. The simple proteins and the mixed cultures of microorganisms, *Saccharomyces cerevisiae* CCM 8191, *Escherichia coli* CCM 3954, *Candida albicans* CCM 8180, *Candida parapsilosis*, *Candida krusei*, *Staphylococcus aureus*, *Streptococcus agalactiae* CCM 6187, *Enterococcus faecalis* CCM 4224, *Staphylococcus epidermidis* CCM 4418 and *Stenotrophomonas maltophilia*, were focused and separated by the method suggested. The minimum detectable number of microbial cells was 5×10^2 to 1×10^3 with on-column UV detection at 280 nm.

Keywords: CIEF; Dynamically modified FS; Spacers; p*I* markers; Proteins and microorganisms; UV detection

1. Introduction

The application of the electromigration techniques [1] in the diagnostic microbiology needs the reliable method, the high run-to-run reproducibility, speed and sensitivity. The wide spectrum of physiological features [2] makes the characterization of microbes by capillary zone electrophoresis (CZE) difficult. The microbes are different in their size, shape and in the composition of the outer membranes, which include the large number of lipids, proteins, etc. The isoelectric points, p*I*'s, of microbes [3] are more appropriate parameters than the electrophoretic mobilities; p*I*'s are practically independent from the measurement conditions [4,5]. Several papers were published showing the successful separation of microorganisms according to their p*I* by capillary isoelectric focusing (CIEF) [1,5,6].

Microorganisms, similarly to bioanalytes, tend to adhere strongly on the surfaces [7,8]. The adsorption of the analytes onto the capillary wall may increase the zone distortion [1,9–11]. Simultaneously, it is necessary to minimize the strong electroosmotic flow (EOF) on the uncoated fused silica (FS) capillary. The suggested solutions include dynamic modification of the inner capillary surface [12,13] by soluble polymers such as hydroxymethylpropyl cellulose, hydroxypropyl cellulose, polyvinyl alcohol, polyacrylamide, poly(acryl-amide-*co*-acrylic acid), polyvinylpyrrolidone, etc. [12,14,15]; coating of the capillary with the hydrophilic polymer such as an acrylamide derivative; and modification of the capillaries by γ-glycidoxypropyltrimethoxysilane [16,17] or the sol–gel technique [18]. The lifetime of the polymeric film on the inner surface of the capillary is often shortened by its degradation due to the strong acids and bases used as the anolytes and catholytes, respectively [12]. At the same time, the adsorbed bioanalytes must be washed out from the wall of the capillary prior to the subsequent run. The rinsing procedure between individual runs was shown to have a strong effect on the reproducibility [19]. Therefore, the application of the

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uncoated dynamically modified FS seems to be the simplest solution.

The poly(ethylene oxide) (PEO) belongs to the group of the hydrophylic uncharged polymers used for the dynamic coating. PEO chains attached to surfaces are reported to reduce the protein and microbial adhesion [7,20,21]. The usage of a diluted PEO solution was demonstrated to increase the efficiency of the separation of the bacteria by CZE [1,14,22–26] or submicron-sized polystyrene particles. [27]. PEO has been also used in capillary electrophoresis as the non-bonded coating for modifying of EOF [28]. The presence of carrier ampholytes in the FS capillary and their adsorption onto the capillary wall mostly decreases EOF as well [15,29,30].

Generally, the capillaries for CIEF are usually preconditioned before each analysis by a mixture of carrier ampholytes [17,31–33]. Electroosmotic displacement of focused protein zones can be achieved by injection of the mixture of proteins and carrier ampholytes in form of a plug at the inlet of the capillary that has been pre-filled with the catholyte. The effect of the adsorption of carrier ampholytes onto uncoated FS capillaries is appreciable [29].

In isoelectric focusing, the resolution strongly depends on the slope of the pH gradient. When the resolution is not compromised by other factors of the process, the gradient manipulation is the main parameter used to improve the resolution [12,15]. The addition of the spacer ampholytes, β-alanine and 6-aminocaproic acid, improves the separation of haemoglobins in the pH gradient 6–8 in Ampholine gels [12,34]. In CIEF, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was added to the commercial carrier ampholytes Pharmalyte 3–10, in order to extend the separation range of pH gradient up to pH 12 [35]. A sandwich injection protocol [29] was used for the effective separation of amphoteric compounds having their p*I* values outside the pH range of the carrier ampholyte.

In this study we suggest the segmental injection of the sample mixture including the segment of spacers, the segment of bioanalytes and the segment of the carrier ampholytes with p*I* markers at the positive side of the inlet of the fused silica capillary that has been pre-filled with the catholyte. For the dynamic coating of the capillary low molecular weight poly(ethylene glycol), PEG 4000, is added to both end electrolytes, catholyte and anolyte, which prevents from the adsorption of bioanalytes and enables to control EOF. In order to obtain reproducible results we also suggest the washing of the capillaries between analyses by the mixture of organic solvents. For the tracing of the pH gradients the low-molecular p*I* markers [16] were used. The mixed cultures of microorganisms and proteins were reproducibly focused and separated by CIEF method suggested.

2. Materials and methods

2.1. Equipment

The capillary isoelectric focusing experiments were carried out using the laboratory-made apparatus [18]. All measurements were performed at the constant voltage (−) 20 kV supplied by the high voltage unit Spellman CZE 1000 R (Plainview, NY, USA). During the experiments, the current decreased from 40 to 60 μA at the beginning of experiment to 3–6 μA at the time of detection, depending on the sampling time interval and the sample solution. The lengths of the fused silica capillaries, 0.1 mm I.D. and 0.25 mm O.D. (Pliva-Lachema a.s., Brno, Czech Republic) were from 250 to 270 mm, with the effective lengths from 180 to 190 mm. The ends of the separation fused silica capillary were dipped in 3 ml-glass vials with the catholyte or the anolyte solutions (CaAn) and electrodes.

The suitable and reproducible linear velocities of focused zones and thus the regulation of EOF were achieved by help of the dynamic modification of the capillary inner surface by poly(ethylene glycol), $M_r = 4000$. The segmental injection of the sample was accomplished by siphoning action achieved by the elevating of the inlet – the anolyte reservoir, relative to the outlet – the catholyte reservoir [18]. The height difference of the reservoirs for the sample injection, Δh, can be adjusted in the range 100–180 mm for 7–60 s.

The on-column UV–vis detector LCD 2082 (Ecom, Prague, Czech Republic) connected to the detection cell by optical fibers (Polymicro Technologies, Phoenix, AZ, USA) was used at the wavelength 280 nm. The light absorption (optical density) of the microbial suspensions was measured using a DU series 520 UV–vis spectrophotometer (Beckmann Instruments, Palo Alto, CA, USA) at 550 nm.

The association between the cells was reversed by the sonication of their suspension in Sonorex, Bandelin electronic (Berlin, Germany). The frequency 35 kHz was used for one minute at the temperature 30 °C. After the sonication the microbial sample was vortexed for 10 min (Vortex-Genie 2, Scientific Industries, Bohemia, USA) and then immediately used.

The CZE experiments with EOF measurement were carried out using laboratory-made apparatus. All measurements were made at (−) 20 kV at constant conditions for the siphoning injection (100 mm, 5 s). The length of the whole separation fused silica capillary (0.1 mm I.D., 0.25 mm O.D.) was 320 mm with 250 mm to the on-column UV–vis detector.

The detector signals were acquired and processed with the Chromatography station for Windows CSW v. 1.5, DataApex s.r.o., Praha, Czech Republic; data were processed by graphic program Sigma-Plot 3.0, Jandel Scientific Software (Point Richmond, CA, USA).

2.2. Chemicals

The strains included in this study, *Saccharomyces cerevisiae* CCM 8191 (*S. cerevisiae*), *Escherichia coli* CCM 3954 (*E. coli*), *Candida albicans* CCM 8180 (*C. albicans*), *Candida parapsilosis* (*C. parapsilosis*), *Candida krusei* (*C. krusei*), *Staphylococcus aureus* (*St. aureus*), *Streptococcus agalactiae* CCM 6187 (*Str. agalactiae*), *Enterococcus faecalis* CCM 4224 (*E. faecalis*), *Staphylococcus epidermidis* CCM 4418 (*St. epidermidis*) and *Stenotrophomonas maltophilia* CCM 1640 (*S. maltophilia*) were obtained from Czech Collection of Microorganisms, Brno, Czech Republic. Bovine serum Albumin ($M_r = 67{,}000$, p*I* 4.9 [36]), Cytochrome *c*, horse heart ($M_r = 12{,}400$, p*I* 9.3 [36]), were from Sigma (St. Louis, MO,

USA). Ribonuclease A from bovine pancreas (M_r = 13,700, p*I* 8.9 [36]) was from Reanal (Budapest, Hungary). The solution of synthetic carrier ampholytes, Biolyte, pH 3–10, was obtained from Bio-Rad Laboratories, CA, USA. The spacers, simple ampholytes, 2-[4-(2-hydroxyethyl)-1-piperazynyl]-ethanesulfonic acid (HEPES, pK_1 = 3.65, pK_2 = 7.5, p*I* = 5.58) and L-aspartic acid (Asp, pK_1 = 1.88, pK_2 = 3.66, p*I* = 2.77) were from Merck, Darmstadt, Germany or Wien-Fischamend, Austria, respectively. Poly(ethylene glycol), M_r = 4000 (PEG 4000) was from Carlo Erba, Milano, Italy. All chemicals were analytical grade. Low-molecular-mass p*I* markers [16], 3.9, 5.3, 6.4, 7.5, 8.5, 10.1, and 10.4, were synthesized in the Institute of Analytical Chemistry Academy of Sciences of the Czech Republic, Brno. Other chemicals were obtained from Pliva-Lachema a.s., Brno, Czech Republic.

2.3. Working solutions

2.3.1. Electrode solutions

For all experiments presented here, 20 mM sodium hydroxide and 100 mM *ortho*- phosphoric acid were used as the catholyte and anolyte, respectively. For the dynamic modification of the capillaries, the solution of final concentration in the range 0–5% (w/v) of PEG 4000 in CaAn was used. For the measurement of the electroosmotic flow, 10 mM phosphate buffers of pH 3–8 and thiourea as an EOF marker were used.

2.3.2. Microbial samples

The strains included in this study were inoculated on blood agar (Hi-Media, Mumbai, India) and stored at −70 °C: *E. coli* CCM 3954, *E. faecalis* CCM 4224, *St. epidermidis* CCM 4418 and *C. albicans* CCM 8180 in Itest KRYOBANKA B (ITEST plus, Hradec Králové, Czech Republic). Before each experiment, the strains were thawed quickly at 37 °C and cultivated on Muller–Hinton's agar (Bio-Rad, Marnes La Coquette, France) at 37 °C or on Nutrient Agar Difco (*S. maltophilia* CCM 1640) from Voigt Global Distribution LLC (MI, USA) at 28 °C, both for 24 h. The microbial cultures were resuspended in the physiological saline solution (PSS). The number of the resuspended microorganisms was estimated by the measurement of the optical density of the suspension. It was measured by a spectrophotometer at 550 nm, according to the calibration curve, which was defined by reference samples. These samples were prepared by resuspension of the microbial culture in PSS. The concentrations of microorganisms in reference samples were measured by dilution and by plating 100 μl of suspension on Muller–Hinton's agar (Bio-Rad). After cultivation at 37 °C for 24 h the colonies were counted.

2.3.3. Segmental injection of the sample

The sample pulse was composed of three parts: The first segment was composed of the solutions of the spacers, 0.25 mM HEPES, 0.35 mM Asp, 20 mM NaOH and 0–2% (w/v) of PEG 4000. The second segment was composed of the solution of proteins, 50 μg ml^{-1}, or suspension of microbes, 10^7–10^9 cell ml^{-1}, in water or in the physiological saline solution of PEG 4000, in the range 0–5% (w/v), respectively; the amount of the proteins injected into the capillary was ranged from 14 to 300 ng, number of microorganisms from 3×10^2 to 10^4 cells. The third segment was composed of the water solution of p*I* markers, 25 μg ml^{-1} of each, their injected amounts are from 7 to 150 ng, and 5% (w/v) of Biolyte 3–10.

Before each segmental injection the capillaries were rinsed minimaly for ten minutes with the mixture of acetone/ethanol, 10:1 (v/v), and then back-flushed with the catholyte for 1 or maximum 2 min. The composition of the rinsing solution was verified experimentally.

3. Results and discussion

3.1. The optimization of CIEF procedure

3.1.1. EOF on the FS dynamically modified by PEG 4000

PEG 4000 was used here for the dynamic modification of the uncoated FS capillary as the prevention of the adsorption of bioanalytes and for the reduction of EOF. Different concentration of PEG 4000 was dissolved in the 10 mM phosphate buffers of pH 3–8, see Fig. 1, curves 1–5. At the concentration of PEG 4000 below 1%(w/v) and pH 8, the electroosmotic flow was diminished approximately by one-eighth of value of μ_{EOF} measured in the non-modified capillary, see curves 2, 3 versus curve 1. The application of 2 and 3% (w/v) of PEG 4000 for the dynamic modification of FS decreases EOF by one-fourth to one-half of

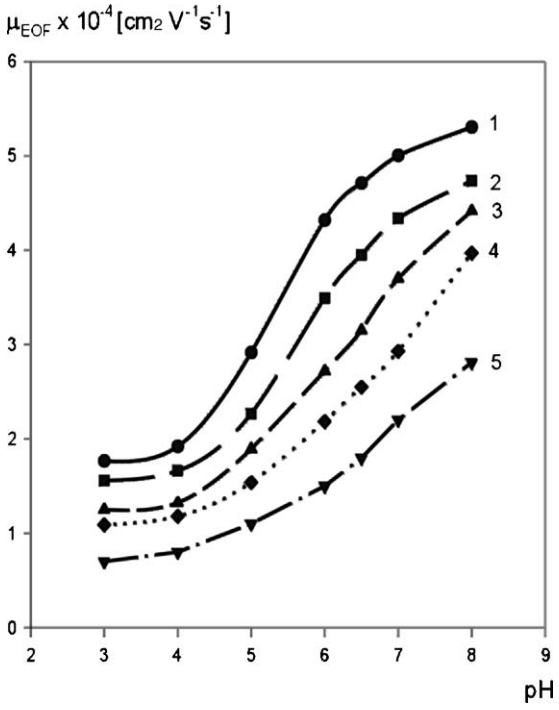

Fig. 1. Dependence of EOF on pH for untreated FS capillary (1) and capillary dynamically modified by PEG 4000 (2–5). Conditions: FS capillaries 0.1 mm I.D., 0.25 mm O.D., length 320 mm (250 mm to the detection window); 10 mM phosphate buffer from pH 3 to 8 with PEG 4000, c_{PEG} [% (w/v)], (2) 0.5, (3) 1, (4) 2, (5) 3; height difference of the reservoirs at the siphoning injection, Δ*h*, 100 mm, time of injection, t_{inj}, 5 s; applied voltage (−) 20 kV; neutral marker of EOF, thiourea; UV–vis detection, λ_{max}, 235 nm.

its original value. That is in agreement with Ref. [1], where high molecular PEO with M_r over 600,000 was used.

The capillaries were washed between each electrophoretic run, see Section 2.3.3. That ensures the high EOF reproducibility on the FS capillary dynamically modified by PEG 4000 which is promising for the reproducible and efficient CIEF with the mobilization of the pH gradient by electroosmosis.

3.1.2. The study of the influence of PEG 4000 in the catholyte and the anolyte on the focusing of pI markers

In addition to the reproducibility the basic requirement for CIEF is the linearity of the pH gradient. From this reason, the capillaries are usually conditioned over night and than before each analysis with the solution of the synthetic carrier ampholytes [17,31–33]. In our initial experiments, see Fig. 2A–D, the FS capillaries were used without previous modification with Biolyte, pH 3–10 and also without the injection of spacers. In the experiment of Fig. 2A, PEG 4000 was not added in the electrode solutions, whereas 1–3% (w/v) of this polymer was dissolved in CaAn in the experiments in Fig. 2B–D. Six low-molecular-mass p*I* markers covering p*I* from 3.9 to 10.1 were used to verify the linearity of the pH gradient. Before each analysis the capillaries were rinsed for ten minutes with the mixture of acetone/ethanol, 10:1 (v/v), and then back-flushed with the catholyte for one minute, for additional information see Section 2.3.3.

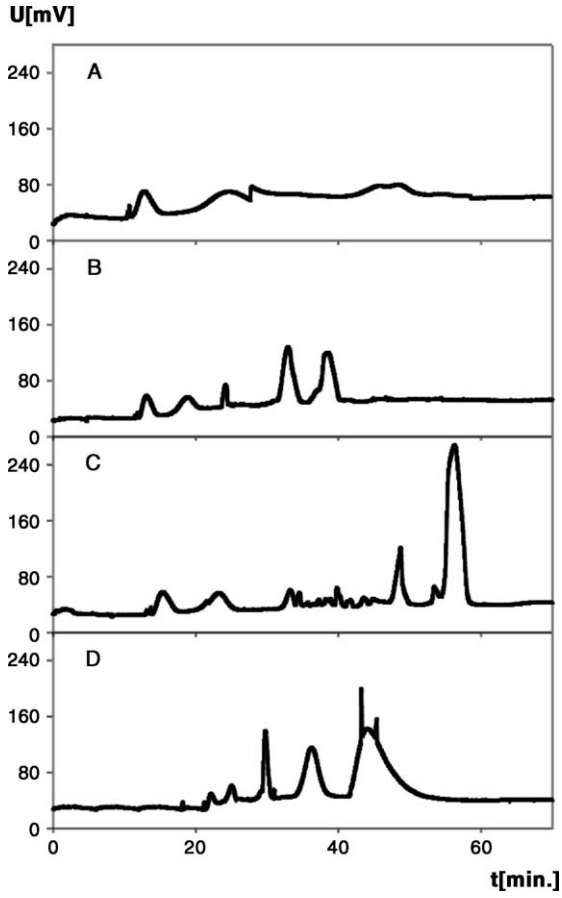

Fig. 2. The influence of the capillary surface pretreatment on CIEF of the low-molecular-mass p*I* markers. Conditions and designations see Fig. 1; FS length 270 mm, 180 mm to the detection cell; anolyte: 100 mM H_3PO_4, catholyte: 20 mM NaOH, $c_{PEG,CaAn}$ [% (w/v)] in both; electropherogram (A) 0, (B) 1, (C) 2; (D) 3; λ_{max}, 280 nm; Δh—Biolyte and p*I* markers, 180 mm, t_{inj}, 35 s (280 nl); p*I* markers, p*I*: 10.1, 8.5, 7.5, 6.4, 5.3, and 3.9, in 5% (w/v) Biolyte; rinsing procedure before each focusing run, 10 min with the solution acetone/ethanol, 10:1, 1 min with the catholyte; migration time, *t* (min).

With the increase in the concentration of PEG 4000 in the electrolytes EOF decreases. It results from the comparison of the migration times of the p*I* markers from 3.9 to 10.1 in electropherograms A (without PEG 4000 in CaAn) – C (2% (w/v) PEG 4000 in CaAn) versus D (3% (w/v) PEG in CaAn) in Fig. 2. In the electropherogram A we can observe only the non-focused zones of the p*I* markers. Five of six p*I* markers are partly focused in electropherograms B and C, where 1 or 2% (w/v) of PEG 4000 were used. At the concentration of 3% (w/v) PEG 4000 in electrolytes the migration time of p*I* markers are decreasing and consequently the pH gradient is narrow. In all experiments, the peak of the acidic p*I* marker 3.9 is missing and the pH gradient can be considered as not linear.

3.1.3. The influence of the addition of PEG 4000 into the catholyte, the anolyte and spacers segment on the linearity of the pH gradient

The injection of the suitable spacers into the capillary significantly influences the linearity of the pH gradient and the reproducibility of CIEF. In our preliminary experiments the composition of the spacer segment was optimized. Thus, the mixture of the injected simple ampholytic electrolytes, 200 nl, consists of 0.25 mM HEPES and 0.35 mM Asp dissolved in 20 mM NaOH, which ensures the linearity of the pH gradient 3–10, see Fig. 3. The second segment is composed of 5%(w/v) solution of Biolyte and low-molecular-mass p*I* markers, 3.9, 5.3, 6.4, 7.5, 8.5, and 10,1. The influence of the change in the concentrations of PEG 4000 in the catholyte and anolyte solutions and in the spacers segment was studied in terms of the dependencies of the migration times of p*I* markers on their isoelectric points, see Fig. 3. The composition of CaAn was the same as in previous experiments, 20 mM NaOH and 100 mM H_3PO_4, respectively. The concentration of PEG 4000 in CaAn, $c_{PEG,CaAn}$, was 0.5 and 2% (w/v), see Fig. 3A and B, respectively. The correlation coefficients, R^2, were chosen for the evaluation of the linearity of the pH gradient. Their values range from 0.998 for the dependencies in Fig. 3A, to 0.999 in Fig. 3B. Generally, good linearity of the pH gradient but different resolution of p*I* markers was achieved at these experimental conditions.

With the increase in $c_{PEG,CaAn}$ EOF decreases and the migration time for the p*I* marker 10.1 increases, see Fig. 3B versus A. Both the minimal changes of the migration times for the p*I* marker 10.1 and significant changes of the migration times for the other p*I* markers are observed with increase in the concentration of PEG 4000 in the spacers segment, see Fig. 3A, curves 1–4 and Fig. 3B, curves 2–4. At the zero concentration of PEG 4000 in the spacers segment, see Fig. 3B, curve 1, the length of the pH gradient is higher than that observed in the other experiments, see curves 2–4 for comparison. The resolution of the zones of the p*I* markers is insufficient for the future focusing of the bioanalytes. With increase in the concentration of PEG 4000 in CaAn

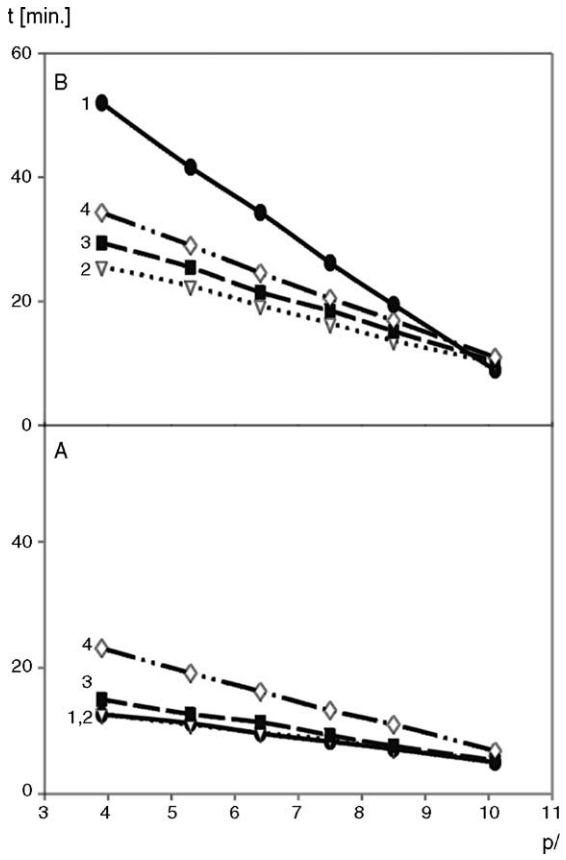

Fig. 3. The influence of the change of the PEG 4000 concentration on the linearity of the pH gradient at segmental injection. Conditions and designations see Figs. 1 and 2; $c_{PEG,CaAn}$ [% (w/v)]—(A) 0.5, (B) 2; Δh—spacers, 180 mm, t_{inj}, 25 s (200 nl); concentration of PEG 4000 [% (w/v)] in the spacer segment, (A and B)—(1) 0, (2) 1, (3) 1.5 and (4) 2.

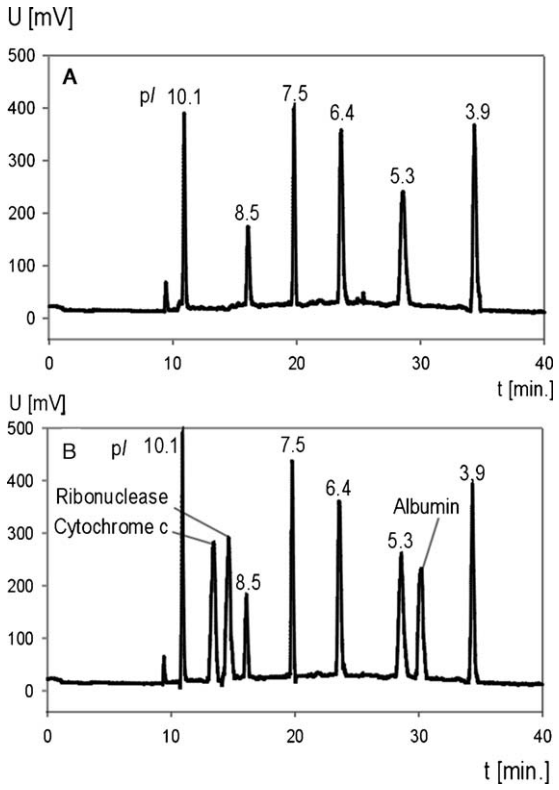

Fig. 4. CIEF of the low-molecular-mass p*I* markers (A) and proteins (B). Conditions and designations see Figs. 1–3; (A) concentration of PEG 4000 in CaAn and spacer segment, 2 [% (w/v)]; (B see A), Δh—proteins, 150 mm, t_{inj}, 15 s (120 nl); proteins, Ribonuclease, Cytochrome *c* and Albumin.

and at the same time in spacers segment the resolution increases and narrow zones of bioanalytes are detected. The optimum concentration of PEG 4000 in the CaAn and in the spacers segment was found around 2% (w/v) for our experiments. Therefore, this concentration of PEG 4000 was used largely during following focusing runs.

3.1.4. CIEF of low-molecular-mass pI markers and proteins

The electropherogram in Fig. 4A shows the focusing run of the sample pulse of segmental structure. The spacers segment consists of HEPES and Asp, the second segment consists of the carrier ampholytes and low-molecular-mass p*I* markers with p*I*'s from 3.9 to 10.1. In the catholyte, anolyte and in the spacers segment 2% (w/v) PEG was dissolved. The acceptable linearity of the pH gradient was achieved after the addition of spacers into the sample segment, see Fig. 4A. Good focusing and narrow peaks of p*I* markers are observed when the rinsing procedure before each focusing run was performed, see Section 2.3. It allows us to use CIEF with mentioned p*I* markers for the estimation of the isoelectric points of the examined bioanalytes by the simple linear interpolation.

In Fig. 4B we can see the examples of the focusing of p*I* markers with p*I*'s from 3.9 to 10.1, 8 ng of each, and proteins, Albumin, Cytochrome *c*, and Ribonuclease, 10 ng each. The simple proteins were used here as examples of the defined biopolymers for comparison with microbial cells. The conditions were the same as in Fig. 4A. The sample pulse was composed of the spacers segment, segment of the bioanalytes, proteins, and segment of the carrier ampholytes along with p*I* markers. The positions of the focused proteins are in good agreement with their p*I* values mentioned in Section 2.2. These results are comparable with the generally used CIEF procedure, where the capillaries are often preconditioned overnight with the mixture of the carrier ampholytes and HPMC [17,31,37,38] before the first use and then before each focusing run. The migration time reproducibilities at the experimentally used conditions were evaluated by the measuring of the relative standard deviation (R.S.D.) of the migration times of p*I* markers. The migration time reproducibilities, RSDs, were lower than 2% in fifty replicated measurements.

3.1.5. CIEF of pI markers without the capillary rinsing before each focusing run

The rinsing of the capillaries with acetone/ethanol mixture significantly influences the migration time of the ampholytic analytes and, simultaneously, the shape of the pH gradient. To demonstrate this phenomenon, the p*I* markers 3.9–10.1 were separated under the identical conditions as in Fig. 4A only without the rinsing of the capillary before each focusing run. The profiles of the pH gradients generated in the capillaries under these conditions are demonstrated in Fig. 5. After the first focus-

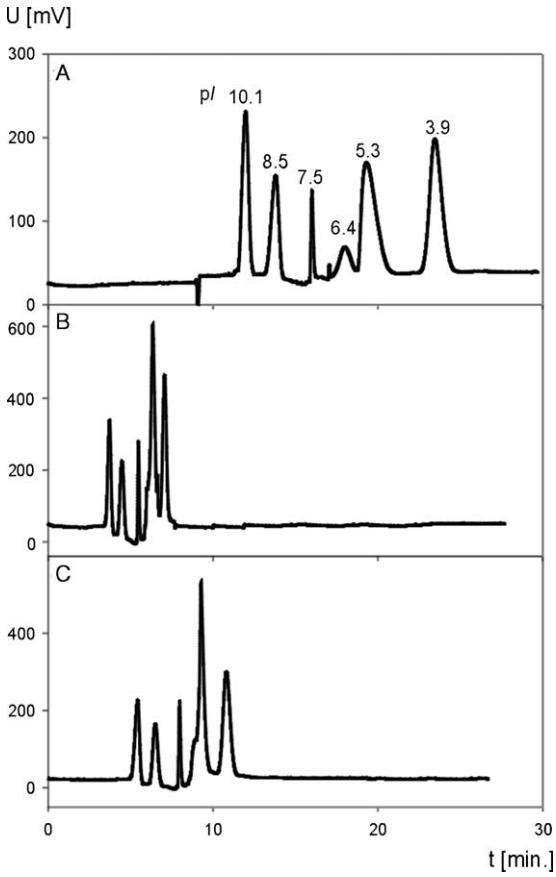

Fig. 5. The influence of the rinsing procedure on CIEF of the p*I* markers. Conditions and designations see Fig. 4A without the rinsing procedure, see Fig. 2, before each focusing run (A–C).

ing run, see Fig. 5A versus A, the migration time of the p*I* marker 10.1, is higher. With increase in the number of the focusing runs without the rinsing of the capillary before CIEF, both migration time of the p*I* marker 10.1 and length of the pH gradient are changed irreproducibly and the resolution of the low-molecular-mass p*I* markers further decreases, see Fig. 5, electropherogram B and C.

3.2. CIEF of microorganisms

3.2.1. Focusing under optimum composition of the catholyte and anolyte solution

The separation of the mixture of the microbes is demonstrated on the series of the electropherograms, see Fig. 6A–E. The optimized compositions of CaAn, 20 mM NaOH, 100 mM H_3PO_4, 2% (w/v) solution of PEG 4000 and spacers—0.25 mM HEPES, 0.35 mM Asp, 2% (w/v) solution of PEG 4000 in 20 mM NaOH, were used here. The isoelectric points of the microbes in the mixture are ranging from 11 (*S. maltophilia* [36]) down to the isoelectric points of fungies, ranging from p*I* 2 to 4.

Most of the microbes were injected into the capillary at their number around 10^7 in 1 ml of the sample suspension, see Fig. 6A, C and E. At 10^9 cells in 1 ml injected into the capillary, whole injected cells in the sample cannot be focused, compare the peak shapes of *C. albicans* in Fig. 6B with A, and peak shapes of *C. parapsilosis* in Fig. 6D with C, c.f. also Ref. [18]. The correlation coefficient R^2 of the linearity of the pH gradient in the range 3.9–10.1 was found 0.99 from 20 measurements. The reproducibility of the migration time of p*I* marker 6.4 was found as 23.7 min (math) 0.24 min, the relative standard deviations of the migration times of microbes R.S.D.'s were found lower than 2%. The lifetime of the capillary dynamically modified with PEG 4000 was at least 150 runs.

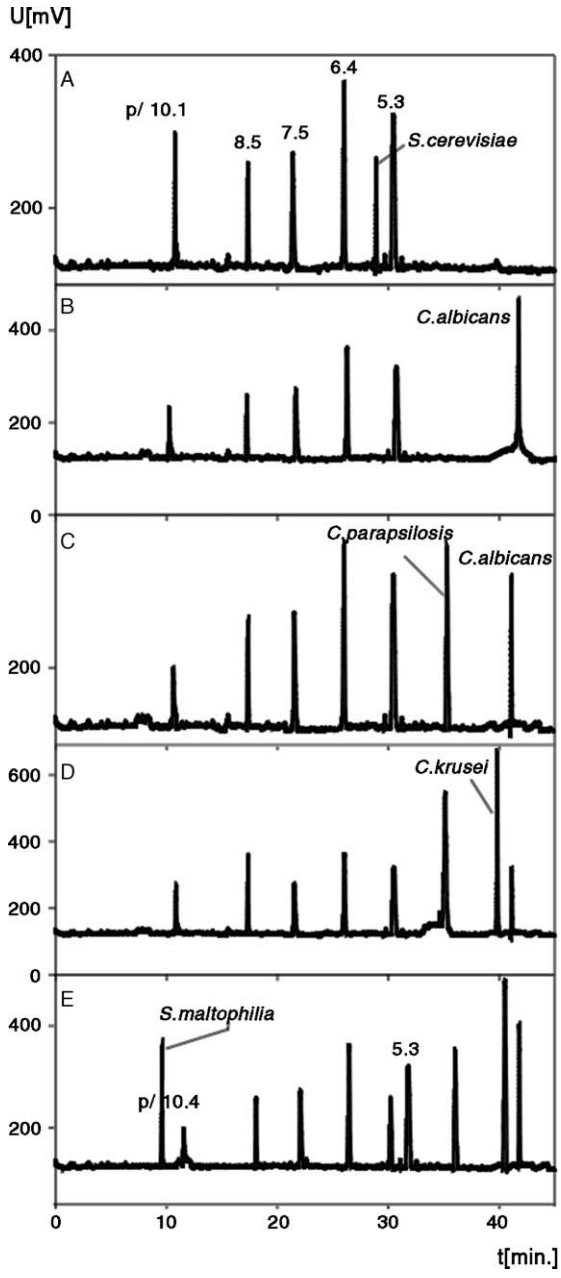

Fig. 6. CIEF of microorganisms at the optimal conditions. Conditions and designations see Fig. 4; FS, length 270 mm, 180 (A–D), 190 mm (E) to the detection cell; p*I* markers, p*I*: 10.1, 8.5, 7.5, 6.4, 5.3; microbial sample, Δh—150 mm, t_{inj}, 12 s (100 nl); (A) *S. cerevisiae*, 10^7 cell ml^{-1}, (B) *C. albicans*, 10^9 cell ml^{-1}, (C) *C. albicans*, *C. parapsilos*, 10^7 cell ml^{-1}, (D) *C. albicans*, *C. krusei*, 10^7 cell ml^{-1}, *C. parapsilosis*, 10^9 cell ml^{-1}, (E, see A, D) and *S. maltophilia*, 10^7 cell ml^{-1}; resuspended in physiological saline solution; (E) p*I* marker 10.4 was used instead of the p*I* marker 10.1.

Therefore, the electropherogram 6E represents CIEF of the microbial sample under above-mentioned optimal conditions and concentration of cells 10^7 in 1 ml of their suspension; that is around 10^3 of injected microbes. The narrow zones of the low-molecular-mass p*I* markers and the cells were obtained from p*I* 10.1 to <3. 9. From p*I* 11 (*S. maltophilia* [36]) to 10.1 the non-linearity of the pH gradient was already observed.

3.2.2. The shortened focusing of the microbes

The decrease in the PEG 4000 concentration in both electrolytes, catholyte and anolyte, to 1.5% (w/v) and in the spacer zone under 2% (w/v) to 0.5% (w/v) led to the decrease in the migration time of the pH gradient, approximately down to one third, compare the results shown in Fig. 7A–C with those in Fig. 6.

The applied concentration of microbes in the sample was 10^7 cell ml^{-1}. In Fig. 7A, *St. aureus* was focused and the narrow peak was obtained. The isoelectric point was found under p*I* 3.9.

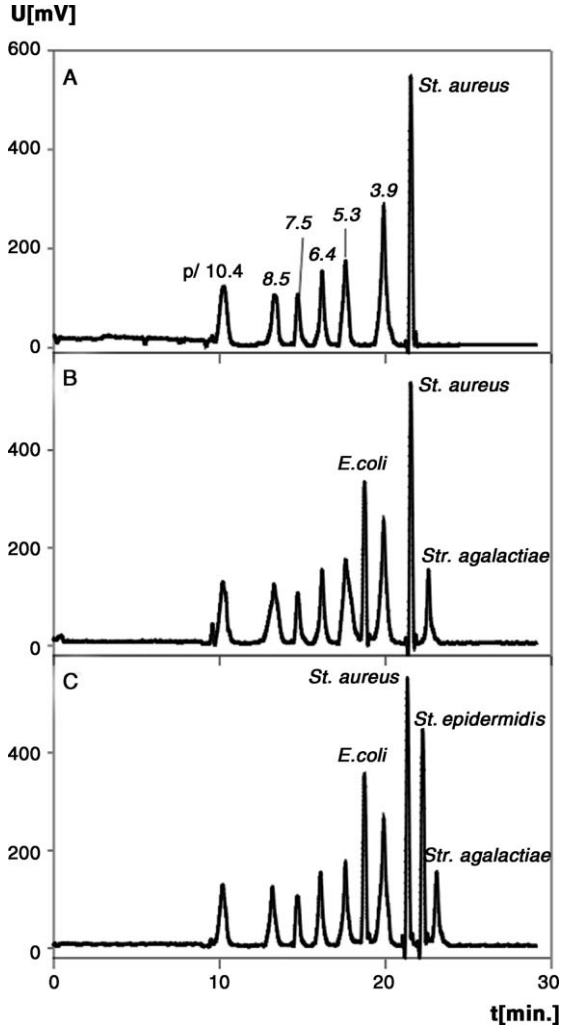

Fig. 7. Shortened CIEF of microorganisms at the modified conditions. Conditions and designations see Figs. 4 and 6; FS 190 mm to the detection cell; concentration of PEG 4000 in CaAn, 1.5 [% (w/v)] and spacer segment, 0.5 [% (w/v)]; microbial sample, (A) *St. Aureus*; (B) *E. coli*, *St. aureus*, *Str. agalactiae*; (C) see B and *St. epidermidis*; number of cells in 1 ml, 10^7, resuspended in physiological saline solution.

In the second electropherogram *E. coli* and *Str. agalactiae* were added to *St. aureus*. The peak of *E. coli* was focused between the peaks of the p*I* markers 3.9 and 5.3 and the strains *Str. agalactiae* and *St. aureus* were focused under p*I* 3.9, see Fig. 7B. *St. epidermidis* was then focused between this two strains, see Fig. 7C. However, the zones of p*I* markers are not fully focused into the narrow zones as in Fig. 6.

4. Conclusions

The proteins and microorganisms were reproducibly focused and separated in the FS dynamically modified with PEG 4000 by CIEF with UV detection in the pH gradient 3–10. In the catholyte and the anolyte as well as in spacers segment 0.5–2% (w/v) PEG 4000 was found adequate. The segmental injection of the sample composed of the spacers segment created by the simple ampholytic electrolytes, HEPES and Asp, as the spacers together with the segment of the mixture of analytes and segment of the carrier ampholytes was used here to achieve suitable shape of pH gradient. For the evaluation of the linearity of the pH gradient the low-molecular-mass p*I* markers 3.9–10.4 were used. The migration time reproducibilities were evaluated by measuring the relative standard deviation of the migration times of p*I* markers. R.S.D.'s were found lower than 2%. The found differences in isoelectric points of cells are promising for the identification of microbes by CIEF method suggested. The lifetime of the capillary dynamically modified with PEG 4000 was at least 150 runs. Many microbial strains have their isoelectric points in the range from 2 to 5, see Ref. [39]. This can be demonstrated and confirmed by our electropherograms. In order to obtain the useful pH gradient in the interval from pH 2 to 4 and thus better separation of the acidic strains, the modified method of CIEF will be necessary to develop.

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