

# Journal of Biomedical Optics

BiomedicalOptics.SPIEDigitalLibrary.org

## **Quantifying creatinine and urea in human urine through Raman spectroscopy aiming at diagnosis of kidney disease**

Cassiano Junior Saatkamp  
Maurício Liberal de Almeida  
Jeyse Aliana Martins Bispo  
Antonio Luiz Barbosa Pinheiro  
Adriana Barrinha Fernandes  
Landulfo Silveira, Jr.

**SPIE.**

# Quantifying creatinine and urea in human urine through Raman spectroscopy aiming at diagnosis of kidney disease

Cassiano Junior Saatkamp,<sup>a</sup> Maurício Liberal de Almeida,<sup>a</sup> Jeyse Aliana Martins Bispo,<sup>b</sup> Antonio Luiz Barbosa Pinheiro,<sup>c,d</sup> Adriana Barrinha Fernandes,<sup>c</sup> and Landulfo Silveira Jr.<sup>c,\*</sup>

<sup>a</sup>Instituto Esperança de Ensino Superior (IESPES), Rua Coaracy Nunes, 3315, Santarém, Pará 68040-100, Brazil

<sup>b</sup>Faculdades Integradas do Tapajós—FIT, Rua Rosa Vermelha, No. 335, Aeroporto Velho, Santarém, Pará 68010-200, Brazil

<sup>c</sup>Universidade Camilo Castelo Branco—UNICASTELO, Biomedical Engineering Institute, Parque Tecnológico de São José dos Campos, Estrada Dr. Altino Bondesan, 500, São José dos Campos, São Paulo 12247-016, Brazil

<sup>d</sup>Federal University of Bahia—UFBA, Center of Biophotonics, School of Dentistry, Av. Araújo Pinho, 62, Salvador, Bahia 40110-150, Brazil

**Abstract.** Due to their importance in the regulation of metabolites, the kidneys need continuous monitoring to check for correct functioning, mainly by urea and creatinine urinalysis. This study aimed to develop a model to estimate the concentrations of urea and creatinine in urine by means of Raman spectroscopy (RS) that could be used to diagnose kidney disease. Midstream urine samples were obtained from 54 volunteers with no kidney complaints. Samples were subjected to a standard colorimetric assay of urea and creatinine and submitted to spectroscopic analysis by means of a dispersive Raman spectrometer (830 nm, 350 mW, 30 s). The Raman spectra of urine showed peaks related mainly to urea and creatinine. Partial least squares models were developed using selected Raman bands related to urea and creatinine and the biochemical concentrations in urine measured by the colorimetric method, resulting in  $r = 0.90$  and  $0.91$  for urea and creatinine, respectively, with root mean square error of cross-validation (RMSEcv) of 312 and 25.2 mg/dL, respectively. RS may become a technique for rapid urinalysis, with concentration errors suitable for population screening aimed at the prevention of renal diseases. © 2016 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.21.3.037001]

Keywords: urea; creatinine; colorimetric method; Raman spectroscopy; partial least squares; urinalysis.

Paper 150695R received Oct. 15, 2015; accepted for publication Feb. 9, 2016; published online Mar. 1, 2016.

## 1 Introduction

The kidneys have a primary role in purifying the blood plasma, removing undesired metabolites such as urea, creatinine, uric acid, and urates by the processes of filtration, tubular secretion, and resorption of some biochemicals. The kidneys also regulate the concentration of body fluid constituents, such as the amount of water and electrolytes in the body, helping regulate blood flow and glomerular filtration.<sup>1</sup>

Body fluids contain vital physicochemical information that is frequently used for clinical diagnosis. The urine is an important body fluid and is obtained easily in a noninvasive way, providing information for the diagnosis of various metabolic diseases.<sup>2</sup> Urinalysis may provide information about the body's metabolism and renal function. Physicochemical constituents present in urine are usually quantified using 24-h urine, a well-established technique that has a disadvantage—the duration of the collection.<sup>3</sup> This can be minimized by using the method of midstream urine, which involves collecting a single urine flow and rejecting the initial jet.

Urea is the major nitrogenated metabolite originating from protein degradation in the body. The kidney is responsible for eliminating 90% of the metabolized urea, while the remainder is excreted by the gastrointestinal tract and skin.<sup>4</sup> The measurement of urea in urine has been widely used to evaluate

nutritional conditions such as low protein intake and food deprivation.<sup>5</sup> Although urea is not specific for the diagnosis of renal disorders in general, it is considered sensitive for the detection of initial renal disorders.<sup>5</sup>

Creatinine is one of the main components of human urine and is a final product of muscle metabolism. The rate of creatinine excretion in urine is almost constant and is used as an internal standard for normalization of the variation of water in urinalysis. Creatinine is the component of urine commonly used for the renal clearance test, which measures the function of renal filtration. It is also valuable in assessing renal impairment<sup>6</sup> and may provide useful information about the health of the kidney.<sup>7</sup>

The standard technique for assessment of urea and creatinine in urine is based on colorimetric measurement. In this process, a specific reagent that reacts with the molecule of interest and has absorption at a given wavelength is employed to identify the desired component.<sup>8</sup> The concentration of the analyte of interest is determined by comparison of the absorption of the reagent added to the sample with the absorbance of a standard solution with known concentrations of analyte and reagent.<sup>9</sup> In immunoassay methods based on colorimetry, reference values for the urea and creatinine in the 24-h urinalysis are 26 to 43 g/24 h for urea<sup>10</sup> and 1.0 to 2.4 g/24 h for men and 0.74 to 1.6 g/24 h for women for creatinine.<sup>11</sup> For isolated urine samples, the reference ranges for these parameters are urea

\*Address all correspondence to: Landulfo Silveira, E-mail: [landulfo.silveira@gmail.com](mailto:landulfo.silveira@gmail.com); [landulfo.silveira@unicastelo.edu.br](mailto:landulfo.silveira@unicastelo.edu.br)

0.9 to 3.0 g/dL, creatinine 28 to 217 mg/dL (female) and 39 to 259 mg/dL (male).<sup>12</sup>

Spectroscopic methods offer several advantages over standard chemical methods for urine assay, including a shorter evaluation time and wider information content.<sup>3</sup> UV-visible colorimetric spectrophotometry is usually employed to determine levels of creatinine in the serum.<sup>9</sup> The use of reagents to evaluate the metabolite of interest is one of the main disadvantages of these methods, since the addition of different amounts of reagent could lead to an error in the assay and a false diagnosis of the actual clinical state of the patient. Automated equipment and well-trained personnel enhance reliability and reduce errors in these methods, but increase costs. Besides, the fact is that some interference may occur with other compounds.

Raman spectroscopy (RS) has been proposed as a suitable technique for identification of chemical constituents present in the urine, such as nitrogen compounds, urea, and creatinine, and their rate of excretion.<sup>13</sup> Raman scattering is based on the interaction of an electromagnetic wave from a laser beam and a molecule, where changes in the polarizability of the electron cloud lead to the inelastic scattering of the incident beam. Thus, Raman bands bring important molecular information related to the vibrational energy of a molecule's chemical bonds.<sup>14</sup> As each molecule has a specific and unique chemical structure, the Raman spectrum is a "molecular fingerprint."<sup>15</sup> Therefore, RS can be used to assess the molecular constitution of a biological tissue or fluid, extracting valuable qualitative (differences in biochemical constitution) and quantitative (differences in concentration) information. The use of near-infrared laser excitation (usually 785 and 830 nm) in tissues and biological fluids has the advantage of low fluorescence emission from biocompounds.<sup>15</sup>

Raman spectra can be used for *in vivo* measurements to monitor analytes in blood, serum, and urine.<sup>14</sup> This noninvasive optical technique has an advantage over traditional biochemical techniques based on its molecular specificity and capacity for quantitative analysis while dispensing with chemicals and reagents.<sup>14</sup> The Raman technique has the potential to be used in urinalysis with the benefit of rapid analysis of individual samples for the early diagnosis of diseases.<sup>5</sup> In addition, the Raman technique requires minimal or no sample preparation, minimizing the potential generation of artifacts, and thus could be an alternative to techniques that require extensive preparation; it is also nondestructive.<sup>16</sup>

RS has been employed to identify cellular components in the urine of patients with neoplasias, such as cells with carcinogenic potential, including urothelial cells and bladder cancer cells,<sup>17</sup> and in the diagnosis of urothelial carcinoma by distinguishing between benign tissue and different degrees of tumor lesions using Raman molecular imaging.<sup>3</sup> RS has been used to identify bacteria commonly found in infections of the urinary tract following suspension in saline solution after being isolated and cultivated in agar gel<sup>18</sup> and directly in the biomass.<sup>19</sup> Raman microspectroscopy and innovative chemometrics have been used to identify the infectious agent in infected urine samples from patients without the need for a prior culture step.<sup>20</sup> Raman analysis was also performed to evaluate glucose concentrations in samples of glucose diluted in water, simulating diluted urine in a toilet bowl.<sup>21</sup>

McMurdy and Berger<sup>2</sup> first used RS with near-infrared excitation (785 nm) to measure the concentration of creatinine in urine samples at clinical levels. Qi and Berger<sup>22</sup> reported

measurements of chemical concentrations in clinical urine samples as well as blood serum samples using liquid-core optical fiber RS to increase the collected signal strength. Wang et al.<sup>7</sup> used surface-enhanced Raman spectroscopy (SERS) applied to a new type of substrate to detect creatinine in the urine of diabetic patients, aimed at early detection of kidney diseases. Bispo et al.<sup>5</sup> used dispersive RS (830 nm) to identify biomarkers (urea, creatinine, and glucose) in the midstream urine of diabetic and hypertensive patients by principal component analysis (PCA) and a discriminating model, suggesting the possibility of diagnosis of clinical complications associated with diabetes and hypertension (HT) through RS. Using the same type of analysis via PCA, RS was also effective in the detection of antibiotics in urine<sup>23</sup> and other metabolites such as biomarkers of nicotine and cotinine for identification of exposure to tobacco smoke (active or passive),<sup>24</sup> and uric acid as indicative of pre-eclampsia in pregnant women.<sup>25</sup> Therefore, it has been demonstrated that RS could be employed in the direct measurement of the concentration of urea and creatinine in human urine samples for biochemical assay.

Due to the multivariate nature of the data obtained by RS, multivariate statistical methods such as the partial least squares (PLS) technique favor a quantitative assessment of the data by correlating various information from the spectrum with changes in the concentration of the sample's biochemical elements, allowing the analysis of data even in the presence of interfering agents.<sup>26,27</sup> These methods consider all relevant spectral information and ignore that noncorrelated with the concentration of analytes of interest, remaining unchanged even with the inclusion of new samples.<sup>26</sup> The evaluation of Raman data using the PLS regression method has been used to measure blood analytes, such as glucose, cholesterol, triglycerides, creatinine, urea, total protein, albumin, and hemoglobin.<sup>27-29</sup>

This study aimed to develop quantitative models using PLS regression applied to the Raman spectra of urine to estimate the concentrations of urea and creatinine in midstream urine of renal asymptomatic subjects, taking the biochemical assay determined by the colorimetric method as the gold standard. The PLS models were then used to estimate the concentrations of urea and creatinine in midstream urine from healthy subjects and from subjects with diabetes and HT, using the Raman spectra from the study of Bispo et al.<sup>5</sup> We attempted to assess the use of RS for urinalysis in midstream urine, providing a tool for obtaining the concentrations of urea and creatinine in urine samples for future control of kidney disease using RS for non-destructive and rapid clinical analysis.

## 2 Materials and Methods

### 2.1 Samples

This work was approved by the Human Research Ethics Committee at the Universidade Camilo Castelo Branco (UNICASTELO) (protocol no. 19690113.3.0000.5494) following Brazilian guidelines for the use of materials of human origin. Subjects were recruited from employees of the Laboratório Celso Matos (Santarém, Pará, Brazil). The samples consisted of urine from 54 volunteers with no renal complaints ( $n = 54$ ), the sample size being estimated using the Krejcie and Morgan estimation.<sup>30</sup> Inclusion criteria were as follows: individuals aged between 18 and 65 years, without history of pre-existing kidney disease or renal failure requiring hemodialysis.

Hypertensive and diabetic individuals with signs of pyelonephritis were excluded from the study.

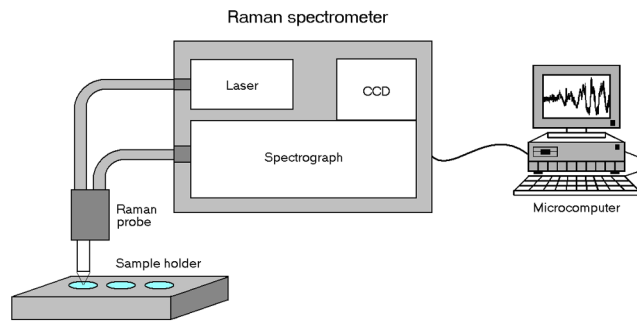
After explaining the goals of the study, each volunteer who agreed to participate read and signed the Consent Terms and answered a questionnaire on lifestyle habits and presence of previous disease and pathologies in close relatives. The urine of volunteers was collected in sterile vials provided by the researcher, after prior hygiene of the external genitalia, using the technique of midstream urine collection, whereby the initial flow is not collected (thus avoiding contamination from the urethra). Urination took place directly into the vial with immediate transport to the laboratory, where the colorimetric biochemical analysis took place to quantify the urea and creatinine in the samples.<sup>9</sup> The colorimetric assay employs reagents to evaluate absorbance in the UV–visible range. Urea was evaluated using the UV kinetic-enzymatic method and creatinine using the modified Jaffé kinetic colorimetric method, using specific reagents (urea/BUN UV—urease/glutamate dehydrogenase, BioSystems S.A., Barcelona, Spain; and creatinine “Mod. Jaffé” 2 reagents, Eurotech Prod. Lab. Serv. Ltda., Goiânia, Brazil)<sup>31,32</sup> and an absorption spectrometer for medical diagnostics (Cobas Modular P, Roche Diagnóstica Brasil Ltda., São Paulo, Brazil). The repeatability and reproducibility for these colorimetric assays are, respectively, 3.3% and 4.3% for urea and 1.3% and 3.6% for creatinine.<sup>31,32</sup>

After biochemical assay, about 10 mL of each sample was placed in a glass screw-cap vial and stored frozen ( $-20^{\circ}\text{C}$ ), and at the end of a week of sample collection, samples were transported refrigerated (dry ice at  $-78^{\circ}\text{C}$ ) to the RS laboratory for spectroscopic analysis, as described below. The storage of samples in a freezer in glass vials for 1 week and transport using dry ice seem to preserve the physicochemical characteristics of urine samples.<sup>33</sup> This study also used the Raman spectra reported by Bispo et al.,<sup>5</sup> who assessed urine samples from normal subjects and diabetic/hypertensive patients without and with complications related to diabetes and HT by means of near-infrared RS and PCA with the aim of identifying potential biomarkers in the urine of diseased compared to healthy subjects. The procedures used to collect, store, and transport the urine samples and conduct Raman signal collection/analysis, including the Raman spectrometer parameters, were the same as those used in Bispo et al.

The spectra of reference samples of urea and creatinine (U5378 and C4255, Sigma-Aldrich Brasil Ltda., São Paulo, Brazil), diluted in 0.9% saline, were also obtained.

## 2.2 Raman Spectroscopy

A dispersive Raman spectrometer (model Dimension P-1, Lambda Solutions, Inc., Massachusetts) was used. In this spectrometer, a diode laser (830 nm) was coupled to a fiberoptic cable (Raman probe), which was used to deliver radiation to the sample and collect the signal scattered by the sample. The probe was positioned at a distance of 10 mm from the urine sample holder, as shown in Fig. 1, and the laser power at the probe tip was adjusted to 250 mW. The light signal scattered by the sample was detected by a spectrometric CCD camera (back-thinned, deep-depleted,  $1340 \times 100$  pixels, Peltier-cooled, working temperature of  $-75^{\circ}\text{C}$ ). The spectrometer used a holographic grating with 1200 lines/mm, providing a spectral resolution of about  $2\text{ cm}^{-1}$  in the useful spectral range of 400 to  $1800\text{ cm}^{-1}$  (fingerprint region).



**Fig. 1** Schematic diagram of the dispersive Raman system used to collect the spectral data from urine samples. Spectra were taken in triplicate. Laser wavelength: 830 nm, laser power at the probe tip: 250 mW, spectral resolution:  $2\text{ cm}^{-1}$ , spectral range: 400 to  $1800\text{ cm}^{-1}$ .

Urine samples were placed on a sample holder made of polished aluminum with holes of 4 mm diameter and about  $100\ \mu\text{L}$  volume in triplicate. The spectra of the urine samples were assessed via fiberoptics, with repeatability of excitation and signal collection geometry, thus it was possible to study the spectral differences related to differences in the biochemical constitution of the urine of different subjects and correlate spectral changes with differences in the concentration of the analytes of interest.

The acquisition and storage of the spectra were performed by a PC microcomputer using the software RamanSoft (version 1.7, Lambda Solutions, Inc., Massachusetts), which controls the exposure time of the detector and the number of acquisitions per sample via USB connection. The exposure time for collecting the spectra was 30 s (3 s and 10 accumulations), resulting in spectra with a signal-to-noise ratio as high as 100 for the Raman peak of urea ( $1006\text{ cm}^{-1}$ ) and 20 for creatinine ( $681\text{ cm}^{-1}$ ). Triplicate spectra of each sample were averaged for the spectral analysis.

The calibration of the spectrometer was checked before data collection. The positions (Raman shift) of the main bands of naphthalene<sup>24</sup> were verified, since the compound has characteristic intense and well-spaced bands in the fingerprint region 500 to  $1700\text{ cm}^{-1}$ . The calibration of the spectral response was performed by the equipment supplier and was checked by measuring the spectrum of a tungsten filament lamp with a National Institute of Standards and Technology traceable spectrum.<sup>34</sup>

Prior to data analysis, spectra were preprocessed. First, background fluorescence was removed by fitting and then subtracting a fifth-order polynomial from the gross spectrum. Then, background-free spectra were subjected to smoothing using the Savitzky–Golay algorithm, decreasing the shot-noise contribution and thus increasing the spectral signal-to-noise ratio. Finally, normalization was performed using the area of the vibrational band of water at  $1660\text{ cm}^{-1}$ . These procedures (calibration, removal of fluorescence emission, smoothing, and normalization) allow the collection of trustworthy and high-quality Raman spectra.

## 2.3 Model for Quantification of Urea and Creatinine Using Partial Least Squares

A multivariate regression model based on PLS was developed for predicting the concentrations of urea and creatinine using the spectral information contained in the Raman spectra and the concentrations obtained via the colorimetric method. The

PLS routine employed the leave-one-out cross-validation approach, where a sample is left out of the model and the concentration of this sample is estimated by modeling using the remaining  $n - 1$  samples with a number of latent variables (LVs).<sup>35,36</sup> The process is repeated  $n$  times for predicting the concentrations of all samples. The number of LVs to be included in the model was chosen from those with the lowest error of cross-validation in the left-out samples. By plotting the biochemical concentration versus the predicted one, the correlation coefficient ( $r$ ) and the root mean square error of cross-validation (RMSEcv)<sup>37</sup> of the model for the dataset were calculated, which was used to estimate the assessing prediction error in the calibration model. Two quantitative models were developed using the software MATLAB 6.0 and the Chemoface toolbox,<sup>38</sup> using selected spectral regions for urea (500 to 560  $\text{cm}^{-1}$ , 960 to 1043  $\text{cm}^{-1}$ , and 1120 to 1192  $\text{cm}^{-1}$ ) and for creatinine (650 to 940  $\text{cm}^{-1}$ ) as predictor variables ( $x$ ) and the concentrations of these biochemicals as predicted variables ( $y$ ).

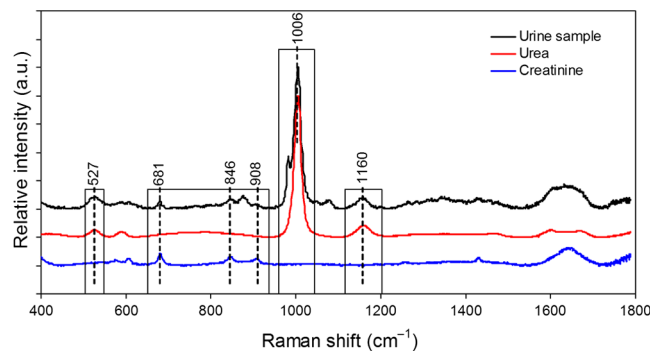
The developed quantitative models have been applied to a Raman dataset composed of spectra of urine from the study of Bispo et al.,<sup>5</sup> aiming to estimate the concentration of urea and creatinine in those samples. In the study of Bispo et al.,<sup>5</sup> spectra were measured in urine samples from healthy subjects ( $n = 18$ ) and from two groups of subjects with diabetes mellitus (DM) and arterial HT, one group ( $n = 20$ ) without complications associated with DM and HT (G1; and therefore at low risk of developing kidney disease), and another group ( $n = 16$ ) with complications such as cardiac disease, cerebrovascular disease, and peripheral vascular disease (G2; and therefore at high risk of developing renal disease), aiming at discrimination of groups using principal components analysis and discriminant analysis. Spectra were measured using the same Raman instrument and acquisition parameters presented here, and the Raman features of urea and creatinine in the same range were used to predict the concentrations from the developed model.

### 3 Results and Discussion

#### 3.1 Quantitative Model to Estimate Urea and Creatinine in Urine

The Raman spectra of a sample of urine from a patient without renal disease and the reference spectra of urea and creatinine diluted in 0.9% saline are presented in Fig. 2. The urine spectrum is dominated by peaks, especially those for urea and creatinine. The peaks at 527, 1006, and 1160  $\text{cm}^{-1}$  indicate the presence of urea and the peaks at 681, 846, and 908  $\text{cm}^{-1}$  creatinine, with attribution assigned to the symmetric stretching peak C—N for the peak at 1006  $\text{cm}^{-1}$  and C—NH<sub>2</sub> and C=O stretching and vibrations of the aromatic ring for the peak at 681  $\text{cm}^{-1}$ ,<sup>39–41</sup> confirming the peaks found in other studies of urine.<sup>3,42,43</sup>

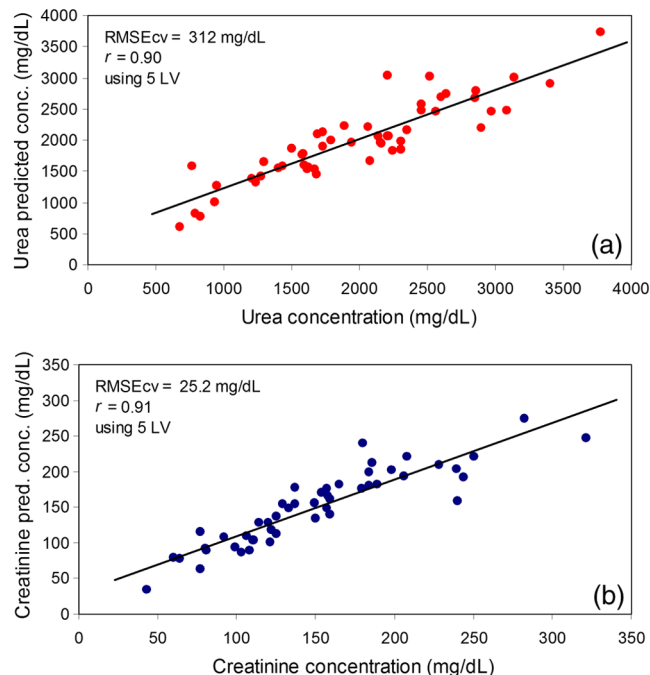
PLS models were constructed in order to estimate the concentrations of urea and creatinine using the spectral information from selected regions and the biochemical concentrations of urea and creatinine in midstream urine measured using the colorimetric method. These models with the predicted concentrations versus the colorimetric concentrations are presented in Fig. 3. In both cases, the PLS models presented higher correlation coefficients and higher RMSEcv when using five LVs. These values were  $r = 0.90$  and  $0.91$ , and



**Fig. 2** Raman spectrum of a sample of urine from a healthy patient and the reference spectra of urea and creatinine diluted in 0.9% saline. Spectral regions inside the boxes were used in the PLS model (500 to 560  $\text{cm}^{-1}$ , 960 to 1043  $\text{cm}^{-1}$ , 1120 to 1192  $\text{cm}^{-1}$  for urea and 650 to 940  $\text{cm}^{-1}$  for creatinine). Wavelength: 830 nm, power: 250 mW, spectral resolution: 2  $\text{cm}^{-1}$ , exposure time: 30 s (3 s, 10 accumulations).

RMSEcv = 312 mg/dL and 25.2 mg/dL, for urea and creatinine, respectively.

McMurdy and Berger<sup>2</sup> found an RMSE of 4.9 mg/dL for estimating the concentration of creatinine in unaltered human urine samples from a calibration dataset. For comparison, the chemical method used by them showed an error of 1.1 mg/dL, indicating that the model based on RS is capable of quantifying creatinine independently of clinical variations among patients.<sup>2</sup> Qi and Berger<sup>22</sup> first demonstrated the use of liquid-core optical fiber RS and multivariate statistics in 61 clinical urine samples (excluding 12 outliers) and found that the error for assaying urea by the standard technique was 42.4 mg/dL, compared to 53.0 mg/dL for the Raman assay. For creatinine, the errors were 5.1 mg/dL for the standard



**Fig. 3** Correlation between concentration of (a) urea and (b) creatinine in midstream urine predicted by the PLS model versus the concentration using colorimetric method. The resulting  $r$  and the RMSEcv error are presented for each biochemical.

assay and 6.8 mg/dL for the Raman assay. These errors were ameliorated when the spectra were corrected by the absorption and scattering coefficients taken with a white light, and the model used the total integration time of 64 s, with errors of 40.4 and 4.3 mg/dL for urea and creatinine, respectively.

In the study conducted by Dou et al.,<sup>42</sup> the concentration of urea, creatinine, acetone, and glucose artificially added to urine samples was analyzed by Raman technique via the measurement of the intensities of the bands at 1013 and 692  $\text{cm}^{-1}$  for urea and creatinine, respectively. The authors found correlation coefficients of  $r = 0.991$  and  $r = 0.998$  for urea and creatinine, respectively. The detection limits were 4.9 and 1.5 mg/mL, respectively, thus demonstrating the potential of near-infrared RS employed in quantitative analysis of glucose, acetone, urea, and creatinine in urine.

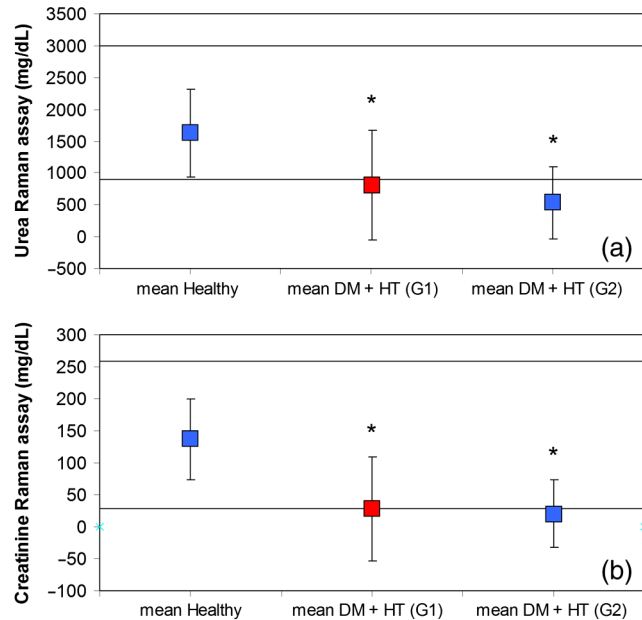
SERS has been proposed for the detection of metabolites in urine. Wang et al.<sup>6</sup> used SERS to evaluate creatinine in samples of artificial and human urine, and found a correlation coefficient of  $r = 0.99$  in artificial urine over the range of 38.4 to 154 mg/dL and  $r = 0.96$  for human urine over the range of 2.56 to 115 mg/dL. In a subsequent study that also evaluated these metabolites by SERS,<sup>7</sup> the same authors observed a good linear correlation for creatinine concentrations over the range from 2.56 to 6.4 g/dL, with a coefficient of determination of  $R^2 = 0.96$ .

The correlation coefficients and the RMSEcv obtained in this work for urea and creatinine in midstream urine ( $r = 0.90$  and  $0.91$ , and RMSEcv = 312 mg/dL and 25.2 mg/dL, respectively) may be considered suitable for screening analysis of clinical samples. In fact, the results achieved by other authors and presented in this work show the feasibility of performing urinalysis rapidly in single urine samples without sample preparation in an attempt to screen for renal disease. This is important as Raman analysis, which needs a low volume of sample, has no need for reagents and no residues to be discarded, and exhibits rapidity in obtaining the Raman spectrum, usefulness for screening samples, and subsequent adequacy of clinical treatment in a rapid way.

### 3.2 Quantitative Analysis of Urine Samples from Diabetic and Hypertensive Patients Using Partial Least Squares

The PLS models for estimating the concentrations of urea and creatinine were applied to the spectra of urine from a group of healthy subjects and two groups of subjects with DM and HT (one group without complications associated with DM and HT—G1, and the other group with complications associated with DM and HT and risks for development of renal disease—G2), the dataset from the study of Bispo et al.<sup>5</sup> These concentrations were estimated by the PLS models using the water-normalized intensities of the Raman peaks of the urine samples at the same spectral regions of urea (500 to 560  $\text{cm}^{-1}$ , 960 to 1043  $\text{cm}^{-1}$ , 1120 to 1192  $\text{cm}^{-1}$ ), and creatinine (650 to 940  $\text{cm}^{-1}$ ), and the mean concentrations are shown in Fig. 4. Large error bars in these groups may be attributed to the existence of subjects with considerable differences in pathological status (clinical samples), and the observance of negative estimated concentrations of creatinine and urea in some samples of G1 and G2 that might be due to noise.

It was found that the group of healthy subjects that were asymptomatic for complications associated with DM and HT, such as renal disease, and had a urea concentration of 1626  $\pm$



**Fig. 4** Mean ( $\pm$ SD) concentrations of (a) urea and (b) creatinine estimated by the PLS model applied to the selected regions of the Raman spectra of urine from Bispo et al.<sup>5</sup> in the groups of healthy subjects and subjects with DM and HT (without clinical complications—G1, and with clinical complications—G2). Symbol \* indicates statistically significant differences between group G1 or G2 versus healthy group (ANOVA,  $p < 0.01$ ). Solid lines present the reference values<sup>12</sup> for urea and creatinine in isolated urine samples.

686 mg/dL (mean  $\pm$  SD) and a creatinine concentration of  $136 \pm 63$  mg/dL, while the group of patients with DM and HT, but without clinical complications (G1), had lower concentrations of  $808 \pm 866$  mg/dL urea and  $28 \pm 81$  mg/dL creatinine. The group of patients with clinical complications (G2) had even lower concentrations of urea and creatinine, at  $533 \pm 561$  mg/dL and  $20 \pm 52$  mg/dL, respectively. Since the reference values for these parameters in isolated urine are urea 0.9 to 3.0 g/dL and creatinine 28 to 217 mg/dL (female) and 39 to 259 mg/dL (male),<sup>12</sup> the mean of G1 is at the limit of the reference value and the mean of G2 is outside the reference value. ANOVA indicated a significant difference between the healthy group and both DM + HA groups ( $p < 0.01$ ). Thus, the results of Bispo et al.,<sup>5</sup> who found differences in the spectral variables among healthy groups and subjects with DM and HT with and without complications, and the results presented in this study, in which the estimated concentrations of urea and creatinine in the DM and HT groups were lower than the concentrations in healthy ones, suggest that the differences in the concentrations of urea and creatinine in healthy patients and patients with DM and HT can be related to the severity of the complications that could lead to a decrease in renal function and culminate with a renal failure, and these differences can be identified using a quantitative RS model applied to single-collection midstream urine. It is important to notice that none of the patients in G2 presented a diagnosis of renal disease at the time of Raman analysis.

To assess the functional status of the kidneys, it is necessary to monitor reliable biomarkers. The use of RS for the quantitative analysis of creatinine concentration in the urine is described in the literature as an option that provides rapid measurement (within 10 s), high sensitivity (limit of detection of creatinine

<0.5  $\mu\text{g}/\text{mL}$ ), and reliability (variation <5%) in urine analysis,<sup>6</sup> together with the evaluation of multiple constituents in the urine.<sup>13,22</sup> In this work, the RMSE values of 312 and 25.2 mg/dL for urea and creatinine in clinical urine samples indicate satisfactory results in terms of using the Raman technique for the screening of large populations using a single-collection sample.

RS has been described as a new and powerful diagnostic tool relative to routine biochemical tests.<sup>2,3,5,7,13,23</sup> The Raman method would allow the simultaneous and rapid identification of biochemical components and their quantitative determination, including glucose, acetone, creatinine, urea, lipids, uric acid, and total blood protein.<sup>22,44</sup> The potential for the characterization of samples associated with the possibility of *in situ* analysis makes this analytical technique a promising tool for the evaluation of biochemical constituents. The results indicate that RS may prove to be a promising technique for the quantification of urea and creatinine in midstream urine, which could be used for the early diagnosis of renal changes at different stages of renal disease. Early diagnosis, with prompt routing to reduce or even stop the progression of renal problems, is among the key strategies to improve clinical outcomes and reduce costs associated with renal diseases.<sup>43</sup> The Raman technique could be an important tool for assessing the risk of developing renal injury in clinical diagnosis, thus providing a better quality of life for many patients due to the early detection of disabling diseases such as renal failure. The results of this study demonstrate that the use of RS is a promising technique in urinalysis for renal evaluation using a single spectrum.

Given its availability at low cost and easy implementation,<sup>45</sup> the technique may become an alternative for existing laboratory methods in the near future and could be applied in the presumptive identification of previously unknown renal lesions, not only for diagnosing renal failure itself but also for diagnosing the risk factors for the development of future renal disease with subsequent clinical referral.

## 4 Conclusion

The PLS model applied to selected spectral regions containing featured peaks of urea and creatinine presented prediction errors of 312 and 25.2 mg/dL for urea and creatinine, respectively, indicating that the concentrations of these compounds could be assayed in midstream urine by using the Raman spectra. The concentration of urea and creatinine estimated using this model was significantly lower in subjects with DM and HT (808  $\pm$  866 mg/dL for urea and 28  $\pm$  81 mg/dL for creatinine) compared to healthy subjects (1626  $\pm$  686 mg/dL for urea and 136  $\pm$  63 mg/dL for creatinine). The prediction errors found in this study indicate that RS may be used to quantify analytical constituents in urine, complementing or even replacing the conventional techniques of biochemical analysis, especially in rapid and costless population screening for kidney disease.

## Acknowledgments

L.S. Jr. thanks the São Paulo Research Foundation (FAPESP) for supporting the acquisition of the Raman spectrometer (Process no. 2009/01788-5). C.J.S. and M.L.A. thank Fundação Esperança, sponsor of the Instituto Esperança de Educação Superior (IESPES) for partly funding this research (Process no. 05/2012). The authors acknowledge the support of the Laboratório Celso Matos in this survey.

## References

1. A. Haase-Fielitz et al., "Novel and conventional serum biomarkers predicting acute kidney injury in adult cardiac surgery—a prospective cohort study," *Crit. Care Med.* **37**(2), 553–560 (2009).
2. J. W. McMurdy and A. J. Berger, "Raman spectroscopy-based creatinine measurement in urine samples from a multipatient population," *Appl. Spectrosc.* **57**(5), 522–525 (2003).
3. A. Shapiro et al., "Raman molecular imaging: a novel spectroscopic technique for diagnosis of bladder cancer in urine specimens," *Eur. Urol.* **59**(1), 106–112 (2011).
4. F. L. Sodr e, J. C. B. Costa, and J. C. C. Lima, "Evaluation of renal function and damage: a laboratorial challenge," *J. Bras. Patol. Med. Lab.* **43**(5), 329–337 (2007).
5. J. A. M. Bispo et al., "Correlating the amount of urea, creatinine, and glucose in urine from patients with diabetes mellitus and hypertension with the risk of developing renal lesions by means of Raman spectroscopy and principal component analysis," *J. Biomed. Opt.* **18**(8), 087004 (2013).
6. T. L. Wang et al., "Semi-quantitative surface enhanced Raman scattering spectroscopic creatinine measurement in human urine samples," *Opt. Quantum Electron.* **37**(13–15), 1415–1422 (2005).
7. H. Wang et al., "Quantitative analysis of creatinine in urine by metalized nanostructured parylene," *J. Biomed. Opt.* **15**(2), 027004 (2010).
8. A. E. Guimarães et al., "Near infrared raman spectroscopy (NIRS): a technique for doping control," *J. Spectrosc.* **20**(4), 185–194 (2006).
9. E. Mohabbati-Kalejahi et al., "A review on creatinine measurement techniques," *Talanta* **97**, 1–8 (2012).
10. A. H. B. Wu, *Tietz Clinical Guide to Laboratory Tests*, 4th ed., Elsevier Saunders, St. Louis, Missouri (2006).
11. W. Junge et al., "Determination of reference intervals for serum creatinine, creatinine excretion and creatinine clearance with an enzymatic and a modified Jaff e method," *Clin. Chim. Acta* **344**, 137–148 (2004).
12. W. Heil and V. Ehrhardt, *Reference Ranges for Adults and Children*, Roche Diagnostics, Rotkreuz (2008).
13. W. R. Premasiri, R. H. Clarke, and M. E. Womble, "Urine analysis by laser Raman spectroscopy," *Lasers Surg. Med.* **28**, 330–334 (2001).
14. E. B. Hanlon et al., "Prospects for *in vivo* Raman spectroscopy," *Phys. Med. Biol.* **45**(2), R1 (2000).
15. L. Silveira et al., "Correlation between near-infrared Raman spectroscopy and the histopathological analysis of atherosclerosis in human coronary arteries," *Lasers Surg. Med.* **30**, 290–297 (2002).
16. J. R. Beattie et al., "Raman microscopy of porcine inner retinal layers from the area centralis," *Mol. Vis.* **13**, 1106–1113 (2007).
17. E. Canetta et al., "Modulated Raman spectroscopy for enhanced identification of bladder tumor cells in urine samples," *J. Biomed. Opt.* **16**(3), 037002 (2011).
18. R. Goodacre et al., "Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks," *Microbiology* **144**(5), 1157–1170 (1998).
19. F. S. D. S. Oliveira, H. E. Giana, and L. Silveira, "Discrimination of selected species of pathogenic bacteria using near-infrared Raman spectroscopy and principal components analysis," *J. Biomed. Opt.* **17**(10), 107004 (2012).
20. S. Kloss et al., "Culture independent Raman spectroscopic identification of urinary tract infection pathogens: a proof of principle study," *Anal. Chem.* **85**(20), 9610–9616 (2013).
21. C. Park et al., "Classification of glucose concentration in diluted urine using the low-resolution Raman spectroscopy and kernel optimization methods," *Physiol. Meas.* **28**(5), 583 (2007).
22. D. Qi and A. J. Berger, "Chemical concentration measurement in blood serum and urine samples using liquid-core optical fiber Raman spectroscopy," *Appl. Opt.* **46**(10), 1726–1734 (2007).
23. R. Kumar et al., "A facile and real-time spectroscopic method for biofluid analysis in point-of-care diagnostics," *Bioanalysis* **5**(15), 1853–1861 (2013).
24. R. Huang, S. Han, and X. S. Li, "Detection of tobacco-related biomarkers in urine samples by surface-enhanced Raman spectroscopy coupled with thin-layer chromatography," *Anal. Bioanal. Chem.* **405**(21), 6815–6822 (2013).
25. B. L. Goodall, A. M. Robinson, and C. L. Brosseau, "Electrochemical-surface enhanced Raman spectroscopy (E-SERS) of uric acid: a

- potential rapid diagnostic method for early preeclampsia detection,” *Phys. Chem. Chem. Phys.* **15**(5), 1382–1388 (2013).
26. A. J. Berger et al., “Multicomponent blood analysis by near-infrared Raman spectroscopy,” *Appl. Opt.* **38**(13), 2916–2926 (1999).
  27. A. M. Enejder et al., “Blood analysis by Raman spectroscopy,” *Opt. Lett.* **27**, 2004–2006 (2002).
  28. D. Rohleder et al., “Comparison of mid-infrared and Raman spectroscopy in the quantitative analysis of serum,” *J. Biomed. Opt.* **10**(3), 031108 (2005).
  29. J. Shao et al., “In vivo blood glucose quantification using Raman spectroscopy,” *PLoS One* **7**(10), e48127 (2012).
  30. R. V. Krejcie and D. W. Morgan, “Determining sample size for research activities,” *Educ. Psychol. Meas.* **30**(3), 607–610 (1970).
  31. Biosystems S/A, “UREA/BUN - UV (urease/glutamato deshidrogenase),” <http://www.imunotech.com.br/bulas/ureia.pdf> (12 March 2014).
  32. Eurotech Prod. Laboratoriais e Serviços Ltda. Creatinina “Mod. Jaffe, “Reagente diagnóstico para determinação quantitativa in vitro da creatinina em soro humano, plasma ou urina em sistemas fotométricos,” [http://www.imunotech.com.br/bulas/rea\\_5073240e63694.pdf](http://www.imunotech.com.br/bulas/rea_5073240e63694.pdf) (12 March 2014).
  33. M. J. Rist et al., “Influence of freezing and storage procedure on human urine samples in NMR-based metabolomics,” *Metabolites* **3**(2), 243–258 (2013).
  34. R. L. McCreery, *Raman Spectroscopy for Chemical Analysis*, John Wiley & Sons, New York, New York (2000).
  35. J. P. A. Martins and M. Ferreira, “QSAR modeling: a new open source computational package to generate and validate QSAR model,” *Química Nova* **36**(4), 554–560 (2013).
  36. P. Geladi and B. R. Kowalski, “Partial least-squares regression: a tutorial,” *Anal. Chim. Acta* **185**, 1–17 (1986).
  37. R. G. Brereton, “Introduction to multivariate calibration in analytical chemistry,” *Analyst* **125**(11), 2125–2154 (2000).
  38. C. A. Nunes et al., “Chemoface: a novel free user-friendly interface for chemometrics,” *J. Braz. Chem. Soc.* **23**(11), 2003–2010 (2012).
  39. R. Keuleers et al., “Vibrational analysis of urea,” *J. Phys. Chem.* **103**, 4621–4630 (1999).
  40. Z. Movasaghi, S. Rehman, and I. U. Rehman, “Raman spectroscopy of biological tissues,” *Appl. Spectrosc. Rev.* **42**(5), 493–541 (2007).
  41. C. Bayrak and S. H. Bayari, “Vibrational and DFT studies of creatinine and its metal complexes,” *Hacetatepe J. Biol. Chem.* **38**(2), 107–118 (2010).
  42. X. Dou et al., “Quantitative analysis of metabolites in urine using a highly precise, compact near-infrared Raman spectrometer,” *Vib. Spectrosc.* **13**(1), 83–89 (1996).
  43. M. G. Bastos, R. Bregman, and G. M. Kirsztajn, “Chronic kidney diseases: common and harmful, but also preventable and treatable,” *Rev. Assoc. Med. Bras.* **56**(2), 248–253 (2010).
  44. S. Uskoković-Marković et al., “Raman spectroscopy as a new biochemical diagnostic tool,” *J. Med. Biochem.* **32**(2), 96–103 (2013).
  45. L. M. Moreira et al., “Raman spectroscopy: a powerful technique for biochemical analysis and diagnosis,” *J. Spectroscopy* **22**(1), 1–19 (2008).

Biographies for the authors are not available.