

# Evaluation of mass spectrometry of urinary proteins and peptides as biomarkers for cats at risk of developing azotemia

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**Objective**—To evaluate proteomic delineation of feline urine by mass spectrometry as a method for identifying biomarkers in cats at risk of developing azotemia.

**Samples**—Urine samples from geriatric cats (> 9 years old) with chronic kidney disease and nonazotemic cats that either remained nonazotemic (n = 10) or developed azotemia (10) within 1 year.

**Procedures**—Optimization studies with pooled urine were performed to facilitate the use of surface enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF-MS) for analysis of the urinary proteome of cats. Urine samples from non-azotemic cats at entry to the study were analyzed via SELDI-TOF-MS with weak cation exchange and strong anion exchange arrays. Spectral data were compared to identify biomarkers for development of azotemia.

**Results**—Low protein concentration in feline urine precluded direct application to array surfaces, and a buffer exchange and concentration step was required prior to SELDI-TOF-MS analysis. Three preparation conditions by use of weak cation and strong anion exchange arrays were selected on the basis of optimization studies for detection of biomarkers. Eight potential biomarkers with an m/z of 2,822, 9,886, 10,033, 10,151, 10,234, 11,653, 4,421, and 9,505 were delineated.

**Conclusions and Clinical Relevance**—SELDI-TOF-MS can be used to detect urinary low-molecular weight peptides and proteins that may represent biomarkers for early detection of renal damage. Further study is required to purify and identify potential biomarkers before their use in a clinical setting. (*Am J Vet Res* 2013;74:333–342)

Proteomics is the analysis of expressed proteins in tissues, cells, or biological fluids and reflects molecular events at the time of sampling. Surface-enhanced laser desorption-ionization time-of-flight mass spectrometry is a high throughput mass spectrometric technique that uses chromatographic surface characteristics (eg, hydrophobicity, pH, and metal affinity) on arrays to facilitate separation of proteins.

Chronic kidney disease is a common condition in geriatric cats. However, plasma creatinine is an insensitive marker of renal damage and substantial renal disease can already be present by the time azotemia is detected. In cats, renal biopsy is not routinely performed

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## ABBREVIATIONS

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CKD	Chronic kidney disease
CV	Coefficient of variance
dH <sub>2</sub> O	Deionized water
EAM	Energy-absorbing matrix
LMW	Low molecular weight
PTC	Proximal tubular cell
ROC	Receiver operator curve
SBP	Systolic blood pressure
SELDI-TOF-MS	Surface-enhanced laser desorption-ionization time-of-flight mass spectrometry
T4	Thyroxine
TFA	Trifluoroacetic acid
UP:C	Urine protein-to-creatinine ratio

and only a limited number of studies have evaluated renal disease with that method.<sup>1–5</sup> Although there are case reports of nephrotic syndrome with primary glomerular injury resulting in marked proteinuria, the most commonly reported histopathologic changes in the latter

stages of feline CKD are tubulointerstitial inflammation and fibrosis.<sup>3,6,7</sup> Studies<sup>8–10</sup> in human patients have determined that proteinuria is associated with both the development and progression of kidney disease. Similarly, recent studies<sup>11,12</sup> have identified that even low-magnitude proteinuria is associated with a lower survival rate in cats with various degrees of azotemia and is a predictor for the development of azotemia. However, although urine total protein and albumin-to-creatinine ratios are of interest on a population basis, they are unlikely to be predictive for individual cats.

In healthy kidneys, LMW proteins (< 40 kDa) and intermediate-molecular-weight proteins freely filtered at the glomerulus are reabsorbed and processed in the PTCs such that urine protein concentration is low.<sup>10,13</sup> When there is evidence of mild tubular dysfunction, the capacity of the PTCs to reabsorb protein is reduced and hence an increased concentration of LMW protein and albumin may be found in the urine. In human patients, various specific LMW proteins such as retinol binding protein and  $\beta_2$ -microglobulin have been investigated as markers of early tubular damage.<sup>14–16</sup> However, they provide an extremely limited representation of the full urinary proteome, and extrapolation of their use in domestic species is limited due to the lack of available cross-reacting antibodies in many instances.<sup>17</sup> Biomarkers are therefore sought that may enable the early detection of cats at risk of developing CKD.

Urine provides a readily available biological fluid in which the proteomic patterns may be indicative of the state of health of the kidneys.<sup>18–22</sup> Surface-enhanced laser desorption-ionization time-of-flight mass spectrometry has been used in human studies<sup>21,23</sup> as a rapid and sensitive method for evaluating the urinary proteome in large numbers of clinical samples. In human studies, SELDI-TOF-MS has been used for biomarker discovery in a variety of urological conditions including detection of early renal injury, transitional cell carcinomas, renal allograft rejection, renal carcinoma, and diabetic nephropathy.<sup>23–28</sup> The purpose of the study reported here was to evaluate SELDI-TOF-MS for delineation of the feline urinary proteome for biomarker discovery in cats at risk of developing azotemia.

## Materials and Methods

### Case selection, sample collection, and storage—

Cats from the 2 first-opinion clinics in central London (Beaumont Animals' Hospital and Peoples' Dispensary for Sick Animals) were enrolled in the study. Cats included in the optimization and validation component of the study had a diagnosis of CKD made on the basis of persistent azotemia (plasma creatinine concentration,  $\geq 2.0$  mg/dL) and were participating in a longitudinal renal monitoring program. For the biomarker discovery component of the study, 20 healthy nonazotemic cats were included and followed up longitudinally for 12 months.

For all cats, a complete medical history was obtained, and physical examination was performed. Systolic blood pressure was assessed with the Doppler technique,<sup>a</sup> and cats with systemic hypertension, defined as an SBP > 170 mm Hg on a single occasion in association with ocular lesions or on multiple occasions if

ocular lesions were not identified, were excluded from the study.

Collection and storage of blood and urine samples were performed with the cat owner's informed consent. The Ethics and Welfare Committee of the Royal Veterinary College approved these protocols. Blood samples were obtained by jugular venipuncture, routinely collected into tubes with lithium heparin, and centrifuged<sup>b</sup> at  $1,000 \times g$  and  $4^\circ\text{C}$  for 10 minutes to yield heparinized plasma, which was used to perform a full biochemical analysis and for assessment of total T4 concentration at an external commercial laboratory.<sup>c</sup> Cats with total T4 concentration > 35 nmol/L (laboratory reference range, 18 to 55 nmol/L) were excluded from inclusion in the study. Urine samples were collected by cystocentesis and were routinely refrigerated for 4 to 6 hours prior to processing. Routine urinalysis was performed on all samples, including measurement of specific gravity, pH,<sup>d</sup> dipstick<sup>e</sup> analysis, and sediment examination. Urine samples with evidence of gross or microscopic hematuria, pyuria, or bacteriuria were excluded. Urine samples were subsequently centrifuged<sup>b</sup> at  $1,000 \times g$  for 10 minutes at  $4^\circ\text{C}$ , and the supernatant was separated on the day of collection. Samples were stored at  $-80^\circ\text{C}$  prior to pooling for use in studies to optimize SELDI-TOF-MS conditions to ascertain the limit of detection and for inter- and intra-array repeatability studies. Samples for analysis as part of the biomarker study were divided into aliquots prior to storage at  $-80^\circ\text{C}$  to prevent the requirement for repeated freeze-thaw cycles. The UP:C was assessed by means of a stored aliquot at an external commercial laboratory,<sup>c</sup> where urine protein concentration was measured via a colorimetric pyrogallol red method and urine creatinine concentration was measured via a colorimetric picric acid method.

Nonazotemic cats included in the biomarker study were reexamined 6 and 12 months after initial examination. Plasma biochemical assessment was performed at each of these visits, and when urine could be obtained, a full urinalysis was also performed. Assessment of total T4 concentration was repeated at the 12-month visit. Cats were excluded from entry to the biomarker study if they were receiving any medications other than ecto- or endoparasitides or if any concurrent medical disease was present. Cats were classified at 12 months according to their renal status as being either nonazotemic (group 1: plasma creatinine concentration, < 2.0 mg/dL) or azotemic (group 2: plasma creatinine concentration  $\geq 2$  mg/dL). No specification was made regarding urine specific gravity in these 2 groups of initially nonazotemic cats.

**Optimization of conditions for use of SELDI-TOF-MS—**Preliminary studies suggested that urine protein concentrations, in particular from nonazotemic or mildly azotemic cats, were too low for direct application of urine to array surfaces. Therefore, a single pooled urine sample, divided into 500  $\mu\text{L}$ /aliquots ( $n = 24$ ), was concentrated and buffer-exchanged<sup>f</sup> 3 times by use of 12 equilibration buffers with incremental differences in pH (50mM sodium acetate buffer<sup>g</sup> with 0.05% Triton [pH, 3.5 to 5.5], 50mM phosphate buffer with 0.05% Triton [pH, 6.0 to 7.5], and 50mM tris buffer with 0.05% Triton [pH, 8.0 to 9.0]). Samples were

centrifuged<sup>h</sup> at 10,000 × *g* at declining temperatures of 18° to 15°C with each buffer exchange cycle. Buffer-exchanged samples were concentrated to a final volume of 50 μL, divided into aliquots to avoid repeated freeze-thaw cycles, and stored at −20°C.

The buffer exchange process was performed in duplicate with and without a protein extraction phase. The protein extraction phase required a 1-hour incubation of the urine sample on ice, with 9.5M urea,<sup>i</sup> 1% dithiothreitol, and 2% CHAPS<sup>j</sup> by means of a urine-to-urea buffer ratio of 8:3. Throughout the present study, the dH<sub>2</sub>O used to prepare buffers was filtered.<sup>k</sup>

Protein concentration for each aliquot was determined.<sup>l</sup> Concentrated buffer-exchanged samples were standardized to a protein concentration of 1 mg/mL with the equilibration buffer of the respective pH immediately before array preparation.

**Manual array handling protocols**—Two array surfaces were evaluated for use with feline urine: weak cation exchange and strong anion exchange.<sup>m</sup> The surface of the weak cation array incorporates a carboxylate group conferring a net negative charge, therefore binding proteins and peptides with a net positive charge at a given pH. The surface of the strong anion array incorporates quaternized ammonium groups that have a net positive charge, therefore binding peptides and proteins that are negatively charged at a given pH.

Array surfaces were manually prepared in accordance with manufacturer's instructions. Briefly, for weak cation exchange and strong anion exchange arrays, spots were equilibrated with 3 μL of the respective buffer (pH of 3.5 to 7.0 for weak cation exchange arrays and pH of 5.5 to 9.0 for strong anion exchange arrays). Arrays were incubated for 5 minutes, and spot surfaces were allowed to dry. All incubations were performed at room temperature (21°C) in a humidified box. Concentrated buffer-exchanged sample was applied to the appropriate spot (2 μL/spot). After 1 hour of incubation, spots were washed 3 times for 5 minutes each with the respective pH binding buffer (3 μL/spot), followed by a final wash with dH<sub>2</sub>O. Arrays were allowed to air-dry before 2 applications of the EAM (sinapinic acid diluted in 50% acetonitrile 0.5% TFA [0.5 μL/spot]). Spots were air-dried between EAM applications and after the final EAM application. The arrays were then protected from light and air and analyzed via SELDI-TOF-MS. All weak cation and strong anion exchange arrays were evaluated in duplicate by use of the concentrated buffer-exchanged sample with and without a 1-hour incubation with urea protein extraction buffer, as previously described.

**SELDI-TOF-MS data acquisition**—Initial data acquisition was generated with a manual-mode SELDI-TOF-MS.<sup>n</sup> Laser intensity, focal point, and matrix attenuation were all evaluated to determine the settings that could optimally be applied to all pH conditions for each array surface. Automated protocols were produced on the basis of these settings. Protein profiles for each array and condition were assessed visually, and optimal conditions for analyzing the feline urinary proteome by weak cation and strong anion exchange arrays were selected through visual assessment of the number and complexity of protein peaks in the spectra.

**Limit of detection**—Pooled urine was used to establish the limit of detection for protein concentration applied to the weak cation and strong anion exchange arrays. Aliquots of urine (500 μL) underwent a protein extraction phase requiring a 1-hour incubation of the sample on ice with 9.5M urea,<sup>i</sup> 1% dithiothreitol, and 2% CHAPS<sup>j</sup> by means of a urine-to-urea buffer ratio of 8:3. Samples were subsequently buffer-exchanged 3 times either at a pH of 8.0 (50mM Tris buffer with 0.05% Triton) for the strong anion exchange array or at a pH of 3.5 (50mM sodium acetate buffer<sup>g</sup> with 0.05% Triton) for the weak cation exchange array and concentrated to a final volume of 50 μL. The protein concentrations of these buffered-exchanged concentrated samples were established.<sup>l</sup> The concentrated samples were serially diluted with their corresponding pH buffer. The strong anion and weak cation exchange arrays were prepared by manually applying serially diluted samples to adjacent spots, according to the manual array preparation method previously described. Arrays were analyzed with SELDI-TOF-MS by the optimized settings. Protein profiles were assessed visually to determine the lowest protein concentration at which clearly defined protein peaks were present.

**Use of SELDI-TOF-MS in cats at risk of developing azotemia**—Urine samples from groups 1 (cats remaining nonazotemic at 12 months) and 2 (cats developing azotemia at 12 months) at entry to the biomarker study were analyzed by SELDI-TOF-MS. Urine samples (500 μL/aliquot for each condition) that had not been subjected to a freeze-thaw cycle were selected and prepared under the following conditions: condition 1, weak cation exchange array buffer-exchanged at a pH of 3.5; condition 2, weak cation exchange array urea protein extraction step and buffer-exchanged at a pH of 3.5; and condition 3, strong anion exchange array urea protein extraction step and buffer-exchanged at a pH of 8.0. All buffer-exchanged, concentrated samples were divided into aliquots to avoid repeat freeze-thaw cycles and were stored at −80°C. For consistency, the same batch of binding buffer at either a pH of 3.5 or pH of 8.0 and the urea protein extraction buffer were used for all samples and for array preparation. One aliquot from each buffer-exchanged concentrated sample was used for the protein concentration assay<sup>l</sup> performed in accordance with the manufacturer's instructions.

**Automated array preparation and data acquisition**—Sample order for analysis by SELDI-TOF-MS was randomized. Immediately prior to array preparation, all buffer-exchanged, concentrated samples were standardized to a protein concentration of 0.2 mg/mL by means of their respective pH binding buffer. The weak cation and strong anion exchange arrays were prepared via an automated robotic preparation system<sup>o</sup> with a bioprocessor unit that allows the simultaneous preparation of 12 arrays/96 samples.

With the bioprocessor gasket in place, arrays were equilibrated with 150 μL of buffer/well (CM10 50mM sodium acetate buffer, 0.05% triton [pH, 3.5], Q10 50mM Tris buffer, and 0.05% Triton [pH, 8.0]) and were incubated at room temperature with shaking for 5 minutes. Binding buffer was then automatically removed

from each well, and the bioprocessor gasket was removed. Spots were dried via capillary action with tissue paper before sample application. Standardized samples were manually loaded into conical-bottom polystyrene 96-well plates<sup>p</sup> (10  $\mu$ L/well). Standardized sample was then applied robotically (2  $\mu$ L/spot) to each array spot and incubated in a humidified box at room temperature for 1 hour. Spot surfaces were allowed to air-dry, the bioprocessor gasket was replaced, and three 5-minute wash cycles, with the respective buffer (150  $\mu$ L/well) and shaking at room temperature, were performed. A final wash with filtered dH<sub>2</sub>O was performed manually after the bioprocessor gasket was removed. Spot surfaces were again dried by capillary action, avoiding any spot-to-spot cross-contamination. The EAM (sinapinic acid<sup>q</sup> diluted in 0.25% TFA and 75% acetonitrile) was applied twice robotically (2  $\mu$ L/spot), with air-drying between matrix applications. This was the minimum volume reliably applied to each array spot via the automated robotic preparation system.<sup>o</sup> Fresh EAM was prepared immediately before use, and arrays were transferred for SELDI-TOF-MS analysis via the optimized protocols for data acquisition.

**Validation of automated array preparation**—A pooled feline urine sample was prepared for weak cation exchange application by buffer exchange and concentration at a pH of 3.5. This sample was standardized

to a protein concentration of 0.2 mg/mL. To examine inter- and intra-array repeatability, the pooled standardized sample was evaluated 6 times on the same weak cation exchange array and 6 times on different weak cation exchange arrays within the same bioprocessor. The samples used to assess inter-array repeatability were analyzed at the same time as the group 1 and 2 samples and randomized as to their location within each array accordingly.

External calibration was performed. A commercial mixed protein calibrant<sup>r</sup> containing 100 pmol/ $\mu$ L of insulin (bovine [5,734.51 Da]), cytochrome c (equine [12,361.96 Da]), apomyoglobin (equine [16,952.27 Da]), aldolase (rabbit muscle [39,212.28 Da]), and albumin (bovine serum [66,430.09 Da]) was applied to a single spot on the first weak cation exchange, and strong anion exchange array and calibration equations were generated and applied to all sample spectra that had been generated via the same sample preparation, array, and data acquisition protocols.

**Statistical analysis**—All statistical analyses of clinical data were performed with computerized software,<sup>s,t</sup> and a value of  $P < 0.05$  was considered significant. Descriptive statistics (median values [25th and 75th percentiles]) were determined to define the biochemical variables and urinalysis results at entry to the study and

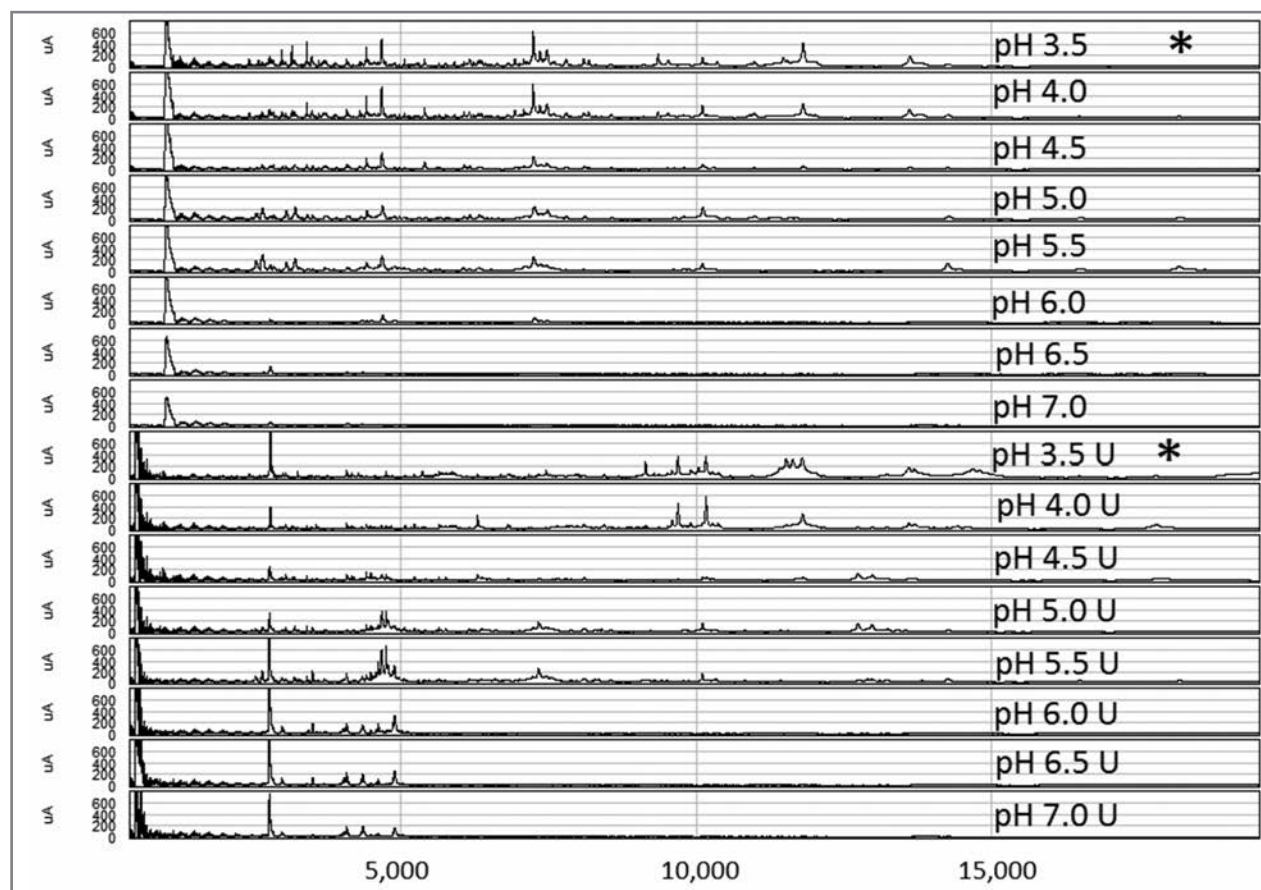


Figure 1—Examples of spectra (m/z, 1,000 to 15,000) generated via SELDI-TOF-MS by use of a weak cation exchange array and a pooled feline urine sample that was buffer-exchanged and concentrated at a pH range from 3.5 to 7.0, with each aliquot standardized to a final protein concentration of 1 mg/mL. The lower 8 spectra were generated after the pooled sample had undergone a denaturation step (indicated by U). Asterisks indicate conditions that were chosen for further analysis. The y-axis values indicate intensity (uA).

at 12 months. A nonparametric Mann Whitney *U* test and Wilcoxon signed rank test were performed to compare variables between group 1 and 2 cats and between variables at entry to the study and at 12 months.

For SELDI-TOF-MS data, automated cluster detection was performed. A cluster represents a single peak that is identified by defined criteria and appears consistently across a specified percentage of spectra in the groups being compared. The first pass detected clusters with a signal-to-noise ratio of > 5.0, a valley depth of > 5.0, and a minimum peak threshold of 20% of spectra. A second pass identified clusters with a signal-to-noise ratio of > 2.0 and valley depth of > 2.0 and also added estimated clusters. By means of computerized software,<sup>14</sup> a Mann-Whitney *U* test was performed to compare clusters between group 1 and 2 cats; a value of *P* < 0.05 was considered significant. Receiver operator characteristics were calculated for each cluster, reaching significance between the 2 groups.

## Results

**Optimization of data acquisition**—The weak cation exchange and strong anion exchange array data were collected between 0 and 100,000 Da with a focal point of 10,000 Da. Matrix attenuation was set as 1,000 Da, and the optimal laser intensity for spectral amplitude was determined as 3,250 and 3,500 for the

weak cation exchange and strong anion exchange arrays, respectively. For all arrays, 1 warming shot was fired at each position with a laser intensity approximately 10% higher than the laser intensity used for data acquisition. Spectra generated from the warming shots were not included in the mean data calculations. One in 4 positions were analyzed on each spot, and 5 laser shots were collected for each position (total shots collected was 530/spot).

**Comparison of peak detection for array surfaces**—Spectra generated with varying pH buffer exchange preparations, both with and without a urea buffer extraction step, were determined for the weak cation exchange and strong anion exchange arrays (Figures 1 and 2). At times when a urea buffer extraction and subsequent concentration step were performed prior to diluting samples to a standardized concentration of 1 mg/mL, the mean CHAPS concentration was 3.5%.

After visual inspection of all spectra, the following conditions were selected as optimal on the basis of peak prevalence and complexity of protein patterns: condition 1, weak cation exchange array with buffer exchange at a pH of 3.5; condition 2, weak cation exchange array with samples undergoing a urea protein extraction and buffer exchange at a pH of 3.5; and condition 3, strong anion exchange array with samples under

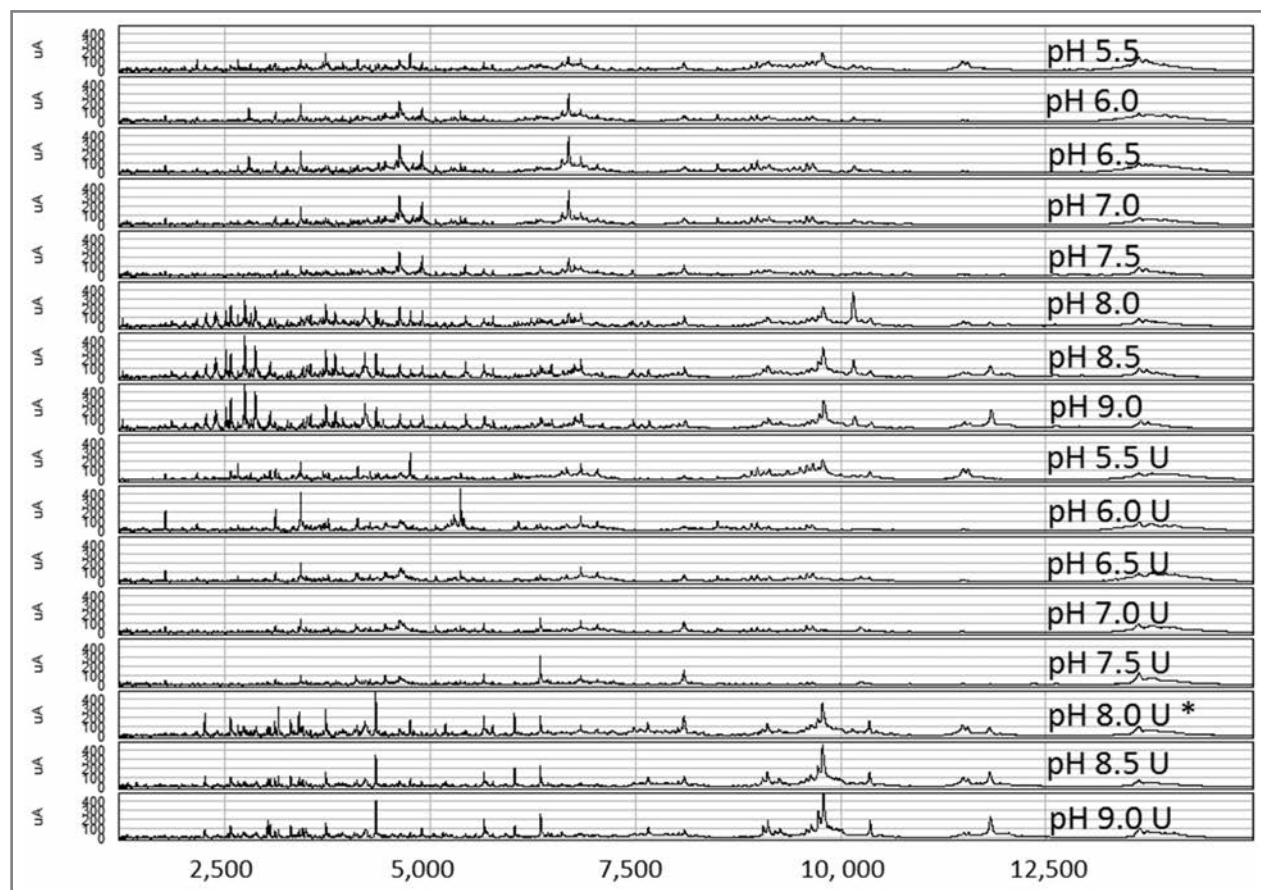


Figure 2—Examples of spectra (m/z, 2,500 to 15,000) generated via SELDI-TOF-MS by use of a strong anion exchange array and a pooled feline urine sample that was buffer-exchanged and concentrated at pH range from 5.5 to 9.0, with each aliquot standardized to a final protein concentration of 1 mg/mL. See Figure 1 for key.

going urea protein extraction incubation and buffer exchange at a pH of 8.0.

**Limit of detection**—Example spectra from the limit of detection study were graphed (Figure 3). A sample protein concentration of 0.1 mg/mL for weak cation exchange and 0.17 mg/mL for strong anion exchange arrays were determined to be the lowest concentrations of protein that still generated spectra with clearly defined protein peaks. From this data, a sample concentration of 0.2 mg/mL was chosen for application of biomarker study samples. This was chosen as the highest concentration that could uniformly be applied to all samples, given the low protein concentration in feline urine, but did not correspond to the concentration that had the highest peak intensity.

**Validation of automated array preparation**—For the intra-array validation, 87 protein clusters were detected, and of these 28 (32%) were common to all 6 spectra. The range of these 28 clusters was 1,023 to 66,116 m/z, and the intensity of these clusters ranged

from 0.9 to 642.9  $\mu$ A. The mean CV of these common clusters was 47.19% (range, 16.05% to 62.08%). The 3 clusters with the greatest intensity were chosen (m/z: 2,820, 9,677, and 10,146) and had an m/z CV of 0.01%. The intensity of these 3 clusters ranged from 107 to 42.9  $\mu$ A, and the mean intensity CV was 42.3%.

For the inter-array validation, 82 protein clusters were detected. Only 22% (18/82) were common to all 6 spectra. For these 18 clusters, the m/z range was 1,044.84 to 66,090 and the intensity ranged from 0.8 to 494.5  $\mu$ A. The mean CV of these common clusters was 51.31 (range, 21.34 to 70.28). The 3 clusters with the greatest intensity were chosen (m/z: 2,820, 9,677, and 10,146) and were found to have an m/z CV of 0.02%. The intensity of these 3 clusters ranged from 90.4 to 494.5  $\mu$ A, and the mean intensity CV was 45.4%.

**Biomarkers for cats at risk of development of azotemia**—Twenty nonazotemic cats were included in the biomarker study, of which 10 remained nonazotemic (group 1) and 10 had developed azotemia (group 2) at 12 months. Clinical data for cats in groups 1 and 2 at

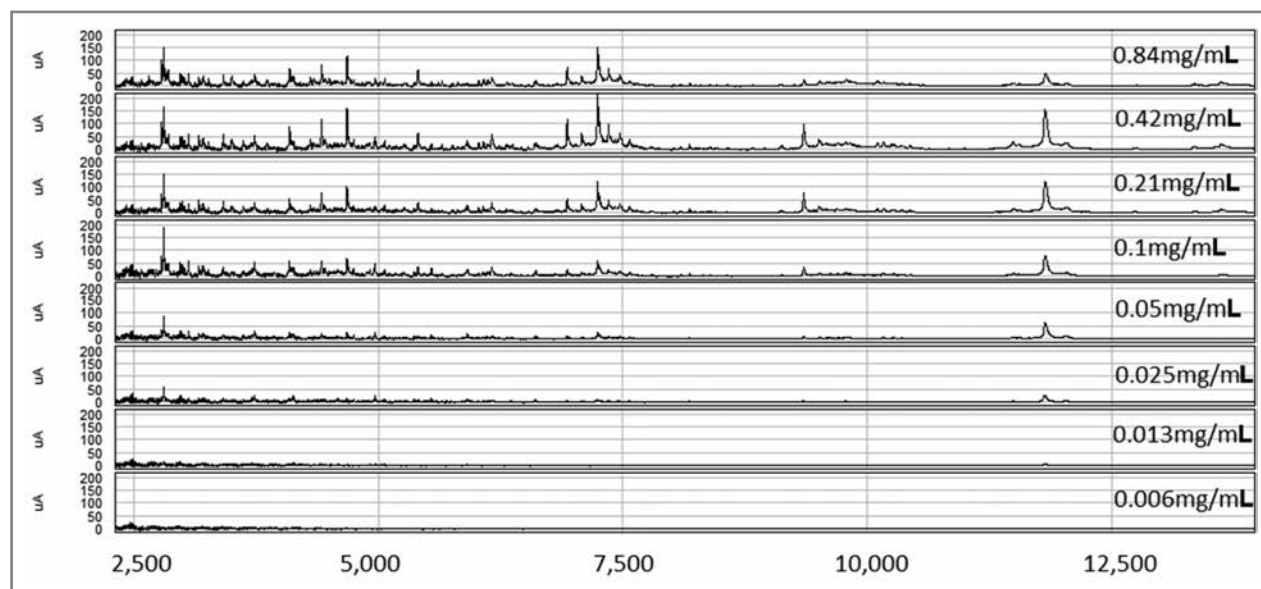


Figure 3—Representative regions of spectra (m/z, 2,500 to 12,500) generated via SELDI-TOF-MS to assess limit of detection by use of a weak cation exchange array and single urine sample that was buffer-exchanged at pH 3.5 and serially diluted.

Table 1—Clinical data collected at entry and after 12 months in a study on the use of mass spectrometry of urinary proteins and peptides as biomarkers for cats at risk of developing azotemia.

Clinical variable	At entry to study			12 months		
	Group 1	Group 2	P value	Group 1	Group 2	P value
No. of cats	10	10	NA	10	10	NA
Age (y)	11.3 (9.6, 12.2)	14.3 (11.0, 15.5)	0.063	12.1 (10.4, 13.2)	15.2 (12.8, 16.1)	0.036
Weight (kg)	4.40 (4.03, 4.93)	4.40 (3.05, 6.00)	0.820	4.36 (4.08, 4.73)	4.50 (2.60, 5.50)	0.685
Creatinine (mg/dL)	1.4 (1.22, 1.68)	1.73 (1.58, 1.77)	0.054	1.40 (1.21, 1.55)	2.17 (2.00, 3.23)	< 0.001
Phosphate (mg/dL)	3.99 (3.04, 4.65)	3.81 (3.60, 4.22)	0.650	4.12 (3.29, 5.58)	4.65 (3.94, 6.48)	0.143
Potassium (mEq/L)	3.70 (3.58, 4.20)	4.05 (3.78, 4.33)	0.138	3.90 (3.68, 4.05)	4.10 (3.78, 4.80)	0.095
SBP (mm Hg)	132 (118, 140)	140 (132, 158)	0.104	128 (113, 144)	144 (117, 170)	0.248
USG	1.044 (1.034, 1.063)	1.039 (1.020, 1.052)	0.495	1.060 (1.046, 1.075)	1.019 (1.014, 1.032)	0.021
UP:C	0.13 (0.11, 0.16)	0.19 (0.13, 0.33)	0.239	0.12 (0.08, 0.20)	0.16 (0.12, 0.30)	0.344

Data are median (25th and 75th percentile) values.

Group 1 = Cats that were nonazotemic at entry to the study and remained nonazotemic for 12 months. Group 2 = Cats that were nonazotemic at entry to the study and developed azotemia by 12 months. NA = Not applicable. USG = Urine specific gravity.

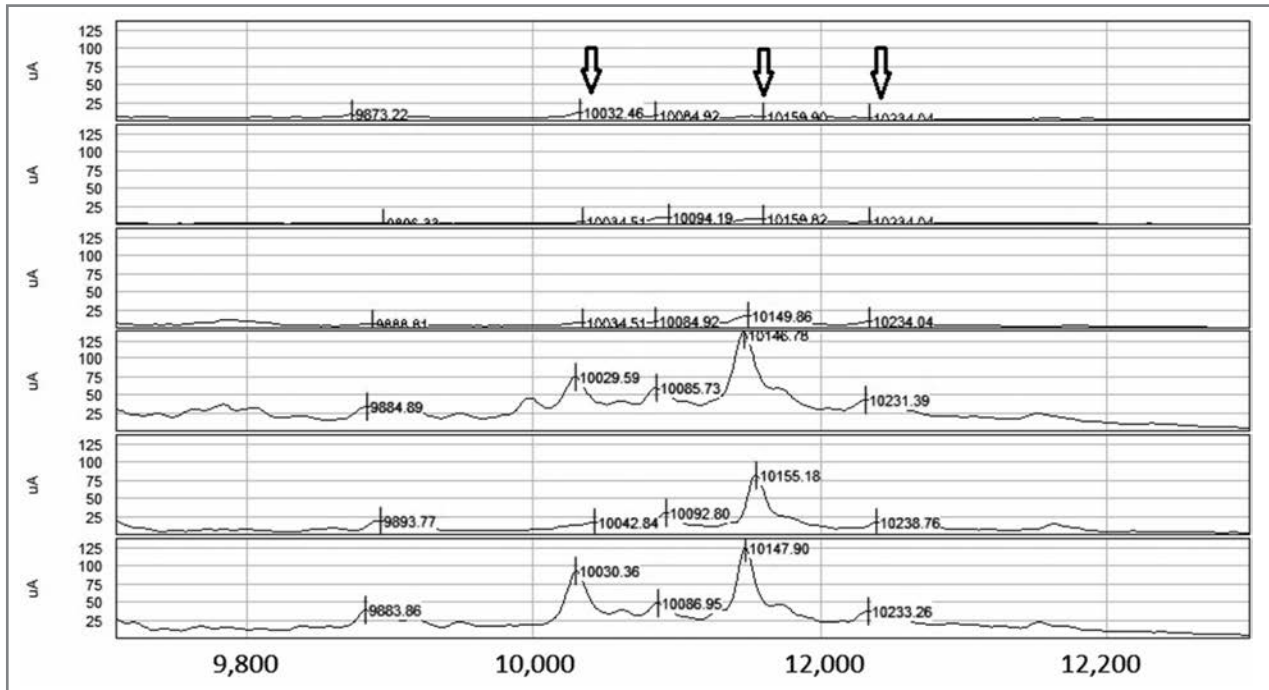


Figure 4—Representative regions of spectra (m/z, 9,000 to 12,200) generated via SELDI-TOF-MS by use of a weak cation exchange array and urine samples that were buffer-exchanged at a pH of 3.5. The upper 3 spectra were generated with urine from 3 cats that remained nonazotemic after 12 months, and the lower 3 spectra were generated with urine from 3 cats that developed azotemia. Arrows indicate examples of clusters that are reduced in intensity in the spectra from cats that remained nonazotemic, compared with cats that became azotemic. Notice 3 clusters that are potential biomarkers with a mean m/z of 10,033, 10,151, and 10,234 in the spectra from cats that developed azotemia. Note that in the upper 3 spectra, an m/z label does not always indicate that a peak was detected. In 6 of 10, 1 of 10, and 6 of 10 nonazotemic cats that remained nonazotemic after 12 months, for clusters with an m/z of 10,033, 10,151 and 10,234, respectively, peak position was estimated.

Table 2—Intensity data for clusters that differed significantly ( $P < 0.05$ ) between groups 1 and 2 in the same cats as in Table 1.

Array condition	Mean m/z	ROC	P value	Fold difference in median intensity
Weak cation exchange (pH, 3.5)	2,822	0.82	0.008	3.19
	9,886	0.82	0.02	2.48
	10,033	0.78	0.05	3.38
	10,151	0.74	0.04	3.04
	10,234	0.78	0.03	2.35
Weak cation exchange urea (pH, 3.5)	11,635	0.78	0.04	3.21
Weak cation exchange urea (pH, 3.5)	4,421	0.82	0.01	2.63
Strong anion exchange urea (pH, 8.0)	9,505	0.74	0.05	2.09

entry to the study and at 12 months were determined (Table 1). There was no significant difference in age, weight, plasma creatinine concentration, phosphorus and potassium concentrations, urine specific gravity, UP:C, or SBP between group 1 and 2 cats at entry to the study. As expected, a significant increase in plasma creatinine ( $P = 0.002$ ) and phosphorus ( $P = 0.019$ ) concentrations was detected for cats in group 2 between entry to the study and the 12-month follow-up visit.

With condition 1, 77 clusters were identified, with 17 common to all spectra. Six clusters with mean m/z values of 2,822, 9,886, 10,033, 10,151, 10,234, and 11,635 determined with the use of condition 1 were significantly different between cats in groups 1 and 2. Examples of the clusters at a mean m/z of 10,033, 10,151, and 10,234 were graphed (Figure 4). With

condition 2, 77 clusters were identified, of which 22 were common to all spectra. However, only 1 cluster for condition 2 (mean m/z, 4,421) was significantly different between groups. With condition 3, 53 clusters were identified, with 23 common to all spectra but of which only 1 cluster (mean m/z, 9,505) was significantly different between groups 1 and 2. The fold difference in median intensity for each cluster in each condition and ROC values was determined (Table 2).

## Discussion

This study determined that LMW proteins and peptides can be delineated in feline urine via proteomic techniques and that the feline urine proteome is more complex than the information ascertained from measurement of either albuminuria or total proteinuria alone. Eight clusters determined with the 3 selected array conditions were significantly different between cats that remained nonazotemic and cats that developed azotemia over a 12-month period.

Clinicopathologic data for the 2 groups of cats were not significantly different at entry to the study. However, some cats that developed azotemia within a 12-month period had higher plasma creatinine concentrations and were older than those cats that remained nonazotemic. Creatinine concentration and a measure of proteinuria, either the urine albumin concentration-to-creatinine ratio or UP:C, have been identified as predictors for the development of azotemia in otherwise healthy nonazotemic geriatric cats, although age in that study was not predictive.<sup>29</sup> There was no significant difference in body weight between groups or visits. How-

ever, because creatinine is produced from muscle, assessment of either body or muscle condition in these cats would have been of interest to ensure that plasma creatinine concentration was not artifactually reduced as a consequence of loss of muscle mass, particularly in the group of cats that went on to develop azotemia.

For all clusters, peak intensity was higher in those cats that developed azotemia (group 2) than in those that remained nonazotemic. Protein concentration for each sample was standardized to 0.2 mg/mL prior to analysis, such that any difference in cluster intensity should represent a genuine difference between groups rather than an excess of filtered protein applied to the array surface. The ROC analysis for clusters identified in this study determined that area under the curve values ranged from 0.74 to 0.82. However, when evaluating the intensity of each cluster, large SDs indicated that there was an overlap between groups and that none of the individual clusters identified clearly discriminated between groups.

Despite SELDI-TOF-MS being a highly sensitive technique, proteomic analysis of feline urine held certain challenges. The low protein concentration precluded direct application of urine to array surfaces. This may reflect differences in underlying renal diseases; for example, humans and dogs are at greater risk of glomerular injury, compared with cats, in which tubulointerstitial inflammation predominates.<sup>3,30</sup> Although direct comparisons among SELDI-TOF-MS studies are difficult to make, it is interesting to note that in the study by Forterre et al<sup>31</sup> that examined dogs with CKD, urine proteins that differed significantly from the controls had m/z values > 10,000 Da, whereas in the study by Nabity et al<sup>32</sup> that examined dogs with X-linked hereditary nephropathy, proteins and peptides with m/z values < 10,000 Da were also identified. Thus, differences in peptides and proteins identified may reflect differences in patient populations, underlying disease processes, sample preparation, and data acquisition.

A further concern with the detection of proteins with an LMW is that they may not represent whole proteins, particularly in urine, which contains endogenous proteases. However, this may also be considered an opportunity because these incomplete proteins may be a rich source of surrogate biomarkers that reflect protease activity within the urine, particularly in the diseased state. There is evidence that fragments of albumin, generated as part of PTC uptake and lysosomal degradation, may be found in urine.<sup>33,34</sup> A study<sup>26</sup> evaluating SELDI-TOF-MS spectra from patients with acute tubulointerstitial renal graft rejection identified a series of clusters with an m/z between 5,269 to 5,550, 7,051 to 7,362, and 10,528 to 11,103, which distinguished these patients from healthy individuals. More recently, it has been reported that all of those clusters represent cleaved products of  $\beta_2$ -microglobulin.<sup>35</sup>

There were a number of limitations to this biomarker study. The group sizes were small, which raises concern regarding extrapolation of candidate biomarkers to the geriatric feline population as a whole. Another concern is the concept of false detection rate. False discovery rate is defined as the expected proportion of false-positive results among declared positive

results.<sup>36,37</sup> By means of highly sensitive techniques such as mass spectrometry, large numbers of potential clusters are identified, which, as in the present study, are individually compared among groups without accounting for multiple comparisons. Although the complexity of urine is substantially less than that of serum or plasma, a proportion of false-positive results would still be expected.

In biomarker discovery studies, variation can be considered to be either biological or technique related and both can have considerable effects on results. In the present study, the authors tried to minimize biological variance through careful selection of cats. However, exclusion of all underlying diseases can never be guaranteed. No attempt was made to assess the inter-individual variation in urine proteome in healthy geriatric cats or to assess the day-to-day variability in the feline urine proteome. Groups were not evaluated for the effect of sex on protein profile. Differences have been found for human male and female urine proteome profiles, but it remains to be investigated whether differences are present among a neutered population of cats.<sup>38</sup> Contamination of urine samples with blood can result in increased peak detection, with the highest intensity peaks representing hemoglobin subunits, which emphasizes the importance of not analyzing hematuric samples.<sup>39</sup> In the study reported here, only samples that had no gross or microscopic hematuria were included and all samples with evidence of a urinary tract infection were excluded. All urine samples were obtained via cystocentesis; therefore, proteins from the urethra and external genitalia were not contaminants.

Technical variation includes variation due to pre-analytical sample processing, the inherent variability of the proteomic technique being applied, instrument performance, and the subsequent calibration of data. Samples in the present study were processed and stored in a standardized manner within the limitations of a veterinary clinic. Several studies have evaluated the effects of urine sample storage and the use of urine protease inhibitors with different array surfaces. Storage of urine at  $-70^{\circ}\text{C}$  and up to 5 repeated freeze-thaw cycles has limited effect on peak detection or intensity with SELDI-TOF-MS, and protease inhibitors are beneficial only for the first 2 hours after sample collection.<sup>38,39</sup> The ratio of TFA:acetonitrile used for dilution of the EAM differed between the optimization and biomarker study. Although the effect of this is likely to be small, this may nevertheless have affected the initial choice of optimized conditions. However, all sinapinic acid was prepared in an identical manner for the biomarker study; therefore, this should not have affected the final study results.

A measure of the technical variation can be obtained by assessing variation in the proteomic profile of replicates of a single sample to evaluate reliability and repeatability. In the present study, samples used in the inter- and intra-array evaluation were all obtained from the same aliquot such that sample preparation factors should not contribute to this variability. To reduce variability as a result of array preparation, a robotic system was used, which meant that samples could be evaluated in fewer assay runs and equilibration and wash steps were standardized. Inter-array samples were randomly



distributed across arrays and also among spots A to H. A study<sup>24</sup> involving the use of human urine found excellent reproducibility of mass determination (CV < 0.05%) with SELDI-TOF-MS, and the inter- and intra-array m/z CVs (0.02% and 0.01% respectively) of the present study were comparable. However, inter- and intra-array peak intensity reproducibility has been less consistent, with CVs obtained with human urine ranging from 14% to 60% for manual-bioprocessor array preparation; reproducibility also depends on the method of peak selection for CV calculation.<sup>24,38,40</sup> The cluster inter- and intra-array intensity CVs in the study reported here were 45.4% and 42.3%, respectively. This indicated that for any potential biomarker, there would need to be at least a 1.5-fold difference in intensity to differentiate the groups, and this was a substantial limitation to this technique for biomarker discovery. Other reasons for increased CVs may include variation in chemical structure of the spot surface, uneven distribution of protein across the spot, the presence of proteins that may interfere with the surface binding of the protein of interest, and variation in the ability of a protein to be ionized and travel in the mass spectrometer. It should also be noted that in the present study, inter- and intra-array variability was performed for 1 array surface and 1 set of conditions only. These values cannot necessarily be extrapolated to other conditions or array surfaces.

Variability in the assessment of clinical samples in the present study also occurred as a result of the preanalytical concentration step, protein quantification, and standardization of the protein concentration to 0.2 mg/mL. However, standardization of the protein concentration prior to application to the array surface enabled direct comparisons to be made between groups, and subjective visual inspection improved the number and complexity of peaks generated. Because of the low sample volume and low protein concentration, it was necessary to apply the concentrated sample directly to the spot surface. This process was always visually inspected, but working with such small volumes carries inherent risk.

Similar to SELDI-TOF-MS studies that involved the use of human urine, the number and distribution of clusters detected were dependent on the array surface and conditions used for sample preparation and were also influenced by the use of a denaturation step.<sup>38,41</sup> Although the use of different conditions and array surfaces can facilitate the detection of particular biomarkers, it inevitably reduces the chance of finding others. Ultimately, as in the present study, financial considerations will always determine the number of array surfaces, conditions, and samples that can be analyzed in a biomarker study.

The present study revealed that feline urine, even in nonazotemic cats, contains many LMW proteins or peptides that may prove to be biomarkers for those cats at risk of developing azotemia. The SELDI-TOF-MS is a semiquantitative method, providing an m/z value and relative intensity value for potential biomarkers. Because only single-charged clusters are selected, the m/z value can be considered equivalent to mass, but further studies are required to separate and identify these prospective

biomarkers. On the basis of the conditions that have been optimized for the preparation of feline urine, the present study provides a basis for power calculations for future SELDI-TOF-MS biomarker studies that use feline urine and also provides important information that may enable fractionation of urine for eventual purification and identification of biomarkers for the development of azotemia.

In feline medicine, the diagnosis of the kidney disease is presently based on assessment of patient's medical history, results of physical examination, evaluation of serum or plasma creatinine concentrations, urine concentrating ability, and magnitude of proteinuria. Biomarkers for the detection of early kidney damage have largely been constrained to those reflecting acute kidney injury and used in a research setting. However, in other fields of veterinary medicine, new biomarker technology is being used to aid practitioners in the diagnosis of occult conditions, such as the use of N-terminal prohormone of brain natriuretic peptide and feline cardiac disease.<sup>42</sup> On the basis of the present study, the ultimate goal would be to develop an autologous quantitative assay for in-clinic use that could be used in conjunction with currently available diagnostic tests to facilitate the early diagnosis of CKD in cats.

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- a. Parks Doppler, model 811B, Parks Medical Electronics Inc, Las Vegas, Nev.
  - b. Mistral 3000, Sanyo-Gallenkamp, Lutterworth, Leics, England.
  - c. Idexx Laboratories, Wetherby, Yorks, England.
  - d. HI 9224 pH meter, Hanna Instruments, Bedfordshire, Leighton Buzzard, England.
  - e. Multistix Urine Chemistry Reagent Strips, Bayer Diagnostics, Newbury, Berkshire, England.
  - f. Vivaspin 500 (3KDa MWCO), Sartorius Mechatronics UK Ltd, Epsom, Surrey, England.
  - g. SIGMA-Aldrich Co Ltd, Gillingham, Dorset, England.
  - h. Multispeed Refrigerated Centrifuge PK121 R, Thermo Scientific, Epsom, Surrey, England.
  - i. Ultrapure Urea 15505.027, Invitrogen Ltd, Paisley, Renfrewshire, England.
  - j. CHAPS 444074J PROLABO, VWR International Ltd, Lutterworth, Leicestershire, England.
  - k. Minisart 0.45 µm (16555) 0297, Sartorius Mechatronics, Epsom, Surrey, England.
  - l. The Better Bradford Assay Kit 23236, PIERCE, Perbio Science UK Ltd, Cramlington, Northumberland, England.
  - m. CM10, Q10 arrays, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, England.
  - n. BioRad Protein Chip SELDI system Enterprise 4000, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, England.
  - o. Biomek 3000, Beckman Coulter (U.K.) Ltd, High Wycombe, Buckinghamshire, England.
  - p. 96 V well microplate, polystyrene 651161, Greiner Bio-One, Stonehouse, Gloucestershire, England.
  - q. Sinapinic acid, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, England.
  - r. ProteoMass Protein MALDI-MS Calibration Kit, MS-CAL3, Sigma-Aldrich Co Ltd., Gillingham, Dorset, England.
  - s. SPSS, version 15.0 for Windows, SPSS Inc, Chicago, Ill.
  - t. GraphPad Prism, version 5.00 for Windows, GraphPad Software, San Diego, Calif.
  - u. Ciphergen Express 3.06 Software, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, England.
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