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# Identification of bacterial uropathogens by preparative isoelectric focusing and MALDI-TOF mass spectrometry

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ABSTRACT

This study describes a new method for simultaneous identification of uropathogens in the case of polybacterial urinary tract infections. The method utilizes recently developed preparative isoelectric focusing (IEF) in cellulose-based separation medium with a subsequent analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Preparative IEF was successfully used for both purification and separation of bacteria, *Escherichia coli* (p*I* 4.6) and *Staphylococcus aureus* (p*I* 3.4), in urine samples. The focused zones of bacteria, localized by the positions of focused colored p*I* markers, were easily collected from the separation media after the IEF analysis and then unambiguously identified by MALDI-TOF MS. The proposed method enables the identification of bacteria in urine specimens when the concentration of individual bacteria is $\geq 10^4$ cells $mL^{-1}$. Another benefit is the viability of bacteria extracted from the collected fractions after preparative IEF.



## 1. Introduction

Urinary tract infections (UTIs) are the most common human infections caused by the presence of bacterial or fungal pathogens in the urinary tract [1,2]. Although the vast majority of UTIs are caused by a single microorganism, the infections may also be polymicrobial [3]. *Escherichia coli* is the most prevalent causative agent of uncomplicated UTIs accounting for about 80% of all infections [2,4]. Other common urinary tract pathogens include *Staphylococcus saprophyticus*, *Klebsiella* species, *Proteus mirabilis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Candida* species [2,5,6]. In addition to these species, an increasing prevalence of *Staphylococcus aureus* in UTIs has been reported recently [7]. UTI can manifest a wide range of symptoms from bacteriuria with limited clinical symptoms to a severe sepsis or septic shock. Therefore, early and accurate identification of the uropathogen (confirmation of UTI is defined as $\geq 10^5$ colony-forming units in 1 mL of urine) is necessary for a timely and appropriate treatment of the infection. Several tests, such as Gram staining, cytometry, microscopy, and urine dipstick testing, are used to make a preliminary diagnosis of UTI [3,8,9]. Bacteriological urine culture is still the "gold standard" for diagnosis; however, this method is time-consuming and expensive.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a fast and reliable technique for identification of various microorganisms including pathogenic bacteria in clinical microbiology laboratories [10–12]. Several MALDI-based methods for identification of pathogens in urine samples have been published in recent years. The suggested methods include various sample preparation steps to get rid of interfering components before MALDI-TOF MS analysis [13–18]. Other methods combine traditional techniques with MALDI-TOF MS providing more reliable pathogen identification [19–22]. However, all these methods often fail in the case of a polymicrobial infection. Moreover, it is difficult to remove all the interfering non-microbial proteins from urine samples, which also limits the MALDI-TOF MS-based identification of uropathogens. In this respect, a proper separation technique could improve the identification ability of the MALDI-TOF MS-based methods.

Since the microorganisms carry charged or chargeable groups on their outer surface, they can be easily separated using electrophoretic techniques [23–27]. In particular, isoelectric focusing (IEF) is suitable technique for both separation and pre-concentration of microorganisms [28–30]. The IEF separates amphoteric analytes according to their different isoelectric points (p*I*) and it also increases concentration of the separated analytes

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during focusing. The bioparticles were separated mainly by capillary format of IEF (CIEF) [29,30]. The major problem regarding CIEF separations is that microbial cells have a strong tendency to form aggregates and to interact with the inner surface of the separation capillary. Nevertheless, several approaches based on the use of different additives (e.g., poly(ethylene oxide) or various detergents), either into the electrophoretic buffers or into the microbial samples, have been proposed to solve this problem [26,27,29]. Although CIEF represents an efficient analytical technique for analysis of various microorganisms, its wider application is limited by the very small volume of the sample injected into the separation capillary. Therefore, CIEF is not suitable for preparative purposes.

The objective of this study was to assess the ability of a new method, a combination of the recently developed preparative IEF in a cellulose-based separation medium [31] with a MALDI-TOF MS analysis, to identify pathogens in urine specimens. The separation medium is composed of cellulose, simple buffers, ethylene glycol, glycerol, nonionic surfactant, and colored p*I* markers. Fibres of the cellulose are approximately 10–20 μm in diameter and 200 μm in length; therefore, the effective pore can be expected to be large enough so particles (e.g., bacterial cells) of μm's dimensions can move independently of their size in the separation medium. In addition, cellulose is inert to the separated analytes and the surfactant prevents the analytes from the aggregation and from their adsorption to the wall of the separation trough. Preparative IEF is a simple method that offers several benefits over other methods: an easy and rapid preparation of the separation medium, good separation efficiency, convenient fraction collection, and easy processing of the collected fractions. This was proved by the analysis of protein samples in our previous study [31]. In this study, preparative IEF was used as a sample preparation technique in the identification of uropathogens by MALDI-TOF MS. For this purpose, urine samples were spiked with *E. coli* and *S. aureus*, significant causative agents of UTIs, and used as model samples simulating infected urine specimens.

## 2. Materials and methods

### 2.1. Chemicals

Sodium chloride, ethylene glycol, glycerol, triton X-100, α-cellulose powder (part No. C8002, average fibre length 200 μm), and amphoteric compounds were purchased from Sigma-Aldrich (Schnelldorf, Germany). Specification and concentrations of the individual amphoteric compounds used as simple buffers in the separation medium is given in Supplementary material (Table S1). Acetonitrile (ACN) was purchased from Fisher Scientific (London, UK). Trifluoroacetic acid (TFA) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). 3,5-Dimethoxy-4-hydroxycinnamic acid (SA) and protein calibration mixture ProMix2 were purchased from LaserBio Labs (Sophia-Antipolis Cedex, France). Colored low-molecular-mass p*I* markers, p*I* 2.0 (green), 2.6 (orange), 3.9 (orange), 5.3 (lavender), 6.2 (red), 7.2 (yellow), 8.0 (orange), 9.0 (yellow), and 10.1 (violet), were developed and synthesized at the Institute of Analytical Chemistry of the CAS, v. v. i. [32–36]. All chemicals were of analytical or MS grade.

### 2.2. Bacterial strains and growth conditions

The bacterial strains used in this study, *E. coli* CCM 3954 and *S. aureus* CCM 4750, were obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). Urine samples from healthy volunteers were tested for the absence of microbes at the Department of Microbiology, St. Anne's University Hospital (Brno, Czech Republic). The strains were stored at −70 °C in Itest cryotubes (ITEST plus, Czech Republic). Before the experiments, the strains were thawed quickly at 37 °C and cultivated on Mueller-Hinton agar (Oxoid, United Kingdom) at 37 °C for 24 h. The cultivated bacteria were suspended in deionized water and the cell concentration was adjusted to $1 \times 10^6$ cells $mL^{-1}$ for MALDI-TOF MS analysis. For the preparative IEF experiments, bacterial suspension in physiological saline solution (PSS) or urine samples spiked with the bacteria were used. The concentrations of bacteria in the samples were in the range of $1 \times 10^3$ to $1 \times 10^9$ cells $mL^{-1}$. When the urine sample contained both types of bacteria, their concentrations were always equal. The concentration of bacterial cells in the suspensions was estimated by the measurement of the optical density of the suspensions using a spectrophotometer at 550 nm, according to the calibration curve, which was defined by reference samples. The number of bacterial cells in the reference samples was measured by serial dilution and plating of 100 μL of the suspension on nutrient agar. After cultivation at 37 °C for 24 h, the colonies were counted. Bacteria collected after IEF were cultured aerobically on Columbia agar base (Oxoid) containing 7% (v/v) defibrinated sheep blood at 37 °C for 24 h.

### 2.3. Safety considerations

Potentially pathogenic bacteria, *E. coli* and *S. aureus*, from risk group 2 of infectious agents were analyzed in this study. Although these pathogens can cause a human disease, they are unlikely to be seriously hazardous to laboratory personnel under normal circumstances. Laboratory exposures rarely cause an infection leading to a serious disease; eff ;ective treatment and preventive measures are available, and the risk of spreading is limited. Therefore, biosafety level 2 needs to be maintained.

### 2.4. Preparative IEF

The preparative IEF device was described in our recent studies [31,32,37]. Briefly, an empty V-shaped plastic trough (17.5 cm long, 0.8 cm high and 1.5 cm wide) was positioned on a power supply and fixed by inserting the working electrodes into the trough. First, 0.8 mL of cellulose-based separation medium [31] was loaded uniformly into the trough, then 30 μl of the p*I* markers solution (p*I* 2.0, 2.6, 3.9, 5.3, 6.2, 7.2, 8.0, 9.0, and 10.1, each of them 5 μg $mL^{-1}$) was loaded into the central third of the trough. The power supply was turned on and the trough was covered to retard the evaporation of water from the separation medium. After two hours, 100 μL of the sample was loaded into the central third of the trough. The sample was either bacterial cells suspended in PSS or the urine sample spiked with the bacteria. A 2-h delay was found necessary for the survival of the bacteria as estimated by the subsequent cultivation of bacterial cells extracted from the collected fractions. The sample was either a cultivated bacterium suspended in PSS or a urine sample spiked with *E. coli*, *S. aureus* or both of them. The IEF device was left running for additional 14 h (overnight) and then the fractions, defined by the positions of colored p*I* markers, were collected and analyzed by MALDI-TOF MS.

### 2.5. MALDI-TOF mass spectrometry

With respect to the cultivated bacteria, 20 μL of the bacterial suspension was centrifuged at 6000 g for 4 min, the supernatant was discarded and the pellet was resuspended in 20 μL of SA solution (20 mg $mL^{-1}$ in ACN/0.1% TFA, 3:2, v/v). Each fraction collected from the IEF trough was suspended in 200 μL of deionized water, the suspension was sonicated for 3 min, then vortexed for 5 min and finally centrifuged at 1000 g for 5 min. The supernatant, containing bacterial cells, was collected and further centrifuged at 6000 g for 4 min. The resulting supernatant was discarded and the pel-

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