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Exploring the possibilities of infrared spectroscopy for urine sediment examination and detection of pathogenic bacteria in urinary tract infections

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Abstract

Objectives: In this study, the possibilities of Fourier-transformed infrared spectroscopy (FTIR) for analysis of urine sediments and for detection of bacteria causing urinary tract infections (UTIs) were investigated.

Methods: Dried urine specimens of control subjects and patients presenting with various nephrological and urological conditions were analysed using mid-infrared spectroscopy (4,000–400 cm⁻¹). Urine samples from patients with a UTI were inoculated on a blood agar plate. After drying of the pure bacterial colonies, FTIR was applied and compared with the results obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Chemometric data analysis was used to classify the different species.

Results: Due to the typical molecular assignments of lipids, proteins, nucleic acids and carbohydrates, FTIR was able to

identify bacteria and showed promising results in the detection of proteins, lipids, white and red blood cells, as well as in the identification of crystals. Principal component analysis (PCA) allowed to differentiate between Gram-negative and Gram-positive species and soft independent modelling of class analogy (SIMCA) revealed promising classification ratios between the different pathogens.

Conclusions: FTIR can be considered as a supplementary method for urine sediment examination and for detection of pathogenic bacteria in UTI.

Keywords: crystals; Fourier-transformed infrared spectroscopy; lipids; soft independent modelling of class analogy; urinary tract infections; urine sediments.

Introduction

Urine sediment examination is an integral part of urinalysis and is used worldwide for diagnosing diseases of the urinary tract [1, 2]. Today, in most clinical laboratories in the developed world, it is mainly performed by automated sediment or flow cytometric analysers [3, 4]. However, when an advanced level of urine sediment examination is required, the use of dedicated microscopy is recommended.

Attenuated total-reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) is a promising analytical method, which is based upon changes in permanent dipole moments during excitation in the mid-infrared region (4,000–400 cm⁻¹). It is a label-free method that can obtain information on the chemical composition of complex biological samples [5]. In this technique, a beam of infrared (IR) light penetrates a particle beyond the reflecting surface and subsequently loses energy at the wavelength absorbed by the sample. The attenuated total reflection is recorded as a function of wavelength, which gives rise to the characteristic spectrum, which can be analysed [5]. The technique has been proven to be useful in a variety of clinical diagnostic and monitoring applications, and can be used to complement traditional laboratory-based analytical devices in a

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compact way with extremely low costs per test [5–7]. FTIR spectroscopy offers qualitative, quantitative and multi-component analyses using biochemical information of molecular composition with a minimum amount of sample and a reduced time needed for sample preparation, even at remote locations [5].

The composition of urine is hypothetically dominated by urea and creatinine [8]. IR spectra of urine samples show distinctive peaks for urea at $1,592\text{ cm}^{-1}$ (C=O stretching vibrations), $1,457\text{ cm}^{-1}$ (C–N stretching vibrations) and $1,154\text{ cm}^{-1}$ (NH_2 deformation vibrations), and weak intensity peaks at $1,334$ and $1,237\text{ cm}^{-1}$ are assigned to creatinine [8]. The amide I band at $1,655\text{ cm}^{-1}$ (mainly C=O stretching vibrations) and amide II band at $1,525\text{ cm}^{-1}$ (N–H bending and C–H stretching vibrations) of the proteins in the spectra of the urine samples are overlapping in the region from $1,500$ to $1,700\text{ cm}^{-1}$ [5, 9]. In case of proteinuria, the absorbance of these typical protein bands increases [10].

The aims of the present study were to explore the possibilities of IR spectroscopy for the examination of urine sediments and for the detection of bacteria causing urinary tract infections (UTIs). Special attention was paid to analysis of elements, where urinary flow cytometry (UFC) is not able to provide clinical information (differentiation of crystals, detection of lipid particles and pure UTI bacterial species).

Materials and methods

Sample preparation

Leukocytes ($n=2$) and hemoglobin ($n=3$) were obtained from ethylenediaminetetraacetic acid (EDTA) blood samples. Leukocytes isolation was done by red blood cell (RBC) lysing based on a difference in osmotic pressure using the erythrocyte lysis (EL) buffer (QIAGEN, Hilden, Germany), while leukocytes remained intact. Hemoglobin preparation was performed by lysing RBCs. After centrifugation (10 min, room temperature [RT], $2095 \times g$), the plasma layer and buffy coat of the EDTA blood samples were removed. Hemoglobin was present in the upper phase after three washing cycles with 5 mL phosphate buffered saline (PBS) pH 7.4 (Thermo Fischer Scientific, Waltham, MA, USA) and three freeze (-80°C)-thaw cycles. Leukocytes and hemoglobin were dried using the Savant SpeedVac Concentrator (Fischer Scientific) (RT, 1 h). A hemoglobin human standard (P/N H7379, lot SLBR9638V) (Sigma Aldrich, Sint Louis, MO, USA) was used.

Urine samples from patients with pyuria (>25 white blood cells [WBCs]/ μL , $n=10$) and hematuria (>25 RBC/ μL , $n=10$) were collected. Five urine samples coming from different healthy subjects were used as controls. The preparation of each urine sample was done in five-fold ($n=25$). Five milliliters urine was transferred into a 15 mL conical centrifuge Falcon tube (Fisher Scientific, Hampton, NH, USA). After

two centrifugation steps, first by using the Allegra X-15R (Beckman Coulter, Brea, CA, USA) (10 min, RT, $1439 \times g$), followed by the Microfuge 18 Centrifuge (Beckman Coulter) (10 min, RT, $669 \times g$), the majority of the supernatant was removed. The precipitate was subsequently dried in a Savant SpeedVac Concentrator (Fischer Scientific) in combination with a refrigerated vapor trap and a vacuum pump (RT, 1 h).

In addition, urine samples from 147 patients with a urinary tract infection caused by a single bacterial species were collected. The following Gram-negative and Gram-positive bacterial pathogens were isolated: *Escherichia coli* (*E. coli*) ($n=15$), *Klebsiella pneumonia* (*K. pneumonia*) ($n=15$), *Klebsiella oxytoca* (*K. oxytoca*) ($n=15$), *Klebsiella aerogenes* (*K. aerogenes*) ($n=7$), *Enterobacter cloacae* (*E. cloacae*) ($n=11$), *Proteus mirabilis* (*P. mirabilis*) ($n=15$), *Pseudomonas aeruginosa* (*P. aeruginosa*) ($n=15$), *Serratia marcescens* (*S. marcescens*) ($n=3$) and *Citrobacter species* ($n=12$) (Gram-negative bacteria); *Enterococcus faecalis* (*E. faecalis*) ($n=15$), *Enterococcus faecium* (*E. faecium*) ($n=15$), *Staphylococcus saprophyticus* (*S. saprophyticus*) ($n=2$) and *Staphylococcus aureus* (*S. aureus*) ($n=7$) (Gram-positive bacteria). All urine samples with the above mentioned causative microorganisms were inoculated using the Walk Away Specimen Processor (WASP) Lab (Copan, Murietta, CA, USA) [11]. The obtained bacteria were subcultured from the Baltimore Biological Laboratory™ (BBL™) Trypticase™ Soy Agar (TSA) II with 5% sheep blood (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) to a fresh agar plate with the same growth medium. The following American type culture collection (ATCC) strains ($n=5$) were inoculated onto the BD™ TSAII with 5% Sheep Blood (BD): *S. aureus* (ATCC 29213, Manassas, VA, USA), *E. coli* (ATCC 25922), *K. pneumonia* (ATCC 700603), *P. aeruginosa* (ATCC 27853) and *E. faecalis* (ATCC 29212). After overnight incubation (Binder GmbH, Tuttlingen, Germany) at 35°C , quality control was assessed by identification of the subcultures and ATCC strains, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) biotyper system (Microflex LT [Bruker, Billerica, MA, USA]) [12]. The bacteria were harvested by carefully scraping the agar plate and transferred to a 2 mL Eppendorf tube (Eppendorf, Hamburg, Germany). The bacteria were dried using the Savant SpeedVac Concentrator (Fischer Scientific) (RT, 1 h).

Urine samples of patients presenting with nephrotic syndrome and lipiduria ($n=5$) were also collected. The alinity c (Abbott, Chicago, IL, USA) assayed cholesterol photometrically by adding 5% Triton X-100 (cholesterol oxidase method) to the urine specimen [13].

Crystal analysis was performed using the pure IR spectra of crystals ($n=4$) presented in the instrument's spectral library (Spectrum 10 software [Perkin Elmer, Waltham, MA, USA]) of the Department of Clinical Chemistry of the Ghent University Hospital, Belgium.

The approval of this study was granted by the Ethical Committee of the Ghent University Hospital (EC2019/1379) on Dec 13, 2019.

Infrared analysis

FTIR spectra of urine sediments and cultured bacteria were obtained using a Perkin Elmer Spectrum Two ATR-FTIR spectrometer (Perkin Elmer), fitted with the universal ATR Accessory (zinc selenide [ZnSe] crystal) and Spectrum 10 software (Perkin Elmer) in the range from $4,000$ to 400 cm^{-1} at a spectral resolution of 4 cm^{-1} (five coadded scans). In order to preprocess the acquired spectral data, several spectral filters were employed to standardize the spectroscopic signals

and to remove irrelevant scatter light using soft independent modeling of class analogy (SIMCA) version 15 software (MKS Data Analytics Solutions, Malmö, Sweden) [7]. The transform techniques (normalizing, differentiating, smoothing and centering) can be used to resolve and locate overlapping bands in complex spectra. In the first derivative, resolution is enhanced as the rate of change of absorbance (A) in function of the wavelength (λ) is examined ($dA/d\lambda$). The second derivative measures alterations in the rate of change of absorbance ($d^2A/d\lambda^2$) [5].

Three models were investigated: M_1 =all Gram-negative ($n=111$) and Gram-positive bacteria ($n=41$), including the ATCC strains; M_2 =Gram-positive bacteria model, namely *E. faecalis* and corresponding ATCC strain ($n=16$), and *E. faecium* ($n=15$); and M_3 =Gram-negative bacteria model with *E. coli* and corresponding ATCC strain ($n=16$), *K. pneumonia* and corresponding ATCC strain ($n=16$), *K. oxytoca* ($n=15$), *P. mirabilis* ($n=15$), and *P. aeruginosa* and corresponding ATCC strain ($n=16$). For the evaluation of the predictive properties of the different models (M_1 , M_2 and M_3), each dataset was divided into a training and test set (external validation). The samples were randomly appointed to the training set (70% of all samples of M_1 and M_2 ; and 85% of all samples of M_3) and test set (30% of all samples of M_1 and M_2 ; and 15% of all samples of M_3). To ensure the inclusion of all samples in the test set at least once, the same procedure was repeated respectively 5 (M_1 and M_2) and 6 (M_3) times with different compositions of both sets [7].

An overview of the data is given by an unsupervised pattern recognition method, namely principal component analysis (PCA) [14, 15]. The multivariate projection method is designed to reduce the dimensionality of data from hundreds of spectral data points into a few orthogonal principal components (PCs). The differences between the bacterial data and clustering of the samples were investigated by PCA analysis to simplify the creation of classification models [7, 14]. SIMCA is a supervised classification technique that models each class of samples separately by defining a number of PCs, which are derived from PCA. By using a cross-validation procedure, SIMCA defines the optimal number of PCs required to describe each training class individually. Classification rules are composed by taking two critical values into account, namely the Euclidean distances towards the SIMCA model (e. g. orthogonal distances) and the Mahalanobis distances, calculated in the space of the scores. Those distances measure the boundaries of the model, determining a limited space around the samples in one specific class. The unknown samples (test set) are assigned to that defined class if the object is situated within the restricted space of the training class [7, 14].

The cholesterol spectrum, as well as the pure IR spectra of the most common crystal forming substances, namely cystine, calcium oxalate monohydrate, ammonium urate and magnesium ammonium phosphate (struvite) were present in the instrument's spectral library Spectrum 10 software (Perkin Elmer).

Statistical analysis

Statistical analysis was carried out using MedCalc version 19 (MedCalc Software, Ostend, Belgium) to assess normality (D'Agostino-Pearson test). To investigate correlations between non-normal continuous variables, Spearman's rho (ρ) was calculated. A p-value < 0.05 was considered *a priori* to be statistically significant.

Results

The mean normalized IR spectra of urine sediments of control subjects, patients with hematuria (mean WBC: 12/ μ L, mean RBC: 182/ μ L) and patients with pyuria (mean WBC: 2,513/ μ L, mean RBC: 65/ μ L) are shown in Figure 1. Distinctive peaks were observed at 1,592 cm^{-1} (urea), 1,457 cm^{-1} (urea), 1,334 cm^{-1} (creatinine), 1,237 cm^{-1} (creatinine) and 1,154 cm^{-1} (urea). The amide I (1,655 cm^{-1}) and II (1,525 cm^{-1}) bands were overlapping in the region from 1,500 to 1,700 cm^{-1} . Table 1 gives an overview of the typical raw IR spectral bands of urine sediment particles. All characteristic molecular assignments (stretching and bending vibrations, symmetrical and asymmetrical vibrations) were retrieved in the dried suspension of isolated hemoglobin and leukocytes (Figure 1). Between the spectra of red blood cells (hemoglobin) and leukocytes, differences were noticeable. One of the significant candidate wavenumbers corresponding to hematuria was 1,768 cm^{-1} as a positive correlation was observed with the standard normal variate (SNV) normalized IR spectrum for the number of RBCs in the urine ($r=0.7090$, $p=0.0334$) (Figure 2A). Significant candidate wavenumbers corresponding to pyuria, namely for the mean centered data, normalized using SNV and converted to the first derivative with a Savitzky-Golay algorithm (9 smoothing points) (Figure 2B), were 1,050 cm^{-1} ($r=0.7580$, $p=0.0230$) and 1,402 cm^{-1} ($r=-0.9150$, $p=0.0060$). For the mean centered data, normalized using SNV and converted to the second derivative with a Savitzky-Golay algorithm (11 smoothing points) (Figure 2C), wavenumbers 1,136 cm^{-1} ($r=0.8790$, $p=0.0084$) and 1,222 cm^{-1} ($r=-0.9030$, $p=0.0067$) were identified as potential discriminative wavenumbers.

The mean normalized IR spectra of urine sediments of control subjects, cholesterol standard, urine sediments with lipid particles (cholesterol concentration of 70 mg L^{-1}) are shown in Figure 3. Lipids were detected at 3,000–2,800, 1,750 and 1,450 cm^{-1} .

The normalized IR spectra of following crystals, namely magnesium ammonium phosphate, cystine, calcium oxalate monohydrate, ammonium urate and drug-induced kidney stone [6] are shown in Figure 4.

Raw absorbance spectra showed high level of similarity between Gram-negative and Gram-positive bacteria (Figure 5, upper spectra). PCA was applied in order to evaluate spectral differences between Gram-negative ($n=111$) and Gram-positive bacteria ($n=41$). Most obvious spectral differences for M_1 were found in the range from 2,970 to 2,820 and 1,772 to 420 cm^{-1} for the mean centered

Figure 1: Mean normalized infrared (IR) spectrum of urinary sediment of control subjects, patients with hematuria and patients with pyuria.

Distinctive peaks were present at $1,592\text{ cm}^{-1}$ (urea), $1,457\text{ cm}^{-1}$ (urea), $1,334\text{ cm}^{-1}$ (creatinine), $1,237\text{ cm}^{-1}$ (creatinine) and $1,154\text{ cm}^{-1}$ (urea). The amide I ($1,655\text{ cm}^{-1}$) and amide II ($1,525\text{ cm}^{-1}$) bands were overlapping in the region from $1,510$ to $1,750\text{ cm}^{-1}$. A dried suspension of hemoglobin (blue line) and isolated leukocytes (green line) was added to the figure. Typical molecular assignments of a biological IR spectrum were indicated on the IR spectrum of hemoglobin and the leukocytes: V=stretching vibrations; δ =bending vibrations; s=symmetrical vibrations and as=asymmetrical vibrations.

data, normalized using SNV and converted to the second derivative with a Savitzky-Golay algorithm (11 smoothing points) (Figure 5A). The score plot showed a clear distinction between the Gram-negative and Gram-positive bacteria (Figure 5B). SIMCA was applied to test whether reliable classification models could be constructed based on the $2,970\text{--}2,820\text{ cm}^{-1}$ and $1,772\text{--}420\text{ cm}^{-1}$ spectral range. A cross-validation procedure showed a 100% classification ratio. External validation resulted in a classification ratio of 97.3%. A second classification model M_2 was developed based on the spectral differences from $1,200$ to 900 cm^{-1} for the mean centered data, normalized using SNV and converted to the second derivative with a Savitzky-Golay algorithm (11 smoothing points) to distinguish the following species of Gram-positive bacteria: *E. faecalis* and *E. faecium* (Figure 6B). The cross-validation procedure showed a 100% classification ratio. External validation resulted in a classification rate of 90.3%. A third classification model M_3 was based on spectral differences from $1,500$ to $1,100\text{ cm}^{-1}$ for the mean centered data, normalized using SNV, converted to the first derivative with a Savitzky-Golay algorithm (9 smoothing points) to distinguish the following species of Gram-negative bacteria: *E. coli*, *K. pneumonia*, *K. oxytoca*, *P. mirabilis* and *P. aeruginosa* (Figure 6A). The cross-validation procedure showed a 96.2% classification ratio. External validation resulted in a classification rate of 74.4%. Identification of all bacteria and ATCC strains were MALDI-TOF MS confirmed with high-confidence identification score values.

Discussion

The present study demonstrates for the first time that IR spectroscopy can be used to study urine sediments and to identify pathogenic bacteria in patients with urinary tract infections. Significant differences in the mean SNV normalized IR spectra of patients presenting with pyuria and hematuria were observed. More in detail, two wavenumbers in the first-order derivative IR spectrum ($1,050$ and $1,402\text{ cm}^{-1}$) and two in the second-order derivative IR spectrum ($1,136$ and $1,222\text{ cm}^{-1}$) were identified in samples with pyuria. The wavenumbers of the first-order IR spectrum are part of the region of symmetrical CO–O–C stretching vibrations of carbohydrates and of the region of symmetrical CH_2 bending vibrations of lipids, respectively. The wavenumbers of the second-order derivative IR spectrum belong to the region of the (a)symmetric phosphate stretching vibrations of the nucleic acids [5]. In contrast to WBCs, no nucleic acids are present in the RBCs [16]. The RBC membrane is a two-layered structure, composed of a cytoskeleton and a lipid bilayer. The lipid bilayer includes various types of phospholipids, sphingolipids, cholesterol and integral membrane proteins [17]. The identified wavenumber $1,768\text{ cm}^{-1}$ represents the ester carbonyl region of the lipids [5, 18].

In urine sediments, lipids may appear as cholesterol crystals, fatty casts, oval fat bodies or as free lipid droplets. This finding is of importance in the case of nephrotic syndrome, where the presence of lipid-containing particles are characteristic of the disease [1, 19]. As lipids are insoluble

Table 1: Urine sediment particles and their typical raw infrared (IR) spectral bands.

Particle	Typical spectral bands (cm ⁻¹) and remarks
Cellular elements	
Leukocytes [5]	Lipids: 2,960 cm ⁻¹ : asymmetrical CH ₃ stretching vibrations; 2,926 cm ⁻¹ : asymmetrical CH ₂ stretching vibrations; 2,854 cm ⁻¹ : symmetrical CH ₂ stretching vibrations; 1,750 cm ⁻¹ : symmetrical C=O stretching vibrations; 1,450 cm ⁻¹ : symmetrical CH ₂ bending vibrations Proteins: 1,655 cm ⁻¹ : amide I band; 1,525 cm ⁻¹ : amide II band, 1,394 cm ⁻¹ : symmetrical COO ⁻ stretching vibrations Nucleic acids: 1,230 cm ⁻¹ : asymmetrical PO ₂ ⁻ stretching vibrations; 1,150 cm ⁻¹ : symmetrical PO ₂ ⁻ stretching vibrations Carbohydrates: 1,060 cm ⁻¹ : symmetrical CO–O–C stretching vibrations
Red blood cells/hemoglobin [5]	Lipids: 2,960 cm ⁻¹ : asymmetrical CH ₃ stretching vibrations; 2,926 cm ⁻¹ : asymmetrical CH ₂ stretching vibrations; 2,854 cm ⁻¹ : symmetrical CH ₂ stretching vibrations; 1,750 cm ⁻¹ : symmetrical C=O stretching vibrations; 1,450 cm ⁻¹ : symmetrical CH ₂ bending vibrations Proteins: 1,655 cm ⁻¹ : amide I band; 1,525 cm ⁻¹ : amide II band, 1,394 cm ⁻¹ : symmetrical COO ⁻ stretching vibrations Carbohydrates: 1,060 cm ⁻¹ : symmetrical CO–O–C stretching vibrations
Microorganisms	
Bacteria (Gram-negative and Gram-positive bacteria) [5, 23]	Same spectral bands as leukocytes, with remarkable higher absorbance in the carbohydrate region
Protein	
Protein [5, 18]	1,655 cm ⁻¹ : amide I band; 1,525 cm ⁻¹ : amide II band, 1,394 cm ⁻¹ : symmetrical COO ⁻ stretching vibrations
Crystals	
Cystine [18]	3,406 cm ⁻¹ : NH group and/or OH vibrations; 3,000 cm ⁻¹ : vibrations of the hydrogen-bonded NH groups and CH/CH ₂ groups; 2,596 cm ⁻¹ : SH group; 1,617 cm ⁻¹ and 1,580 cm ⁻¹ : antisymmetric vibrations of the CO groups; 1,500–1,200 cm ⁻¹ : symmetrical vibrations of the CO groups; 454 cm ⁻¹ : S–S group
Calcium oxalate [18]	3,477–3,047 cm ⁻¹ : OH stretching vibrations of the hydrate water; 1,615 cm ⁻¹ : antisymmetric stretching vibrations with shoulder at 1,655 cm ⁻¹ ; 1,316 cm ⁻¹ : symmetrical CO stretching vibration; 947 and 882 cm ⁻¹ : OH libration vibrations; 780 and 517 cm ⁻¹ : CO deformation vibration; 657 and 593 cm ⁻¹ : OH libration vibrations
Ammonium urate [18]	3,005 cm ⁻¹ : combination of OH, NH, C–NH and CH vibrations; 1,666 cm ⁻¹ : CO band (Amide I); 1,600–1,200 cm ⁻¹ : number and relative intensities are in range below 800; 599 cm ⁻¹
Magnesium ammonium phosphate [18]	2,320 cm ⁻¹ : marked decrease in intensity due to OH stretching vibrations; 2,910 cm ⁻¹ : minimum of broad band due to superimposed OH and NH stretching vibrations; 1,616 cm ⁻¹ : OH deformation vibration of water of crystallization; 1,465 and 1,430 cm ⁻¹ : ammonium ion; 998 cm ⁻¹ : minimum of a strong band assigned to the stretching vibration of the phosphate group; 890 cm ⁻¹ ; 756 cm ⁻¹ ; 571 cm ⁻¹ : deformation vibration of the PO ₄ group
Lipiduria	
Cholesterol [18]	3,800–3,060 cm ⁻¹ ; 3,010 cm ⁻¹ : C–H stretch; 2,954 cm ⁻¹ , 2,926 cm ⁻¹ and 2,852 cm ⁻¹ : C–H stretching in CH ₃ and CH ₂ ; 2,868 cm ⁻¹ ; 1,736 cm ⁻¹ : C=O stretching in esters; 1,630 cm ⁻¹ : amide I; 1,540 cm ⁻¹ : amide II; 1,516 cm ⁻¹ ; 1,470 cm ⁻¹ ; 1,452 cm ⁻¹ : CH ₂ bending; 1,390 cm ⁻¹ ; 1,340 cm ⁻¹ ; 1,310 cm ⁻¹ ; 1,236 cm ⁻¹ : amide III, 1,164 cm ⁻¹ , 1,108 cm ⁻¹ and 1,070 cm ⁻¹ : C–O–C stretching in esters; 1,046 cm ⁻¹ ; 1,030 cm ⁻¹ ; 938 cm ⁻¹ ; 924 cm ⁻¹ ; 846–400 cm ⁻¹

in water, conventional quantitative chemical analysis of lipids in native urine is not easy [20]. Urine flow cytometry does neither allow detection nor qualification of these particles [4]. As lipid particles showed a well-defined IR spectrum with typical characteristics (absorbance at 3,000–2,800, 1,750 and 1,450 cm⁻¹), the quantitative

determination of lipids in urine sediments is an advantage of the IR spectroscopic technique [5, 18]. The lipid bands were dominated by the (a)symmetrical CH₂ and CH₃ stretching vibrations (3,000–2,800 cm⁻¹), symmetrical C=O stretching vibrations (1,750 cm⁻¹) and symmetrical CH₂ bending vibrations (1,450 cm⁻¹) [5, 18].

Figure 2: Infrared (IR) spectrum of hematuria and pyuria.

(A) Mean standard normal variate (SNV) normalized IR spectrum of hematuria and pyuria. Zoomed IR spectrum of the significant candidate wavenumber, 1768 cm^{-1} , for number of RBCs in urine samples with hematuria is shown on the right. (B) Mean spectral differences in the first derivative of pyuria and hematuria. Zoomed first-order derivative IR spectrum of the significant candidate wavenumbers, 1050 and 1402 cm^{-1} , for number of WBCs in urine samples with pyuria is shown on the right. (C) Mean spectral differences in the second derivative of pyuria and hematuria. Zoomed second-order derivative IR spectrum of the significant candidate wavenumbers, 1136 and 1222 cm^{-1} , for number of WBCs in urine samples with pyuria is shown on the right.

Figure 3: Mean normalized infrared (IR) spectrum of urinary sediments of control subjects, cholesterol standard (spectrum is derived from the instrument's spectral library) and urine samples with lipid particles. Typical molecular lipid assignments of a biological IR spectrum are indicated: V: stretching vibrations; δ : bending vibrations; s: symmetrical vibrations; as: asymmetrical vibrations.

Physiological and pathological urine crystals are characterized by unique IR spectra, which can be easily distinguished. Unknown (drug) crystals can be compared with data from a spectral library [6]. In urine flow cytometry, sample dilution and heating of the samples to 37°C have a negative effect on crystal analysis [3].

Gram staining is normally used to classify bacteria, based on their cell wall composition. Gram-positive bacteria have cell walls of several layers of peptidoglycan containing chains of teichoic acid. In contrast, Gram-negative cell walls are composed of a single thin layer of peptidoglycan and an outer membrane with

Figure 4: Normalized infrared (IR) spectrum of drug-induced kidney stone (sulfapyridine/acetyl-sulfapyridine) [6], magnesium ammonium phosphate, cystine, calcium oxalate monohydrate and ammonium hydrogen urate (spectra derived from the instrument's spectral library).

Figure 5: Model M_1 .

(A) Mean spectral differences in the second derivative from 2,970 to 2,820 cm^{-1} and from 1,772 to 420 cm^{-1} between Gram-negative bacteria (green line) and Gram-positive bacteria ($n=41$, blue line). (B) Score plot showing clustering of the Gram-negative bacteria (green dots) and Gram-positive bacteria (blue dots), based on principal component analysis in the 2,970–2,820 cm^{-1} and 1,772–420 cm^{-1} spectral range.

lipopolysaccharide components. The periplasmic space is situated between the cell wall and plasma membrane. The outer membrane with lipopolysaccharide molecules is attached to the cell wall by murein lipoproteins. No differentiation of bacteria on species level can be made using this staining technique [21]. As raw data of Gram-negative and Gram-positive bacteria showed a high level similarity,

chemometric data analysis was necessary to unveil spectral differences (M_1) in our study. Spectral differences were revealed in the range from 2,970 to 2,820 cm^{-1} and 1,772 to 420 cm^{-1} (second-order derivative), which covers all important molecular assignments of a biological IR spectrum for lipids, proteins, nucleic acids and carbohydrates of Gram-negative and Gram-positive bacteria [5]. IR data of

Figure 6: Model M_2 and M_3 .

The upper Figure (A) shows the mean standard normal variate (SNV) normalized absorbance spectra for Gram-negative bacteria (M_3), namely *E. coli* (black line), *K. pneumoniae* (blue line), *K. oxytoca* (red line), *P. mirabilis* (yellow line) and *P. aeruginosa* (green line) and (B) shows the mean SNV normalized absorbance spectra for Gram-positive bacteria (M_2), namely *E. faecalis* (black line) and *E. faecium* (blue line). Typical molecular assignments of a biological infrared (IR) spectrum are indicated on the mean SNV normalized IR spectra: V: stretching vibrations; δ : bending vibrations; s: symmetrical vibrations; as: asymmetrical vibrations. The lower Figure (A) shows the first derivative of the mean spectra of the Gram-negative bacteria in the spectral range from 1,500 to 1,100 cm^{-1} and (B) shows the second derivative of the mean spectra of the Gram-positive bacteria in the spectral range from 1,200 to 900 cm^{-1} .

most common pathogenic Gram-positive (*E. faecalis* and *E. faecium*: M_2) and Gram-negative (*E. coli*, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis* and *P. aeruginosa*: M_3) bacteria for urinary tract infections were classified on species level using SIMCA. The spectral range from 1200 to 900 cm^{-1} covered the region of symmetrical CO–O–C stretching vibrations of carbohydrates due to the presence of peptidoglycans for the classification of the Gram-positive bacteria (second-order derivative). Classification of the Gram-negative bacteria (first-order derivative) was done using the spectral region from 1,500 to 1,100 cm^{-1} , which covered symmetrical CH_2 bending vibrations of lipids, symmetrical COO^- stretching vibrations of proteins, (a) symmetrical PO_2^- stretching vibrations of nucleic acids and a part of the symmetrical CO–O–C stretching vibrations of carbohydrates. The results of the external validation were respectively 97.3% (M_1), 90.3% (M_2) and 74.4% (M_3), which were lower regarding the cross-validation results (M_1 : 100%, M_2 : 100% and M_3 : 96.2%), especially for the Gram-negative bacteria. This is probably due to the limited amount of urine samples, which gave an underestimated classification ratio.

In conclusion, the present study highlights the diagnostic possibilities of FTIR spectroscopy for analysis of urine sediments, detection of lipiduria and differentiation

of cultured bacteria. In order to analyse the IR spectra in detail, the use of chemometric methods is recommended [22]. FTIR spectroscopy should mainly be regarded as a supplementary technique, in addition to pattern recognition based on urine flow cytometry [22]. As water strongly absorbs IR light, the technique cannot be applied on native urine samples. The required sample volume (5 mL) is less than the standard amount required for microscopic urine sediment analysis [2]. The drying process is not really time-consuming and this sample preparation step makes the described technique very sensitive. The drying process is necessary to eliminate the intense water absorption. The water absorption bands are minimized, while the spectral bands of the possible lipids, proteins, nucleic acids and carbohydrates of the biological analysed material are maximized [5]. Reproducibility of spectra is excellent. In addition, we believe that this technique can probably be used in developing countries as it is easy-to-use, reagent-free and cost-effective [7]. Detection of the pathogenic bacteria in UTI by analysing dried urine using IR spectroscopy would simplify and accelerate the identification of the species because no cultured media of the urine is needed, however it excludes the possibility to perform susceptibility testing. Next to bacteria causing UTI, multiple other bacterial species and complex biological material

can be present in urine. This should be taken into account when setting up a model for bacterial classification directly on the urine sediment.

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