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Separation of phenotypically indistinguishable *Candida* species, *C. orthopsilosis*, *C. metapsilosis* and *C. parapsilosis*, by capillary electromigration techniques

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ABSTRACT

At the current state of laboratory diagnostics, methods for fast identification of phenotypically indistinguishable species are difficult or inaccurate. An example is represented by *Candida parapsilosis*, which is the second most common yeast species isolated from bloodstream infections. *C. parapsilosis* comprises a complex of three genetically distinct groups. Genotypes II and III have been designated as the separate species *Candida orthopsilosis* and *Candida metapsilosis*, phenotypically indistinguishable. The considerable genetic variability of these newly described yeasts species has caused difficulties in the development of molecular techniques for their precise identification. Similarly, the detection of biofilm formation, which is considered as an important yeast virulence factor, is accompanied by difficulties. In this study we optimize the first precise and reproducible method for the separation and possible identification of *C. orthopsilosis*, *C. metapsilosis* and *C. parapsilosis* as well as the detection of their ability to form biofilm. The method is based on capillary isoelectric focusing and capillary electrophoresis with UV detection. In capillary isoelectric focusing, very narrow pH gradients were established. With such gradients, differences in isoelectric points of biofilm-negative and biofilm-positive species calculated from the migration times of the selected p*I* markers were below 0.03 p*I* units. In the capillary zone electrophoresis narrow zones of the cells of *Candida* species were detected with sufficient resolution. The values of the isoelectric point and the migration velocities of the examined species were independent on the origin of the tested strains. Capillary isoelectric focusing was examined also for the separation and detection of the cultivated biofilm-negative *C. parapsilosis* in the blood serum.

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1. Introduction

A phenotype is defined as any observable characteristic or feature of a microorganism, such as its morphology, biochemical or physiological properties or its behavior in the environment. Identification of phenotypically indistinguishable species by conventional laboratory methods is difficult, time-consuming and variable in its success. Inadequacy in proper species identification limits its effectiveness for proper diagnosis and therapy in the clinical practice [1]. An example may involve the *Candida* species. Specifically, *Candida parapsilosis* is the second most common yeast species isolated from bloodstream infections and belong to the important nosocomial pathogens [2,3]. *C. parapsilosis* isolates are more genotypically heterogeneous than those of other *Candida* species. Recently, it was found that *C. parapsilosis* comprises a complex composed of three phenotypically indistinguishable however genetically distinct groups (groups I, II, and III) [4] from which groups II and III were designated as new species *Candida orthopsilosis* and *Candida metapsilosis*. The strains of the group I are considered *C. parapsilosis* sensu stricto [4]. Data on the isolation frequency of *C. orthopsilosis* and *C. metapsilosis* are just starting to be released [5–7]. The prevalence of these species is significant and differences observed in their susceptibility profiles could have therapeutic importance [6,8].

Another factor that should be taken into account when distinguishing the yeast specimen is their ability to form biofilm on host tissue or indwelling medical devices [9]. The biofilm-positive (+) *C. parapsilosis* strains were associated with significantly higher mortality rates of patients with candidaemia than biofilm-negative (−) ones [10]. It appears that none of the clinical *C. orthopsilosis* isolates were found to form biofilm layer in vitro on the internal wall of microtiter plate wells and the ability of *C. metapsilosis* strains to

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form biofilm is lower than those observed for *C. parapsilosis.* This finding confirms a reduction of the virulence potential at these two species [7,11].

There are currently no reliable phenotypic tests to distinguish *C. parapsilosis* from *C. orthopsilosis* and *C. metapsilosis* [4,7]. The differentiation among *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* has been successfully performed using various molecular techniques including random amplification of polymorphic DNA (RAPD) analysis [5,7,12], nucleotide sequence analysis [5,13–16], amplification fragment length polymorphism analysis [7] and restriction fragment length polymorphism (RFLP) analysis of genomic DNA [17,18], the multilocus sequence typing (MLST) [4,17], melting curve of random amplified polymorphic DNA (McRAPD) [19] etc. Also matrix-assisted laser desorption/ionisation–time of flight mass spectrometry (MALDI–TOF MS) spectra has been proven to be suitable for the identification of the yeasts in question [20]. However, a successful identification requires a database with a sufficient number of the spectra of the appropriate reference strains.

In summary, only a few methods have been developed for distinguishing the particular yeast specimen. Unfortunately, these methods are laborious, costly and they are not available in most of routine laboratories. Moreover, the detection of phenotypic expression of the ability to form biofilm is complicated and the result evaluation poises certain problems. Therefore, it is essential to develop new methods to address these shortcomings.

The differences in the physico-chemical properties of the cell surfaces, e.g., the values of the cell surface charge, may result in different electromigration behavior. Capillary isoelectric focusing (CIEF) and capillary electrophoresis (CZE) could be appropriate tools [21–28] for the efficient separation of the phenotypically indistinguishable microorganisms according to their isoelectric points, p*I*'s, or mobilities and for improvement of their characterization. Recently, the isoelectric points of different strains of *Candida* species were determined [25,29,30] in the expected pH range from pH 1.5 to 4.5 which is characteristic for most microorganisms [31,32]. Here, the isoelectric point for the strains of biofilm-negative *C. parapsilosis*, was determined as 3.8 and for biofilm-positive as 3.6 [29]. In the case of the studied phenotypically similar strains of *Candida* species – *C. orthopsilosis* and *C. metapsilosis,* their p*I*'s will be located probably near the p*I*'s of the *C. parapsilosis* biofilm-negative or biofilm-positive.

The IEF separations of species with slight p*I* differences less then several hundredths of pH units were reached under certain conditions. The separation of Hemoglobins Hb A from Hb A_{1c} may be an classic example [33,34] where the separations with $\Delta pI < 0.03$ pH units were demonstrated. They were separated by CIEF [35,36] using focusing mixture consisted of commercial carrier ampholytes, short-chain liquid polyacrylamide and an equimolar mixture of two electrolytes [35]. The wide pH gradient course is traced by several p*I* markers [33,34]. For more precise p*I* determination, narrow pH gradients are required with the use of a minimum of two appropriate p*I* markers that closely bracket the p*I*'s of analyte cells. This allows precise and reproducible p*I* values and minimizes errors resulting from curvature of the pH gradient [33,34,37].

The possibilities to separate and to compare the electromigration behavior of the phenotypically indistinguishable microorganisms using CIEF and CZE in the example of the above-mentioned *Candida* species are newly communicated in this paper. We used experiences from previous works [25,29,38,39] where the reproducible results and the smooth pH gradient in the narrow pH range were achieved by CIEF using segmented injection. Some difficulties such as the adsorption of microorganisms onto the capillary wall [29,40–42] were solved based upon different surface properties of the cells during CE separation, as achieved by using appropriate buffer solutions additives [29,40,43–46], e.g. poly(ethylene glycol) (PEG) [22,24,29,38,47–50].

2. Materials and methods

2.1. Chemicals

Poly(ethylene glycols) (M_r 4000 and 10,000) were from Aldrich (Milwaukee, WI, USA). The high resolution ampholyte, pH 2–4, ampholyte pH 3–4.5 and 2-morpholino-ethanesulphonic acid monohydrate (MES), 3-morpholino-propanesulphonic acid (MOPS), N-[tris-(hydroxymethyl)-methyl]-3-amino-2-hydroxy-propansulphonic acid (TAPSO) were from Fluka Chemie GmbH (Buchs, Switzerland). The solution of synthetic carrier ampholytes, Biolyte, pH 3–10, from Bio-Rad Labs (Hercules, CA, USA). N-(2-acetamido)-2-aminoethansulphonic acid (ACES) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES) were from Merck (Darmstadt, Germany). L-aspartic acid (Asp) was from LOBA Chemie (Wien, Austria), other spacers-tartaric (Tart), malic (Mal), formic (Form), succinic (Suc), acetic (Acet), pivalic (Piv), glutamic (Glu) and nicotinic (Nic) acids, as well as the non-ionogenic tenside Brij 35 were from (Sigma, St. Louis, MO, USA). The specifications [51,52] of the used spacers, simple ampholytic electrolytes, are described in Ref. [39]. The low-molecular p*I* markers, p*I* = 3.3 (compound No. X, p*I* 3.33 ± 0.04 pH units), 3.7 (compound No. XIV, p*I* 3.65 ± 0.06 pH units), 3.9 (compound No. XV, p*I* 3.90 ± 0.12 pH units) and 4.7 (compound No. XVII, p*I* 4.71 ± 0.04 pH units) [53] and 4.0 [25] for tracing of the pH gradient 3.3–4.7, 3.3–3.7, 3.7–4.0, 3.8–4.0, 3.6–4.0 and 4-morpholinyl acetic acid (MAA) [53] were synthesized in the Institute of Analytical Chemistry Academy of Sciences of the Czech Republic, v. v. i., Brno. All chemicals were analytical grade.

2.2. Microbial samples

All the strains included in this study, biofilm-negative (*C. orthopsilosis* BC7, *C. orthopsilosis* PH93, *C. metapsilosis* PH87, *C. metapsilosis* PH89, *C. metapsilosis* PH92, *C. parapsilosis* BC8, *C. parapsilosis* BC9 and *C. parapsilosis* BC111) as well as biofilm-positive (*C. metapsilosis* PH85, *C. metapsilosis* PH86, *C. metapsilosis* FS58, *C. parapsilosis* BC11, *C. parapsilosis* BC12 and *C. parapsilosis* BC16) were clinical strains obtained from Collection of Microbiology Institute, Masaryk University and St. Anna University Hospital (Brno, Czech Republic). The reference strains of *C. orthopsilosis* MUCL49939 and *C. metapsilosis* MUCL46179, both biofilm-negative, were obtained from Belgian Co-ordinated Collections of Microorganisms – (Agro) Industrial Fungi & Yeasts Collection (Louvain-la-Neuve, Belgium). The reference strains of biofilm-positive *C. parapsilosis* CCM 8260 was obtained from Czech Collection of Microorganisms (Brno, Czech Republic).

The identification of C. *orthopsilosis*, *C. Metapsilosis* and *C. parapsilosis* strains was confirmed by sequencing. Briefly, PCR amplification of the ITS2 region was performed with the primer pair UNF1a and UNF2 published previously [54]. Purified cycle sequencing products were subjected to a sequencing procedure on ABI Prism 3100 Avant (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Sequences were aligned with sequences in the GenBank BLAST database (http://www.ncbi.nlm.nih.gov/blast) and the highest-scoring (homology at least 99%) species were recorded.

2.3. Cell cultivation

The yeast strains included in this study were stored at −70 °C in Itest cryotubes (ITEST plus, Hradec Králové, Czech Republic). Before each experiment, the strains were thawed quickly at 37 °C and cultivated on Sabouraud dextrose agar (HiMedia, Mumbai, India) at 37 °C for 24 h. The microbial cultures were re-suspended in physiological saline solution (PSS). The concentrations of the re-

suspended microorganisms were estimated by the measurement of the optical density of the suspension by means of spectrophotometer at 550 nm, according to the calibration curve, which was defined by reference samples. The numbers of microorganisms in the reference samples were controlled by serial dilution and plating of 100 μL of the suspension on Sabouraud dextrose agar. After the cultivation at 37 °C for 24 h, the colonies were counted.

2.4. Biofilm formation

Biofilm formation in these strains was detected by a modification of the Microtiter Plate Method (MTP) described by Shin et al. [55]. Briefly, yeasts were grown in Yeast Nitrogen Base medium Difco (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) containing 5×10^{-2} mol L^{-1} glucose (YNBg) at 37 °C in an orbital shaker at 300 rpm. Cells were harvested after 24 h, washed twice in a phosphate-buffered saline (PBS) and adjusted to the optical density of 0.8 at 520 nm. The wells of the 96-well flat-bottomed polystyrene tissue culture microtiter plates (Nunc, Roskilde, Denmark) containing 180 μL of YNBg were inoculated with 20 μL of standardized yeast cell suspension. After 24 h of incubation at 37 °C, the wells were emptied and washed three times with sterile PBS. The adherent biofilm layer was stained with 1% (w/v) crystal violet for 20 min. For the spectrophotometric assessment, the bound dye was eluted with 200 μL of 33% glacial acetic acid per well. The absorbance (A_{595}) of each well was measured at 595 nm, ref. filter 690 nm. The assessment of biofilm formation was performed three times in three wells for each strain. The wells whose A_{595} was higher than the mean A_{595} of the negative controls (wells containing only sterile medium) plus 3 × Standard Deviation were considered biofilm-positive.

2.5. Safety

The CZE and CIEF runs dealt with separation of potentially pathogenic microorganisms from risk group 2 of infectious agents. These pathogens can cause human or animal disease but, under normal circumstances, they are unlikely to be seriously hazardous to laboratory personnel. Laboratory exposures rarely cause infection leading to serious disease; effective treatment and preventive measures are available, and the risk of spread is limited. Therefore, biosafety level 2 is necessary to maintain. In accord with the regulations, protective gloves were worn and the inner and outer surface of the capillaries, as well as any material contaminated by the microorganisms, were disinfected using ethanol or other disinfecting solutions, such as per acetic acid.

2.6. CZE and CIEF instrumentation, procedures

CZE and CIEF experiments were carried out using the laboratory-made apparatus [39] at constant voltage (−) 20 kV on the side of the detector supplied by high voltage unit Spellman CZE 1000 R (Plainview, NY, USA). The lengths of the fused silica capillaries (FS), 0.1 mm I.D. and 0.25 mm O.D. (Agilent Technologies, Santa Clara, CA, USA) were 350 mm, from 150 to 200 mm to the detector. The ends of the fused silica capillary were dipped in 3 mL-glass vials with the background electrolytes, BGE. During the CIEF experiments, the current decreased from 40 to 60 μA at the beginning of the experiment down to 3 or 6 μA at the time of detection, depending on the sampling time interval and the sample solution.

The on-column UV–vis detector LCD 2082 (Ecom, Prague, Czech Republic) used in the experiment was connected to the detection cell by optical fibers (Polymicro Technologies, Phoenix, AZ, USA) 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 sample injection was performed by siphoning action achieved by elevating of the inlet reservoir on the side of the anode relative to the outlet reservoir, side of the cathode. The yeast clusters were either left intact, or were de-agglomerated by sonication of the microbial suspension in the ultrasound bath Sonorex (Bandelin electronic, Berlin, Germany) before injection of the cells into the capillary. The sonication was performed for 1 min at the temperature 25 °C and at frequency 35 kHz. Between the separation runs the sample suspensions were vortexed by TTS 3 digital yellow^{line} (IKA works, Wilmington, DE, USA). The detector signals were acquired and processed with the Chromatography data station Clarity (DataApex, Prague, Czech Republic).

In the CIEF experiment, sodium hydroxide and H_3PO_4 were used as the catholyte or the anolyte, respectively, with addition of ethanol (EtOH), PEG 10,000, PEG 4000 or Brij 35. In the CZE phosphate buffer (pH 8.4) with addition of EtOH, PEG 4000 or PEG 10,000 were used as BGE. Before each injection the capillaries were rinsed with EtOH for 5 min, and then back-flushed with the catholyte (CIEF) or phosphate buffer (CZE) for 2 min. The rinsing procedures were carried out hydrodynamically.

The CIEF separation was carried out using the segmental injection of the sample pulse into the capillary [38,39]. The sample was injected in three parts-segment of the spacers, solution of the selected simple ampholytic electrolytes, 15×10^{-5} mol L^{-1}, dissolved in 2×0^{-2} mol L^{-1} NaOH, segment of the sample mixture of yeast (see Section 2.2) and the segment of the mixture of 5% (w/v) water solution of commercial carrier ampholytes, Biolyte, pH 3–10, ampholyte pH 2–4 and pH 3–4.5 and low-molecular p*I* markers (0.5–1.0 mg mL^{-1}) for the tracing of the used pH gradients. The blood serum used at the separation of the real samples was from St. Anna University Hospital (Brno, Czech Republic). The detailed compositions of a BGE and a sample pulse used in the CIEF and CZE are shown in Table 1.

The height differences of the reservoirs at the injection of the segments were 100 mm, the injection time, $t_{inj.}$, of the segment of spacers were from 15 to 25 s, the sample segment, 10–16 s (35–65 nL), and segment of carrier ampholytes and p*I* markers, 35–45 s.

3. Results and discussion

Initial CIEF experiments were conducted with biofilm-negative *C. orthopsilosis* (strain BC7) and both biofilm-negative and biofilm-positive species of *C. metapsilosis* (strains PH87 and PH86) and *C. parapsilosis*, (strains BC8 and BC12) on the basis of our earlier published results [25]. The separation of the similar yeasts was achieved with a pH gradient in the range from 3.3 to 4.7. Further details regarding the separation conditions are found in Table 1 and Refs. [29,38,50–53]. Under these experimental conditions for all tested *Candida* species, only two peaks were detected: one at an isoelectric point of 3.6 for biofilm-positive strains and one at an isoelectric point of 3.8 for the biofilm-negative strains, similar to results in Refs. [25,30]. The p*I* values of the tested yeast strains were calculated from the migration times of the appropriate p*I* markers and their isoelectric points. As the resolution of p*I* markers differing by one tenth of a p*I* unit is sufficiently large, the ability to monitor differences of isoelectric points of a few hundredths of a p*I* unit are possible). According to Refs. [34,56], resolution and precision of 0.1 pH unit can be routinely achieved, whereas a resolution of 0.01 pH unit could be possible only under certain conditions. In earlier experiments [25], each strain was tested fourteen times and the relative standard deviations (RSD's) in the migration times were always under 1.6%. In subsequent CIEF experiments reported in this paper, see Figs. 1–5, the goal involved identification of optimal composition of the sample pulse and BGE to separate tested

Table 1
Composition of the background electrolytes and sample pulse at CIEF and CZE.

pH gradient pH range	Background electrolytes		Sample pulse		
			CIEF		
	Catholyte	Anolyte	Segment of the simple ampholytic electrolytes	Sample segment	Segment of commercial carrier ampholytes and p*I* markers
	Additives				
3.3–4.7 [25]	4×10^{-2} mol L^{-1} NaOH	0.1 mol L^{-1} H_3PO_4	Nic, Asp, Glu, Mes, Aces, MOPS, TAPSO, MAA, HEPES [25,39]	8×10^7 cells mL^{-1} in 3% (v/v) EtOH 2% (w/v) PEG 10,000, 15×10^{-3} mol L^{-1} NaCl	The ratio of carriers: 1:1:5 p*I* markers, p*I* values 2.7, 3.3, 3.7, 3.9, 4.0, 4.7
	0.6% (w/v) PEG 10,000 and 1% (v/v) EtOH				
3.7–4.0	2×10^{-2} mol L^{-1} NaOH	0.1 mol L^{-1} H_3PO_4	Glu, Asp, HEPES	1×10^7 cells mL^{-1} in 3% (v/v) EtOH 2% (w/v) PEG 10,000 15×10^{-3} mol L^{-1} NaCl	The ratio of carriers: 1:1:6 p*I* markers, p*I* values 3.7, 3.9, 4.0
	0.1% (w/v) PEG 10 000 and 1% (v/v) EtOH				
3.3–3.7					The ratio of carriers: 1:2:5 p*I* markers, p*I* values 3.3, 3.7
3.8–4.0	2–4 $\times 10^{-2}$ mol L^{-1} NaOH	0.1 mol L^{-1} H_3PO_4	Tart, Mal, Form, Suc, Acet, Piv, Glu, Nic	4–8 $\times 10^7$ cells mL^{-1} in 15×10^{-3} mol L^{-1} NaCl or 0.3% (w/v) PEG 10,000 and PSS or blood serum	The ratio of carriers: 1:3:4 p*I* markers, p*I* values 3.9, 4.0
	0.3% (w/v) PEG 10,000 or 0.3% (w/v) Brij 35, 1 or 3% (v/v) EtOH				
3.6–4.0	4×10^{-2} mol L^{-1} NaOH	0.1 mol L^{-1} H_3PO_4		8×10^7 cells mL^{-1} 1% (w/v) PEG 4000 15×10^{-3} mol L^{-1} NaCl	The ratio of carriers: 1:3:5 p*I* markers, p*I* values 3.7, 3.9, 4.0
	0.3 or 0.6% (w/v) PEG 4000 and 1% (v/v) EtOH				
			CZE		
	1×10^{-2} mol L^{-1} phosphate buffer (pH 8.4)	8×10^7 cells mL^{-1} PSS with or without addition of 1% (w/v) PEG 10,000			
	0.6% (w/v) PEG 4000 or 0.3% (w/v) PEG 10,000 and 3% (v/v) EtOH				

yeast species from one another more precisely for reliable strain identification.

3.1. CIEF in the pH gradients within the pH range 3.7–4.0 and 3.3–3.7

In the subsequent experiments three biofilm-positive and/or three biofilm-negative strains of each of the studied *Candida* species, see Section 2.2, were independently separated by CIEF or by CZE for the comparison of their isoelectric points or for assessment of their migration times. In order to maintain the clarity the following text and images the code designation of the *Candida* strains were not used. However, for comparison in all figures the strains used at the separation in Fig. 1 are depicted.

In Fig. 1A each of the tested strains was separated by CIEF in the pH gradient in the pH range 3.7–4.0, see Table 1. Different from the preliminary CIEF experiments – 2×10^{-2} mol L^{-1} NaOH was used as the catholyte and in both electrolytes, in the catholyte and the anolyte, 0.1% (w/v) PEG 10,000 was dissolved. According to the required pH gradient in the acidic pH range [38,39] the spacer segment was composed only of Glu, Asp and HEPES. Based upon the results of the preliminary experiments, the ratio of carrier ampholytes Biolyte, pH 3–10, ampholyte pH 2–4 and pH 3–4.5 in the segment was increased for ampholyte pH 3.0–4.5 to 1:1:6. As a result, there was an increase in migration times and slight indication of the separation of the studied *Candida* species between the p*I* markers p*I* 3.7 and 3.9.

Similarly, at the separation of the biofilm-positive yeasts the ratio of the carrier ampholytes was changed to 1:2:5 in behalf of ampholyte 2–4. Two peaks between the p*I* markers 3.3 and 3.7 were detected, see Fig. 1B. The isoelectric points for biofilm-negative and biofilm-positive *C. parapsilosis* were again determined as 3.8 and 3.6. The approximate values of p*I's* for both biofilm-negative *C. orthopsilosis* and *C. metapsilosis* and biofilm-positive *C. metapsilosis* were found higher by several hundredths of pH unit, see Fig. 1A and B. The differences between the p*I*'s calculated from at least five measurements were found less than 0.03 units of pH.

3.2. CIEF in the pH gradients within the pH range 3.8–4.0

In order to minimize the error resulting from the curvature of the pH gradient [33,34,37] two appropriate p*I* markers, p*I* 3.9 and 4.0, were added to the segment of the carrier ampholytes. Furthermore, strains of biofilm-negative *C. parapsilosis,* the isoelectric point of which has repeatedly been determined as 3.8 [25,30], were used for the tracing of the narrow pH gradient at

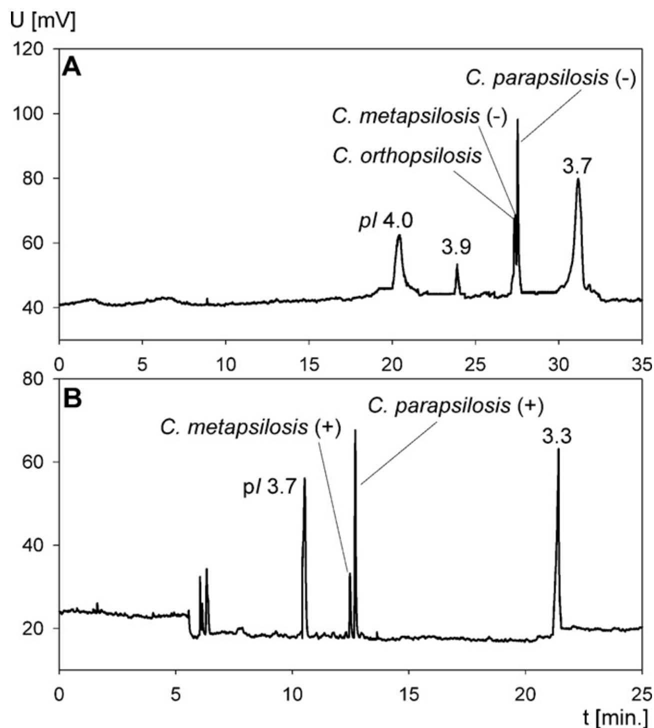

Fig. 1. Separation of the *Candida* species by CIEF with UV detection in the pH gradient pH range 3.7–4.0 (A, biofilm-negative) and 3.3–3.7 (B, biofilm-positive). A and B – see Table 1; FS capillary 200 mm to the detection cell; *t* [min], the migration time.

the separation of the biofilm-negative species, *C. orthopsilosis* and *C. metapsilosis*. The ratio of commercial carrier ampholytes in their segment was changed to 1:3:4 and the spacer segment was composed of more acidic compounds [38,39], see Table 1. In both, the catholyte (4×10^{-2} mol L^{-1} NaOH) and the anolyte 1% (v/v) EtOH was only dissolved.

In addition to the peaks established for *C. parapsilosis* (No. 3 in Fig. 2a–c), two peaks corresponding to biofilm-negative *C. orthopsilosis* (No. 1 in Fig. 2a) and to *C. metapsilosis* (No. 2 in Fig. 2b) were detected and their isoelectric points were determined around 3.83

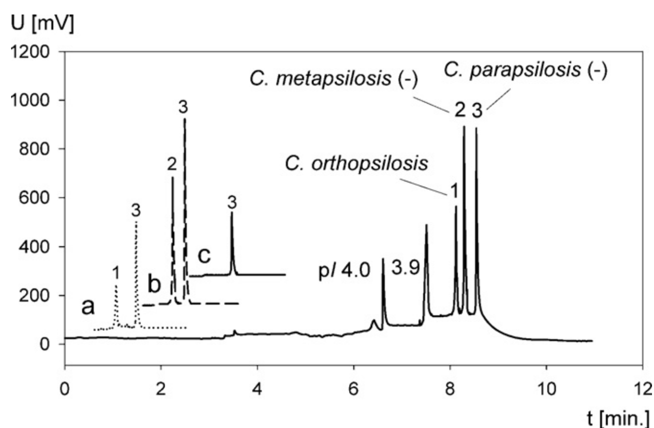

Fig. 2. Optimization of the CIEF of the biofilm-negative *Candida* species, pH gradient pH range 3.8–4.0, with UV detection. Conditions and designations, see Fig. 1 and Table 1; the catholyte, 4×10^{-2} mol L^{-1} NaOH, 1% (v/v) EtOH, were dissolved in both, the catholyte and the anolyte; the sample of yeasts – 8×10^{7} cells mL^{-1}, except 4×10^{7} cells mL^{-1} of *C. orthopsilosis* (peak No. 1) and 6×10^{7} cells mL^{-1} of *C. parapsilosis* (peak No. 3) in a; 6×10^{7} cells mL^{-1} of *C. metapsilosis* (peak No. 2) in b; 3×10^{7} cells mL^{-1} of *C. parapsilosis* (peak No. 3) in c, were re-suspended in water solution of 15×10^{-3} mol L^{-1} NaCl; FS capillary 150 mm length to the detection cell; t_{inj} – the spacer segment, 15 s, the segment of the sample, 16 s, carrier ampholytes and p*I* markers together, 40 s.

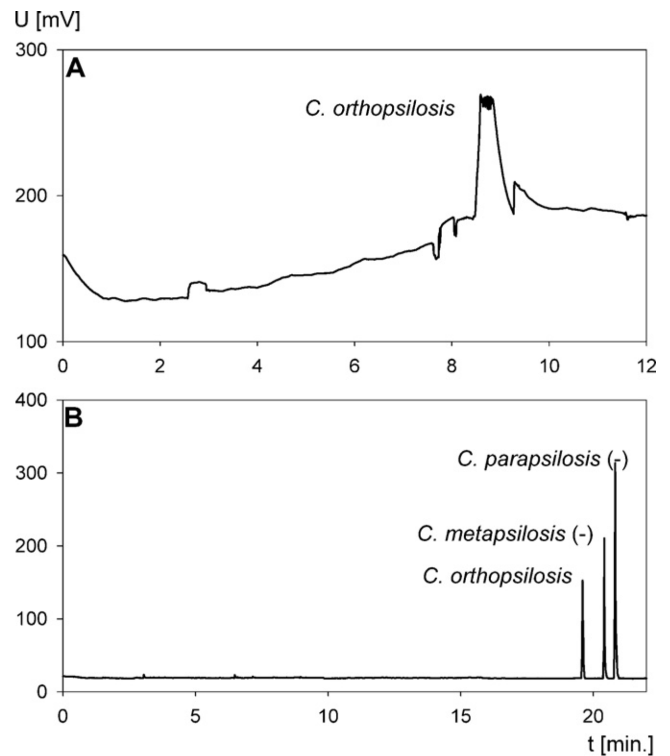

Fig. 3. CIEF of the biofilm-negative *Candida* species, pH gradient pH range 3.8–4.0, with UV detection re-suspended in PSS. Conditions and designations, see Fig. 2 and Table 1; the catholyte, 2×10^{-2} mol L^{-1} NaOH, 3% (v/v) EtOH, (A) 0.3% (w/v) PEG 10,000 or (B) 0.3% (w/v) Brij 35 were dissolved in both, the catholyte and the anolyte; the samples of yeasts were re-suspended in PSS and 0.3% (w/v) PEG 10,000; t_{inj} – the spacer segment, (A) 15 s, (B) 25 s, the segment of the sample, 10 s; (A) sample of *C. orthopsilosis* was not sonicated before injection.

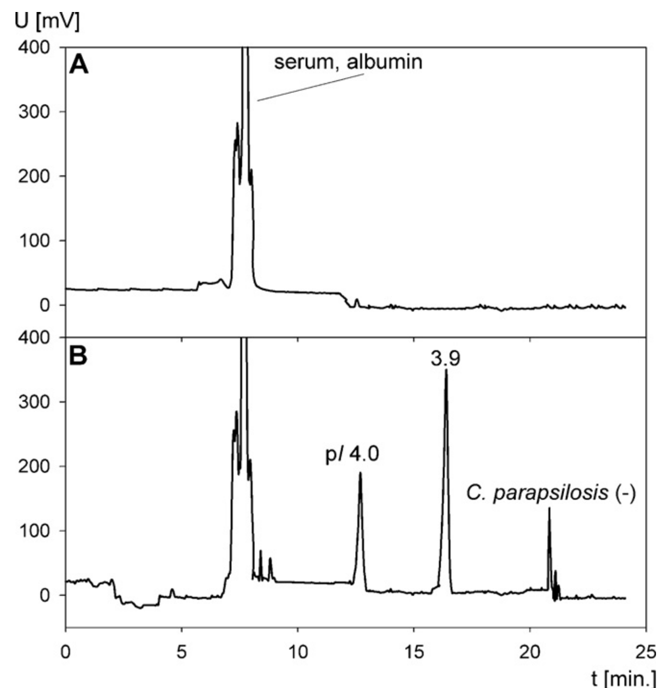

Fig. 4. CIEF of the biofilm-negative *C. parapsilosis* in the blood serum, pH gradient pH range 3.8–4.0, with UV detection. Conditions and designations, see Figs. 2 and 3 and Table 1; the catholyte, 2×10^{-2} mol L^{-1} NaOH, 0.3% (w/v) Brij 35 was dissolved in both, the catholyte and the anolyte; the samples of yeasts – (B) 3×10^{7} cells mL^{-1} of *C. parapsilosis*, biofilm-negative were re-suspended in blood serum; t_{inj} – the spacer segment, 25 s, the segment of the sample, 10 s.

Table 2
The date were obtained from the separations depicted in Fig. 2[a] or in Fig. 5A[b].

No. of measurement	Migration times, and p*I*'s of *Candida* species									
	Biofilm-negative						Biofilm-positive			
	orthopsilosis		*metapsilosis*		*parapsilosis*		*metapsilosis*		*parapsilosis*	
	t [min]	p*I*	*t* [min]	p*I*	*t* [min]	p*I*	*t* [min]	p*I*	*t* [min]	p*I*
1	7.99	3.83	8.16	3.81	8.37	3.80	12.41	3.63	12.84	3.60
2	8.19	3.83	8.41	3.81	8.61	3.80	12.38	3.63	12.78	3.60
3	7.96	3.84	8.17	3.82	8.35	3.81	12.25	3.62	12.68	3.59
4	8.18	3.83	8.39	3.81	8.59	3.80	12.08	3.63	12.41	3.60
5	7.98	3.82	8.19	3.80	8.38	3.79	12.30	3.63	12.74	3.60
6	8.14	3.83	8.35	3.81	8.55	3.80	12.12	3.64	12.50	3.61
7	8.21	3.83	8.41	3.81	8.63	3.80	12.21	3.63	12.65	3.60
8	8.22	3.84	8.40	3.82	8.62	3.81	12.18	3.63	12.56	3.60
9	7.96	3.83	8.17	3.81	8.34	3.80	12.05	3.64	12.46	3.61
10	8.05	3.83	8.30	3.81	8.51	3.80	12.01	3.63	12.41	3.60
Mean *t*	8.09	3.83	8.29	3.81	8.50	3.80	12.19	3.63	12.60	3.60
RSD (%)	1.4	0.01	1.3	0.01	1.6	0.01	1.4	0.01	1.6	0.01

Repeatabilities of the migration times and p*I* of *Candida* species, biofilm-negative[a] and biofilm-positive[b], by their CIEF separation

and 3.81, respectively, for all strains of particular species, see Fig. 2. Although shorter capillary was used here for the separation of monitored yeasts better resolution at the same maximum RSD's of the migration times and isoelectric points (under 1.6 or 0.01%, respectively) was achieved for at least ten measurements for each studied strain, see Table 2. Similarly to the p*I* values of p*I* markers given in Section 2.1, here given p*I* values of yeasts bear the error of several hundredths of p*I* unit.

The future practical application of this technique depends on whether it can be used for the separation of phenotypically similar species directly from complex matrices, e.g. body fluids. For very simplified demonstration of the physiological environment the yeast samples were re-suspended directly in PSS (Fig. 3A and B) or in the blood serum (Fig. 4A and B). The composition of the electrolytes had to be optimized by adding 3% (v/v) EtOH and 0.3% (w/v) PEG 10,000 into the catholyte (2×10^{-2} mol L^{-1} NaOH) and the anolyte.

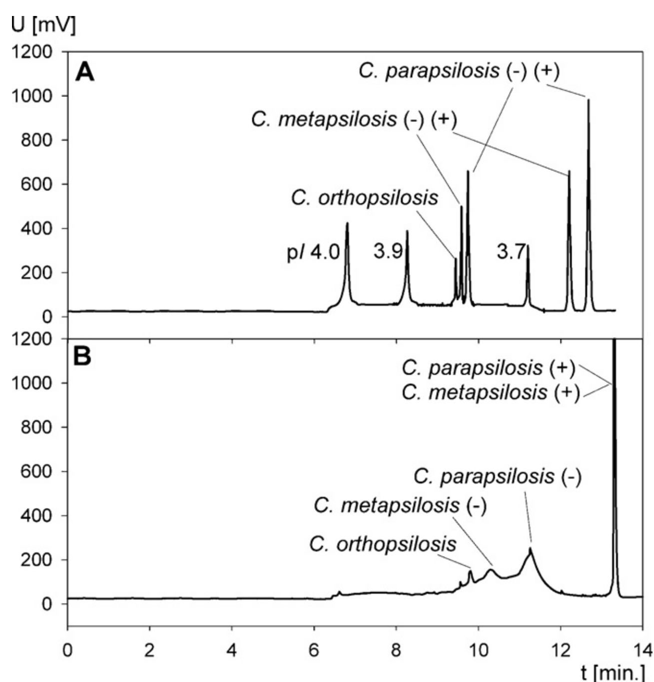

Fig. 5. CIEF of both, biofilm-positive and biofilm-negative species of *Candida* in the pH gradient in the pH range 3.6–4.0. Conditions and designations, see Fig. 2A and Table 1; (A) 0.3 and (B) 0.6% (w/v) PEG 4000; t_{inj} – the spacer segment, 25 s, the segment of the sample, 16 s.

A pre-requisite for successful separation of cells is appropriate sample preparation. In the experiments in Fig. 3A *C. orthopsilosis* was re-suspended in PSS with dissolved 0.3% (w/v) PEG 10,000 and the microbial sample was not sonicated before injection, the clusters were not de-agglomerated. Therefore, the cells and their agglomerates were focused and detected separately.

Simultaneously, reduction of the electroosmotic flow due to the addition of electrolyte additives influenced the migration time of *C. orthopsilosis.* Chloride ions are responsible for the high sample conductivity and undesirable dispersion. In general, the problem of enormous zone dispersion may be reduced by transient isotachophoretic migration mode [57] which is integrated into the focusing process. It is necessary to set the experimental conditions that would allow the separation of the biofilm-negative yeasts without the undesirable dispersion. Therefore, PEG 10,000 from the catholyte and the anolyte was replaced by the non-ionogenic tenside Brij 35 in the next experiment, see Fig. 3B. For better clarity the pH gradient was not traced here by the p*I* markers. The identical separation conditions were used for separating blood serum, see Fig. 4A, leading to the detection of a significant peak of serum albumin (p*I* around 4.7). In Fig. 4B *C. parapsilosis* (−), 3×10^7 cells mL^{-1}, was re-suspended in the blood serum and sonicated before injection. The pH gradient was traced by the p*I* markers p*I* 3.9 and 4.0. Narrow peaks of the separated cells were now detected. The migration time of *C. parapsilosis* (−) correspond to with its migration time in Fig. 3B like as the migration time calculated for the peak of the p*I* marker p*I* 3.9. However, the linearity of the pH gradient above pH 4 estimated from p*I* of albumin from blood serum is not maintained as we can see from Fig. 4B.

3.3. CIEF of examined yeasts species in the pH gradient within the pH range 3.6–4.0

The separation of the five very similar *Candida* species can serve as an illustrative example that a slight change in the composition of the electrolytes can affect the CIEF separation of *Candida*, see Table 1. In the first experiment, the catholyte and the anolyte were modified by adding 0.3% (w/v) PEG 4000 together with 1% (v/v) EtOH; the dilution of PEG 4000 was doubled to 0.6% in the second experiment. The composition of the segment of carrier ampholytes was optimized to 1:3:5. The pH gradient in the selected pH range was traced by the p*I* markers p*I* 3.7, 3.9 and 4.0 together with the biofilm-positive *C. parapsilosis* in which the isoelectric point was repeatedly determined as 3.6 [25]. The isoelectric point about 3.63 was determined for biofilm-positive strains of *C. metapsilosis* correspond to the findings in Fig. 1B. RSD's of the migration times under

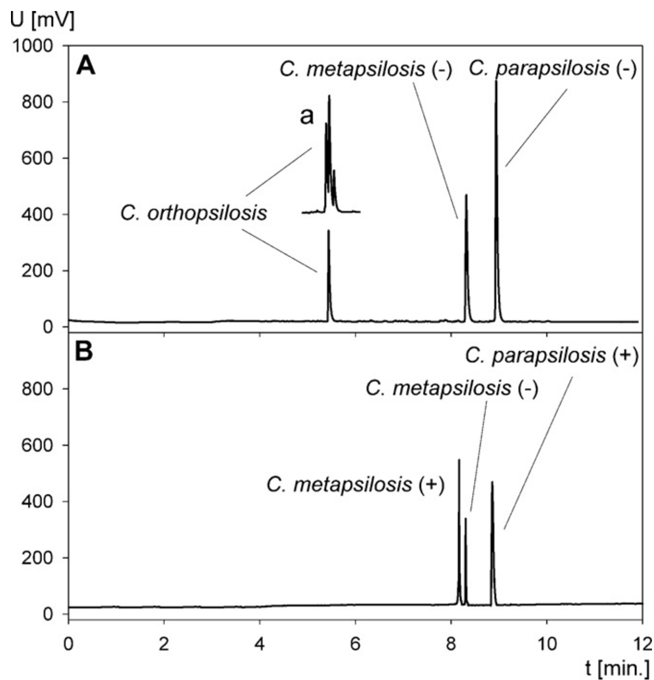

Fig. 6. CZE of *Candida* species, biofilm-negative (A and B) and biofilm-positive (B), with UV detection. Conditions and designations, see Fig. 2A and Table 1; (A) 0.6% (w/v) PEG 4000 and (B) 0.3% (w/v) PEG 10,000; sample of yeasts without (A) or with (B) the addition of 1% (w/v) PEG 10,000; time of sonication before injection, 1 min (A and B) or without sonication (Aa); t_{inj}, 16 s.

1.6% were also achieved for at least ten measurements for each studied strain, see Table 2.

Similarly as in Ref. [30] quantitative reproducibility of the measurements was found with the correlation coefficient, $R = 0.99$ for the dependence of the peak areas on the number of injected cells of the *Candida* species for all of studied strains. Therefore, comparing the electropherograms illustrates that the resolution of the biofilm-negative *Candida* species in Fig. 2 is better than in Fig. 5A. It may be assumed that the lower peak areas of the monitored analytes in Fig. 5A than in Fig. 2 were caused by the partial adsorption of the cells onto the capillary wall during their CIEF. After increase in PEG 4000 concentration in the catholyte and the anolyte the migration times of the biofilm-negative biofilm-positive strains of the studied species were increased, the strains of the biofilm-negative species were almost completely adsorbed onto the capillary and the strains of the biofilm-positive species were not separated. For more clarity the pH gradient was not traced by the p*I* markers.

3.4. CZE of Candida species

The separation of the biofilm-negative *Candida* species by CZE, see Table 1, is depicted in Fig. 6A. Agglomerates of yeast are necessarily disrupted by sonication before separation similar to the analysis by CIEF. Otherwise, they are separated by different fractions of the sample according to the agglomerate surface charge, see Fig. 6Aa. Based on the results depicted in this Fig. 6A similarity was found between *C. metapsilosis* and *C. parapsilosis* migration velocities compared to the greater migration velocity of *C. orthopsilosis* for all studied strains. A similar sequence of peaks was found also by CIEF. The finding is probably related to stronger tendency of *C. metapsilosis* strains to form biofilm than that of *C. parapsilosis.* Simultaneous separation of biofilm-positive and biofilm-negative strains of *C. metapsilosis* and biofilm-positive *C. parapsilosis* was achieved from a single mixture, see Fig. 6B. However, the separation of the biofilm-positive strains in enabled by adding 1% PEG 10,000 into the sample, see Table 1; otherwise, only a single peak was detected.

4. Conclusions

The phenotypically indistinguishable species of biofilm-negative *C. orthopsilosis* and both biofilm-negative and biofilm-positive *C. metapsilosis* and *C. parapsilosis* are possible to separate by capillary electromigration techniques. At CIEF the examined yeast species were separated according to their isoelectric points, which differ by down to few hundredths of the isoelectric point units for both biofilm-negative or biofilm-positive strains, respectively. Careful control of experimental conditions, the composition of the sample pulse and the catholyte and the anolyte, have been adapted for the separation purposes. Small changes in the optimized conditions influence the linearity of the pH gradient in the reference pH range or peak shapes. The properly chosen separation conditions allow us to separate the biofilm-negative *C. parapsilosis* from the blood serum. In the CZE separation, the peaks of the biofilm-negative or biofilm-positive *Candida* species are probably organized according to the physical–chemical similarity of the observed yeasts, *C. metapsilosis* vs. *C. parapsilosis* and their ability to form the biofilm. All results have been verified on three strains of each studied *Candida* species and their biofilm-positive and biofilm-negative variants. The suggested electromigration techniques can be useful at the rapid separation and identification of the cultivated phenotypically indistinguishable species in future thanks to the reproducibility of the migration times and good separation of the microorganisms.

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References

[1] D. Campa, A. Tavanti, F. Gemignani, C.S. Mogavero, I. Bellini, F. Bottari, R. Barale, S. Landi, S. Senesi, J. Clin. Microbiol. 46 (2008) 209.
[2] S.A. Messer, R.N. Jones, T.R. Fritsche, J. Clin. Microbiol. 44 (2006) 1782.
[3] L.N. Miranda, I.M. van der Heijden, S.F. Costa, A.P.I. Sousa, R.A. Sienra, S. Gobara, C.R. Santos, R.D. Lobo, V.P. Pessoa Jr., A.S. Levin, J. Hosp. Infect. 72 (2009) 9.
[4] A. Tavanti, A.D. Davidson, N.A. Gow, M.C. Maiden, F.C. Odds, J. Clin. Microbiol. 43 (2005) 284.
[5] S. Kocsube, M. Toth, C. Vagvolgyi, I. Doczi, M. Pesti, I. Pocsi, J. Szabo, J. Varga, J. Med. Microbiol. 56 (2007) 190.
[6] A. Gomez-Lopez, A. Alastruey-Izquierdo, D. Rodriguez, B. Almirante, A. Pahissa, J.L. Rodriguez-Tudela, M. Cuenca-Estrella, Antimicrob. Agents Chemother. 52 (2008) 1506.
[7] A.L. Tavanti, A. Hensgens, E. Ghelardi, M. Campa, S. Senesi, J. Clin. Microbiol. 45 (2007) 1455.
[8] E. van Asbeck, K.V. Clemons, M. Martinez, A.J. Tong, D.A. Stevens, Diagn. Microbiol. Infect. Dis. 62 (2008) 106.
[9] F. Růžička, V. Holá, M. Votava, R. Tejkalová, Folia Microbiol. 52 (2007) 209.
[10] M. Tumbarello, B. Posteraro, E.M. Trecarichi, B. Fiori, M. Rossi, R. Porta, K. de Gaetano Donati, M. La Sorda, T. Spanu, G. Fadda, R. Cauda, M. Sanguinetti, J. Clin. Microbiol. 45 (2007) 1843.
[11] J.W. Song, J.H. Shin, D.H. Shint, S.I. Jung, D. Cho, S.J. Kee, M.G. Shin, S.P. Suh, D.W. Ryang, Med. Mycol. 43 (2005) 657.
[12] P.F. Lehmann, D.M. Lin, B.A. Lasker, J. Clin. Microbiol. 30 (1992) 3249.
[13] S. Iida, T. Omak, T. Oguri, K. Okuzumi, A. Yamanaka, M.L. Moretti-Branch, K. Nishimura, Y. Mikami, Nippon Ishinkin Gakkai Zasshi 46 (2005) 133.
[14] M. Kato, M. Ozeki, A. Kikuchi, T. Kanbe, Gene 272 (2001) 2751.
[15] D.M. Lin, L.C. Wu, M.G. Rinaldi, P.F. Lehmann, J. Clin. Microbiol. 33 (1995) 1815.
[16] J. Nosek, L. Tomaska, A. Rycovska, H. Fukuhara, J. Clin. Microbiol. 40 (2002) 1283.
[17] E.C. van Asbeck, K.V. Clemons, A.N. Markham, D.A. Stevens, Mycoses 52 (2009) 493.
[18] H. Mirhendi, B. Bruun, H.C. Schønheyder, J.J. Christensen, K. Fuursted, B. Gahrn-Hansen, H.K. Johansen, L. Nielsen, J.D. Knudsen, M.C. Arendrup, J. Med. Microbiol. 59 (2010) 414.
[19] R. Plachý, P. Hamal, V. Raclavský, J. Microbiol. Methods 60 (2005) 107.

[20] G. Marklein, M. Josten, U. Klanke, E. Muller, R. Horre, T. Maier, T. Wenzel, M. Kostrzewa, G. Bierbaum, A. Hoerauf, H.-G. Sahl, J. Clin. Microbiol. 47 (2009) 2912.
[21] E. Kenndler, D. Blaas, TrAC Trends Anal. Chem. 20 (2001) 543.
[22] D.W. Armstrong, G. Schulte, J.M. Schneiderheinze, D.J. Westenberg, Anal. Chem. 71 (1999) 5465.
[23] V. Košťál, E.A. Arriaga, Electrophoresis 29 (2008) 2578.
[24] M.J. Desai, D.W. Armstrong, Microbiol. Mol. Biol. Rev. 67 (2003) 38.
[25] M. Horká, F. Růžička, V. Holá, K. Šlais, Electrophoresis 30 (2009) 2134.
[26] E. Kłodzinska, M. Szumskia, K. Hrynkiewicz, E. Dziubakiewicz, M. Jackowski, B. Buszewski, Electrophoresis 30 (2009) 3086.
[27] B. Buszewski, M. Szumskia, E. Kłodzinska, R. Jarmalaviciene, A. Maruska, J. Chromatogr. A 1216 (2009) 6146.
[28] Z. Liu, S.S. Wu, J. Pawliszyn, J. Chromatogr. A 1140 (2007) 213.
[29] M. Horká, F. Růžička, V. Holá, K. Šlais, Electrophoresis 28 (2007) 2300.
[30] F. Růžička, M. Horká, V. Holá, A. Kubesová, T. Pavlík, M. Votava, J. Microbiol. Methods 80 (2010) 299.
[31] M.N. oses, P.G. Rouxhet, J. Microbiol. Methods 6 (1987) 99.
[32] B.A. Jucker, H. Harms, A.J.B. Zehnder, J. Bacteriol. 178 (1996) 5472.
[33] K. Shimura, Electrophoresis 30 (2009) 11.
[34] P.G. Righetti, J. Chromatogr. A 1037 (2004) 491.
[35] M. Conti, C. Gelfi, A.B. Bosisio, P.G. Righetti, Electrophoresis 17 (1996) 1590.
[36] S. Molteni, H. Frischknecht, W. Thormann, Electrophoresis 15 (1994) 22.
[37] K. Shimura, W. Zhi, H. Matsumoto, K. Kasai, Anal. Chem. 72 (2000) 4747.
[38] M. Horká, F. Růžička, J. Horký, V. Holá, K. Šlais, J. Chromatogr. B 841 (2006) 152.
[39] M. Horká, F. Růžička, V. Holá, K. Šlais, Anal. Bioanal. Chem. 385 (2006) 840.
[40] Z. Zhao, A. Malik, M.L. Lee, Anal. Chem. 65 (1993) 2747.
[41] M. Huang, M. Bigelow, M. Byers, LC–GC Internat. 9 (1996) 658.
[42] X. Ren, P.Z. Liu, M.L. Lee, J. Microcolumn. Sep. 8 (1996) 529.
[43] D. Corradini, J. Chromatogr. B 699 (1997) 221.
[44] T. Rabilloud, Electrophoresis 17 (1996) 813.
[45] E. Szökö, Electrophoresis 18 (1997) 74.
[46] X.-W. Yao, F.E. Regnier, J. Chromatogr. 632 (1993) 1853.
[47] A. Roosjen, H.J. Karper, H.C. van der Mei, W. Norde, J. Busscher, Microbiology 149 (2003) 3239.
[48] A. Razatos, Y.L. Org, F. Boulay, D.L. Elbert, J.A. Hubell, M.M. Sharma, G. Georgiou, Langmuir 16 (2000) 155.
[49] H.J. Kaper, H.J. Busscher, W. Norde, J. Biomater. Sci. Polym. Ed. 14 (2003) 313.
[50] M. Šťastná, K. Šlais, Anal. Bioanal. Chem. 382 (2005) 65.
[51] T. Hirokawa, M. Nishino, N. Aoki, Y.K.T.Y. Sawamoto, J.-I. Akiyama, J. Chromatogr. A 271 (1983) D1.
[52] F. Acevedo, J. Chromatogr. A 545 (1991) 391.
[53] M. Šťastná, M. Trávníček, K. Šlais, Electrophoresis 26 (2005) 53.
[54] M. Dendis, R. Horváth, J. Michálek, F. Ruzicka, M. Grijalva, M. Bartos, J.P. Benedik, Clin. Microbiol. Infect. 9 (2003) 1191.
[55] J.H. Shin, S.J. Kee, M.G. Shin, S.H. Kim, D.H. Shin, S.K. Lee, S.P. Suh, D.W. Ryan, J. Clin. Microbiol. 40 (2002) 1244.
[56] H. Kobayashi, M. Aoki, M. Suzuki, A. Yanagisawa, E. Arai, J. Chromatogr. A 772 (1997) 137.
[57] L. Křivánková, P. Pantůčková, P. Gebauer, P. Boček, J. Čáslavská, W. Thormann, Electrophoresis 24 (2003) 505.