Comparative Urine Analysis by Liquid Chromatography–Mass Spectrometry and Multivariate Statistics: Method Development, Evaluation, and Application to Proteinuria

research articles

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We describe a platform for the comparative profiling of urine using reversed-phase liquid chromatography-mass spectrometry (LC-MS) and multivariate statistical data analysis. Urinary compounds were separated by gradient elution and subsequently detected by electrospray lon-Trap MS. The lower limit of detection (5.7-21 nmol/L), within-day (2.9-19%) and between-day (4.8-19%) analytical variation of peak areas, linearity (R^2 : 0.918–0.999), and standard deviation for retention time (<0.52 min) of the method were assessed by means of addition of seven 3-8 amino acid peptides (0-500 nmol/L). Relating the amount of injected urine to the area under the curve (AUC) of the chromatographic trace at 214 nm better reduced the coefficient of variation (CV) of the AUC of the total ion chromatogram (CV = 10.1%) than relating it to creatinine (CV = 38.4%). LC-MS data were processed, and the common peak matrix was analyzed by principal component analysis (PCA) after supervised classification by the nearest shrunken centroid algorithm. The feasibility of the method to discriminate urine samples of differing compositions was evaluated by (i) addition of seven peptides at nanomolar concentrations to blank urine samples of different origin and (ii) a study of urine from kidney patients with and without proteinuria. (i) The added peptides were ranked as highly discriminatory peaks despite significant biological variation. (ii) Ninety-two peaks were selected best discriminating proteinuric from nonproteinuric samples, of which 6 were more intense in the majority of the proteinuric samples. Two of these 6 peaks were identified as albumin-derived peptides, which is in accordance with the early rise of albumin during glomerular proteinuria. Interestingly, other albumin-derived peptides were nondiscriminatory indicating preferential proteolysis at some cleavage sites.

Keywords: urine • proteomics • liquid chromatography-mass spectrometry • biomarker • bioinformatics • multivariate statistical analysis

1. Introduction

Biofluids such as serum, plasma, whole blood, and urine are routinely used for diagnostic purposes and in comparative studies. Urine seems a more suitable biofluid than blood and its derivatives (plasma or serum), because it can be obtained in large quantities by noninvasive sampling. Analytical advantages of urine analysis are the less complex sample pretreatment due to the much lower protein content, the relatively small size, and higher thermodynamic stability of urinary

peptides/proteins,¹ and the lower complexity and intermolecular interactions compared to proteins in serum.^{2,3} The majority of pathological changes in human organs may well be reflected in urine. In this way, urine analysis can aid in disease diagnosis, treatment monitoring, and prognosis.⁴ Urine, on the other hand, is prone to larger biological variations than the blood compartment, as it samples the metabolic end products from the organism destined for excretion. Factors affecting the concentrations of these products in humans, for example, age and gender,⁵ can be controlled by careful matching, whereas this is much harder to do for the influence of factors of cultural and dietary nature.6 One of the major challenges in biomarker research using urine is thus the large natural variation in the concentration of peptides, proteins, and metabolites, which requires careful normalization of the measurements.7

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A frequently used analytical technique to profile urinary compounds for biomarker research is liquid chromatographymass spectrometry (LC-MS). The advantages of LC-MS are high sensitivity and the feasibility of detecting thermolabile, water soluble compounds without the need for chemical derivatization. Analysis of urine by LC-MS has been applied in metabolomics,^{8,9} proteomics,^{4,10–15} and peptidomics,^{16–24} as well as in drug metabolism research. LC-MS is sensitive to matrix effects due to the interference of matrix components with the ionization of analytes (ion suppression).²⁵ The use of internal standards and standardization of the injected amount are therefore necessary for quantitative analyses. Comparative profiling using stable isotope-labeled standards²⁶ or dye-labeled proteins as internal standards²⁷ can overcome these limitations but have proven to be laborious and expensive, making a wellcontrolled, label-free quantitative method as described here of particular interest for clinical studies, where large series of samples have to be processed. A quantitative method for profiling complex biological samples without the need for labeling or spiking with internal standards seems feasible as long as there is linearity of signal versus concentration and a high degree of reproducibility of sample processing and the LC-MS platform.28

When profiles of urinary compounds are compared, it may be insufficient to normalize the data based on a single compound like creatinine, as this may not be representative of the wide range of molecules with different physicochemical properties (e.g., molecular weight, pK_a , and hydrophobicity). Since the urinary creatinine concentration, which is widely employed to correct for concentration differences and to express the urinary clearance of blood components, is mainly determined by age, gender, muscle mass, kidney function, exercise, and diet, it is questionable whether this should be the only standard when it comes to accurate data about renal clearance.⁵ To base normalization on a broader molecular basis, we have therefore compared normalization of the injected volume of urine based on a fixed amount of creatinine with a multicompound normalization strategy based on the area under the curve of the chromatogram at 214 nm (AUC $_{214}$).

Data processing prior to multivariate statistical analysis is critical for comparing LC-MS data sets that may reach 10⁷ to 108 data points per analysis or even more, in case highresolution mass spectrometry is used. Data processing has the goal to correct for unwanted variations in data sets by, for example, correcting shifts in retention time and by discriminating meaningful data points from background, noise, and spikes,²⁹⁻³¹ thus, generating a peak matrix suited for further processing by peak matching, missing peak allocation, and, finally, statistical analysis. Even after data processing, there is a need for a further reduction in dimensionality, since the complexity of biological samples like urine generates a great number of significant peaks that generally exceeds the number of analyzed samples with the inherent risk of overfitting the data. One way of further reducing the number of dimensions in the data is by using a regularized linear discriminant classifier method like the nearest shrunken centroid (NSC) algorithm.³² Visual inspection of patterns in the high-dimensional data space is possible through the use of further dimension-reducing techniques such as principal component analysis (PCA).³³ For an extensive review and references on data (pre-) processing and multivariate statistical methods, we refer to the review of Listgarten and Emili.34

In the present work, we describe the development of a simple, rapid, and robust reversed-phase LC electrospray (ESI) Ion Trap MS platform to reproducibly profile urinary compounds. The platform was evaluated using peptides, which are frequently present at nanomolar concentrations in urine. Dedicated data processing and multivariate statistical approaches were developed and applied to the data to obtain proof-of-principle on several aspects of the method such as the following: (i) the ability to discriminate groups of urine samples based on the absence or presence of standard peptides at approximately 2-10 times the lower limit of detection (LOD; nM range), and (ii) the differentiation between the contribution of analytical and biological variation to the final result. Finally, the platform was tested in a preliminary study comparing urine samples from hospitalized patients with and without proteinuria. Using multivariate statistical analysis of LC-MS profiles of urinary compounds is likely to aid in diagnostics, monitoring of disease activity, and therapy. It may also be of great value in forming new hypotheses about disease mechanisms and the effect of therapeutic interventions.

2. Experimental Procedures

Further detailed information is available in Supporting Information.

2.1. Chemicals. Acetonitrile (ACN) (HPLC-S gradient grade), ultrapure water (18.2 M Ω cm), trifluoroacetic acid (TFA) 99% spectrophotometric grade, and formic acid (FA) 98–100% pro analysis were used for reagent preparation. A peptide stock solution [peptide, concentration in mmol/L; VYV, 0.29; YGGFL (leucine enkephalin), 0.20; DRVYIHPF (angiotensin II), 0.10; YPFPGPI (β -casomorphin 7), 0.16; YPFPG (β -casomorphin 5), 0.21; GYPPT (gluten exorphin A5), 0.19; and YGGWL (gluten exorphin B5), 0.20] was used for addition experiments and internal standardization.

2.2. Sample Preparation. Urine samples were stored at -20 °C, thawed, mixed, acidified with 1% TFA, and centrifuged to remove precipitate (5 min at 1500g and 4 °C). The supernatant was diluted 1:1 with 0.2% FA in 10% ACN and stored at 4 °C until analysis. The injection order on the LC–MS was randomized. A pooled urine sample was prepared by mixing equal volumes of urine from seven apparently healthy adults (creatinine: 10.6 mmol/L).

Urinary creatinine concentration and total protein content were assayed on an autoanalyzer (MEGA, Merck, Darmstadt, Germany). The study protocol was in agreement with local ethical standards and the Helsinki declaration of 1964, as revised in 2004.

2.3. Reversed-Phase HPLC-MS. All LC-MS analyses were performed on an 1100 series capillary HPLC system equipped with a cooled autosampler (4 °C), a UV detector ($\lambda = 214$ nm) and an SL ion trap mass spectrometer (Agilent Technologies). Urine samples were desalted on an Atlantis dC₁₈ precolumn $(2.1 \times 20 \text{ mm}, 3 \mu \text{m} \text{ particles and } 10 \text{ nm pore diameter})$ using 0.1% FA in 5% ACN at a flow rate of 50 μ L/min for 16 min. Urinary compounds were back-flushed from the precolumn onto a thermostated (25 °C) Atlantis dC18 analytical column $(1.0 \times 150 \text{ mm}, 3 \,\mu\text{m} \text{ particles}, 30 \text{ nm pores})$ and separated in 90 min at a flow rate of 50 μ L/min during which the percentage of solvent B (0.1% FA in ACN) in solvent A (0.1% FA in ultrapure H₂O) was increased from 5.0 to 43.6% (0.43%/min). During these 90 min, UV absorption and positive mode MS spectra were acquired. Settings for ESI and mass analysis were as follows: 16.0 psi N₂; drying gas, 6.0 L/min N₂; T, 325 °C; cap. voltage, 3.8 kV; skimmer, 57.5 V; cap. exit, 190.7 V; oct. 1, 4.12 V; oct. 2, 2.49 V; oct. RF, 190.7 V; lens 1, -4.9 V; lens 2, -37.7 V; trap drive, 52.5; scan speed, 5500 m/z s⁻¹; 50 ms accumulation time or 30 000 ions; scan range, 100–1500 m/z; Gaussian acquisition filter (width 0.1 m/z) of each scan; rolling average of 5 spectra. Spectra were saved in the centroid mode. Following the gradient, both columns were washed with 85% B for 5 min and equilibrated with 5% B for 10 min prior to the next injection.

Internal standards were injected onto the precolumn prior to injection of the urine samples. Between each injection, the injection system was cleaned with 70% ACN and filled again with 0.1% FA in 5% ACN. The amount of internal standard (IS) peptides injected varied per experiment (see Results for more details).

2.4. Tandem Mass Spectrometry and Database Searching. NanoLC ESI quadrupole time-of-flight (Q-TOF) MS/MS (API QSTAR Pulsar i LC/MS/MS System [Applied Biosystems, MDS Sciex, Framingham, MA]) was used in the Auto-MS² mode (precursor ions >100 counts, 1+ to 4+ ions; spray voltage 2350V; varying collision energies). Using mascot.dll scriptprocessing in Analyst QS 1.1, build 9865 (Applied Biosystems, MDS Sciex) MS spectra were deconvoluted with respect to charge state and isotopes. The resulting spectra were saved in mascot (Matrix Science, London, U.K.) generic file format and submitted to an in-house version of the MASCOT³⁵ search engine (v1.9.05) for UniProt (release 7.7) queries and to a Webbased version of the Phenyx36 search engine (v2.1) for UniProt Sprot (r. 48.8 of 10-Jan-2006) gueries. Enzyme settings were based on chymotrypsin-, trypsin-, and caspase-like proteolytic activity of the 26S proteasome in renal cells³⁷ allowing "half cleavages (Phenyx)" of peptide bonds. Search outcomes were evaluated at a significance level of a probability-based Mowse score for MASCOT of ≥ 61 (P < 0.001) and at an ACscore of ≥ 5 for Phenyx. The latter is the sum of the best scores per valid peptide sequence.

The identities of discriminatory peaks, as selected by multivariate statistics, from Ion-Trap and quadrupole-TOF MS data, were confirmed by comparing mass spectra and relative retention times using bracketing between added standard peptides. MS/MS data from these discriminatory peaks were processed using MASCOT scripts to obtain a peak list of fragment ions suitable for MS/MS ion search. Prior to subsequent database queries, these peak lists were tagged and added to the original MS/MS peak list used for protein identification to evaluate whether they originated from one of the previously identified proteins. A peak was considered to be identified if the Phenyx *z*-score \geq 4, *P* \leq 0.0001, and retention time of the peptide were within 0.05 min and 0.01 amu of the respective discriminatory peak. If the peak/peptide did not reach these criteria, manual conversion of the monoisotopic multiply charged fragment ions to monoisotopic singly charged fragment ions was performed prior to another round of searches using MASCOT and Phenyx.

Further detailed information is available in Supporting Information.

2.5. Data Processing and Analysis.

2.5.1. Data Analysis for Method Evaluation. LC–MS chromatographic data were analyzed with Data Analysis software for LC/MSD Trap, version (3.2 build 121) (Bruker Daltoniks, Bremen, Germany). Peak areas and intensities of the spiked peptides described in Table 1 were obtained from the respective smoothed and baseline-subtracted extracted ion chromato-

Table 1. Peak Area and Intensity Variation of Peptides Addedto Urine after Normalization of the Injected Amount toCreatinine or UV-Area

	peptide	Creatini	Creatinine $[CV^b (\%)]$		$AUC_{214}^{a} [CV^{b} (\%)]$		
no.	sequence	area	intensity	area	intensity		
1	VYV	30.1	22.2	23.3	20.7		
2	GYYPT	19.3	16.2	13.2	11.9		
3	YPFPG	19.2	15.1	11.3	11.6		
4	DRVYIHPF	13.7	13.3	25.6	23.3		
5	YGGFL	21.5	16.3	5.9	6.1		
6	YGGWL	11.5	11.1	7.1	9.7		
7	YPFPGPI	20.4	20.1	15.4	17.6		
$AUC_{TIC}{}^{c} \\$			38.4		10.1		

^{*a*} Area under the curve of the UV-chromatogram ($\lambda = 214$ nm) between 30 and 80 min retention time. ^{*b*} Coefficient of variation. ^{*c*} Area under the curve of the total ion chromatogram between 30 and 80 min retention time.

grams (EIC). One cycle of smoothing with a Gaussian algorithm at a width of 1.8 points preceded baseline subtraction with a flatness of 1. The AUC_{214} was calculated between 30 and 80 min retention time, which corresponds to 14-64 min for mass spectrometric data acquisition (MS data acquisition was started 16 min later than the gradient program) during gradient elution.

Univariate statistical analyses were performed with the Statistical Product and Service Solutions package version 11.5 (SPSS, Inc., Chicago, IL).

2.5.2. Data (Pre-)Processing. For processing and multivariate statistical analysis, the original Bruker Daltoniks LC–MS data files were converted into ASCII-format with the Bruker Data Analysis software. For further data analysis, Matlab (version 7.0.0.19920, Mathworks, Natick, MA) and the PLS toolbox (version 3.5.2, Eigenvector Research, Inc., Wenatchee, WA) were used.

Initially, the nominal m/z ratios were rounded to the nearest integer to 1 amu bins (instead of the original 0.1 amu in the acquired data) according to Radulovic et al.,38 which is adapted to the accuracy of the ion trap (± 0.3 amu). Binning reduced the amount of data by roughly a factor of 10, and it also partially corrected for the slight shift in m/z values as a result of trap overfilling occurring during peak elution. For signal filtering and background reduction, data was first smoothed using a moving average filter (3-scan header width, 2 cycles). A modified M-N rule was applied for peak detection in which a predefined baseline T (30% of trimmed mean) was multiplied by M (set at 2) to set the threshold (1000 counts), which should be exceeded by the peak intensity for at least N (set at 5) consecutive observations in the time dimension.³⁸ If T was lower than 1000 counts, $M \times 1000$ was used as a threshold for that bin. For each bin, we included the m/z bin value, intensity of the data point with the highest intensity, and the mean retention time of the three data points with the highest intensity to generate the peak list.

We used a similar approach as Radulovic et al.³⁸ to obtain optimal settings for *M* and *N*. *M* (1.5–4) and *N* (4–8) were applied to two blank LC–MS runs and two LC–MS runs of the pooled urine sample. The settings at which the ratio between the number of peaks (between 30 and 80 min and broader than 0.3 min at baseline × *N*) in the sample and in the blank was the highest and at which a minimal number of peaks was extracted from the noise in the blank chromatogram were used.

Time alignment of the chromatograms was not needed, because the median (range) standard deviation of the retention time of the standard peptide peaks was only 0.37 (0.23-0.52)

min. One-dimensional peak matching and missing peak allocation in different samples was realized by using a sliding windows technique in which similar m/z bins are evaluated for peaks proximate in time (step size, 0.1 min; search window, 1.0 min; maximal accepted difference of centroided retention time within a group of matched peaks, 0.75 min). Missing peak allocation was performed by extracting the background in given m/z bins at the mean retention time of the other identified peaks. The generated final peak matrix, created from the peak matrices of the individual samples, consisted of a peak(*row*) – sample(*column*) – intensity(*value*) matrix. This final peak matrix was used for multivariate statistical analysis.

All data preprocessing work was done on a personal computer equipped with a +3600 MHz AMD processor and with 4 GB of RAM.

2.5.3. Classification and Multivariate Statistical Methods. To select the most discriminating peaks, we applied the NSC classification algorithm.^{32,39} NSC regularizes data whereby classspecific centroids are "shrunk" toward the overall (nonclassspecific) centroid, which has the effect of eliminating the influence of the most weakly correlated peaks, thereby reducing the capacity to overfit.34 This algorithm is used to select peaks that are relevant for the discrimination of the predefined classes in conjunction with permutation tests to validate the classification algorithm⁴⁰ using leave-one-out cross-validation (LOOCV) to avoid overfitting due to one outlier. The optimal shrinkage value was the value at which LOOCV showed the lowest classification error. In LOOCV, one observation per class is iteratively omitted from the data set that is used to construct the classification model, which is then used to classify the omitted observation as case or control. Variables selected at the highest shrinkage value and lowest LOOCV error were employed for construction of the final classification model. The selected peaks were then analyzed and visualized by plotting the first two principal components obtained after PCA.33 As a measure for class separation, the Mahalanobis distance (MD) was calculated.⁴¹ We consider an MD above the cutoff of 6.0, corresponding to a difference of 6 sigma between the mean centroids of the classes,⁴⁰ as indicative for significant class separation.

3. Results

3.1. Method Development and Evaluation.

3.1.1. Optimization of RP-HPLC. Resolution between the spiked peptides (see Experimental Procedures) was optimized by varying gradient steepness and flow rate in a central composite design. The goal was to achieve optimal resolution within a maximal runtime of 90 min and a flow rate of 50 μ L/ min, which is adapted to the ESI-source and the column diameter of 1 mm. Optimal resolution was obtained at a gradient steepness of 0.43% solvent B/min. Runs of urine samples of 6 healthy adults without the added peptides assured that there were no detectable peaks in the respective EIC at the retention times of the added peptides. A typical chromatogram of 9.6 μ L of urine spiked with 0.25–0.73 pmol of standard peptides under these conditions is shown in Figure 1A. For two peptides (DRVYIHPF and YPFPGPI), the mono- and diprotonated ions were extracted and combined into one trace, whereas for the other peptides, only the monoprotonated ions were observed and thus extracted (Figure 1B). Figure 1C shows the corresponding chromatographic trace at 214 nm of the inline UV-detector.



Figure 1. Analysis of a pooled urine sample spiked with nanomolar concentrations of internal standard peptides. (A) Smoothed and baseline-subtracted base peak chromatogram of a pooled urine sample (9.6 μ L injected, equivalent to 50 nmol creatinine) co-injected with 5 μ L of 1:2000 diluted peptide stock solution, after optimization of the resolution by varying gradient steepness in a central composite design. (B) Smoothed and baselinesubtracted extracted ion chromatograms (EIC) of the seven peptides used for optimization and internal standardization (same sample as shown in panel A). Peptide peaks correspond to *n*-times the lower limit of detection (LOD): peptide ($n \times \text{LOD}$) 1, VYV (3.9); 2, GYYPT (2.3); 3, YPFPG (4.6); 4, DRVYIHPF (2.2); 5, YGGFL (9.5); 6, YGGWL (3.5); and 7, YPFPGPI (7.3); the absolute values for the LOD can be found in Table 2. The calculated LOD for VYV appears to be lower than the LOD that was derived from the signal-to-noise ratio of the peak shown in Figure 1B. Peptide m/z values and retention times derived from the analysis of diluted stock solutions were used to extract and appoint peaks to the standard peptides spiked into urine. (C) Smoothed and baseline-subtracted UV chromatogram ($\lambda = 214$ nm) of the same urine sample as shown in panel A.

3.1.2. Normalization of Injected Amount of Urine to Creatinine or the AUC214. Since ESI is sensitive to matrix effects leading to ion-suppression,²⁵ it is important to standardize the injected amount of urine in comparative analyses. Assuming that matrix effects are proportional to the injected amount, two methods of normalization were investigated: (i) to a given amount of creatinine (50 nmol) and (ii) to a fixed AUC₂₁₄ value $(1.02 \times 10^5 \text{ absorbance units [AU]}; \text{ see below for the calcula-}$ tion). Creatinine was chosen, because of its frequent use in clinical chemistry, and the UV-area at 214 nm between 30 and 80 min (AUC₂₁₄) was chosen, because it normalizes the injected amount based on a large number of compounds, which is likely to be more representative of the overall sample composition. Moreover, normalizing to the AUC₂₁₄ rather than to the AUC of the total ion chromatogram (AUC_{TIC}) avoids confounding effects resulting from ion suppression, since extinction coefficients are characteristic of each compound as long as the linear range of the UV-detector is not exceeded. By starting integration at 30 min, we avoided the contribution of very small and very hydrophilic compounds, for example, small hydrophilic organic acids, to the AUC₂₁₄.

To investigate which normalization method of the injected amount (injection volume) was preferable, 10 different 24-h urine samples of children (male/female 5:5; age range 6.8–13.6 years) with creatinine levels ranging from 2.0 to 10.6 mmol/L were analyzed. The initial sample volume injected onto the column was equivalent to 50 nmol creatinine, and the AUC₂₁₄ between 30 and 80 min was a measure of the overall amount of compounds injected. From this area, a new injection volume was calculated keeping the 'injected AUC₂₁₄' constant at 1.02 \times 10⁵ AU, which corresponds to an average amount of 50 nmol creatinine for the 10 urine samples. Five microliters of the 1:500 diluted peptide stock solution (1.0–2.9 pmol per peptide) was co-injected to evaluate which method of standardization resulted in the most repeatable peak areas for each peptide.

Overall, peptide MS signals showed less variation when the injected volume was normalized to a fixed AUC₂₁₄ value as compared to a constant amount of creatinine (Table 1). Similarly, the AUC_{TIC} between 30 and 80 min (dead time between UV detector and ion source was approximately 5 s), which reflects all of the MS-detected urinary compounds, improved with respect to the coefficient of variation (CV) from 38% when normalized to creatinine to 10% when using the AUC₂₁₄ value (Table 1). Generally, these results indicate that adjusting the injected amount of urine based on the AUC₂₁₄ is preferable to minimize variations in peak area and intensity due to matrix effects, although this seems component-dependent. A drawback of using the UV-area is that one compound eluting at 35.0 min retention time determined a large portion (median [range]; 44% [14–62%]) of the total AUC₂₁₄.

3.1.3. Method Evaluation. The linearity and lower limit of detection (LOD) of the optimized LC–MS method were determined by duplicate analyses of pooled urine samples spiked with peptides at concentrations of 0 (n = 5), 10, 25, 50, 100, 150, 250, and 500 nmol/L (calculated for YGGFL). Calibration curves were based on the respective smoothed and baseline subtracted EIC. Least-squares linear regression analysis was employed. The LOD is calculated from the intercept of the *y*-axis with the upper limit of the 95% confidence interval of the calibration curve.⁴²

The method exhibited good linearity (all curves P < 0.001) with R^2 values ranging from 0.918 to 0.999 for peak area and intensity over the concentration range (data not shown). The

Table 2. Lower Limits of Detection (LOD) of Seven Peptides

 Used as Internal Standards

		m/z values		
		of EICs ^a	LOD	LOD
	peptide	$([M + H]^{1+},$	(concentration)	(amount)
no.	(sequence)	$[M + 2H]^{2+})$	(nmol/L)	(fmol injected)
1	VYV	380.2	19^{b}	186^{b}
2	GYYPT	600.3	21	204
3	YPFPG	580.3	12	113
4	DRVYIHPF	1046.5, 523.8	12	114
5	YGGFL	556.3	5.7	54
6	YGGWL	595.3	15	143
7	YPFPGPI	790.4, 395.7	5.7	55

^{*a*} Extracted ion chromatogram. ^{*b*} The reported LOD, calculated from the intercept of the *y*-axis with the upper limit of the 95% confidence interval of the calibration curve, appears to be lower than the LOD derived from the signal-to-noise ratio of peak VYV depicted in Figure 1B.

Table 3. Within-Day (n = 5) and Between-Day (5 days, n = 5) Variation of Internal Standard Peptides Added to a Pooled Urine Sample

		within-day			between-day			
	amount	area	intensity	RT ^a	area	intensity	RT	
peptide	injected	CV^b	CV	SD^{c}	CV	CV	SD	
(sequence)	(pmol)	(%)	(%)	(min)	(%)	(%)	(min)	
VYV	0.73	12	11	0.26	18	14	0.49	
	2.9	6.0	12	0.50	7.2	9.5	0.40	
GYYPT	0.48	16	11	0.42	19	17	0.35	
	1.9	7.2	9.3	0.43	12	14		
YPFPG	0.53	12	7.3	0.25	13	10	0.37	
	2.1	3.2	4.4	0.25	6.5	8.7		
DRVYIHPF	0.25	19	12	0.22	5.3	6.0	0.43	
	1.0	7.4	13	0.25	8.1	9.1		
YGGFL	0.50	6.2	5.8	0.00	6.2	8.3	0.44	
	2.0	2.9	3.1	0.26	7.3	7.8	0.44	
YGGWL	0.50	3.0	4.5	0.27	4.8	5.3	0.47	
	2.0	3.5	3.6		6.6	6.8	0.47	
YPFPGPI	0.40	6.5	8.3	0.00	8.5	6.6	0.52	
	1.6	2.9	4.4	0.29	5.2	7.0		

^{*a*} RT, retention time. ^{*b*} CV, coefficient of variation. ^{*c*} SD, standard deviation.

LOD ranged from 5.7 to 21 nmol/L, depending on the peptide (Table 2). This corresponds to an injected amount of 54-204 fmol per peptide (Table 2). Figure 1B shows the EIC of the respective peptides at the calculated 2–10 times their LOD (i.e., 0.25-0.73 pmol injected or 50-145 nmol/L). The calculated LOD for VYV appears to be lower than the LOD that was derived from the signal-to-noise ratio of the peak shown in Figure 1B.

The within-day and between-day analytical variation of the method were determined by repetitive analyses (n = 5) of the pooled urine sample (equivalent to 50 nmol creatinine) injected together with 5 μ L of a low (1:2000 diluted peptide stock solution, 0.25–0.73 pmol) and a high (1:500 diluted peptide stock solution, 1.0–2.9 pmol) amount of added peptides on the same day and on five consecutive days, respectively. Outliers were removed ($\alpha = 0.05$) using the Dixon test⁴³ and Cochran's maximum variance test.⁴⁴ Overall, the within-day and between-day variation ranged between 2.9 and 19% for the various peptides (Table 3).

3.2. Data Processing and Multivariate Statistical Analysis of LC–MS Profiles of Urinary Compounds.

3.2.1. Data Processing and Analysis. Binning from an instrumental resolution of 0.1 to 1 amu reduced the size of the data set to reach an acceptable data processing and analysis time on a normal desktop computer (see Experimental Proce-



Figure 2. Description of the analytical variation in urine profiles from a pooled urine sample analyzed six times. (A) The principal component (PC) analysis plot using the complete peak matrix of 10 029 peaks shows overlap between repetitions of the blank (purple ♦) and spiked (gray ★; 2-10 times lower limit of detection of standard peptides) pooled urine sample. Together, PC 1 and PC 2 explain only 30.39% of the variation between the samples. (B) The biplot depicts the same blank (purple ♦) and spiked (gray ★) samples as in panel A together with 17 of the 10 029 most discriminating peaks (red v) selected by the nearest shrunken centroid classification method at a leave-one-out cross-validation error of 0. Arrows indicate peaks that do not belong to the added peptides. In contrast to panel A, PC 1 and PC 2 now explain a much larger portion (85.65%) of the variation between the samples. Ellipses circle groups of samples belonging to the same class, i.e., blank and spiked.

dures for specifications). The M-N rule proved to be a simple, fast, and robust peak picking algorithm, which generated a peak list within 1 h per sample. The input, that is, the number of samples, determined the time required for peak matching, missing peak allocation, and construction of the final peak matrix (see Experimental Procedures for details).

3.2.2. Multivariate Statistical Comparison of Pooled Urine Samples To Assess the Effect of Analytical Variation. LC–MS data from repetitive analyses (n = 6) of 11.1 μ L injections of blank pooled urine (AUC₂₁₄ = 1.02 × 10⁵ AU) and of the same urine spiked at 2–10 times the LOD (n = 6) were processed as described before to obtain a final matrix containing 10 029



Figure 3. Description of the analytical and biological variation in urine profiles from six healthy individuals. (A) The principal component (PC) analysis plot using the complete peak matrix of 14 234 peaks shows overlap between the blank (blue •) and spiked (green ■; 2-10 times lower limit of detection of standard peptides) urine samples of six apparently healthy adults, and six repetitive analysis of a blank (n = 6; purple \blacklozenge) and a spiked (n =6; gray \star) pooled urine sample. Co-localization (red ellipses) of the blank and spiked samples from the same individual (for 5 out of 6 individuals) and the pool suggests biological variation to be the main determinant of the observed variation between samples. Unsupervised PC analysis obviously provides insufficient discriminatory power to detect the variation caused by spiking. Together, PC 1 and PC 2 explain 45.74% of the variation between the samples. (B) The biplot depicts the same blank (blue ●) and spiked (green ■) samples as in panel A together with 16 of the 14 234 most discriminating peaks (red **v**) selected by the nearest shrunken centroid classification method at a leave-oneout cross-validation error of 0. PC 1 in the biplot explains 78.51% of the variation between blank (blue ●) and spiked (green ■) samples. Two peaks (X and Y) could not be related to the added peptides.

peaks. This peak matrix was used to construct a PC score plot of the two first principal components (PC 1 and PC 2) best explaining the total variance of the data (Figure 2A). When all 10 029 peaks were used, the plot showed a strong overlap between the blank (purple \blacklozenge) and spiked (gray \bigstar) group, which was confirmed by an MD of 0.43 (nonsignificant).



Figure 4. Univariate comparison of peak areas and intensities of peptides/peaks discriminating blank from spiked urine samples from different individuals. Graphs represent univariate paired comparisons (*P*-values) of peak area (A) and intensity (B) of smoothed and baseline-subtracted extracted ion chromatograms from raw LC-MS data of the peptides/peaks selected by the nearest shrunken centroid classification method. Bars represent the mean values (error bars: ± 1 SD). The median (error bars: minimum-maximum) is presented for the area of 356.5 *m/z* and the intensity of peptide VYV. Comparison was performed with the Wilcoxon signed ranks test (nonparametric) and the paired Student's *t*-test (parametric) dependent on the normality of the distribution (Shapiro-Wilk's test).

Application of the NSC classification method yielded 17 highly discriminatory peaks (red \mathbf{v}) out of the original peak matrix at a shrinkage value of 3.0 with a LOOCV (see Experimental Procedures) error of 0 (Figure 2B). Fourteen (82%) of these 17 peaks could be traced back to the added standard peptides based on m/z values and retention times. These 14 peaks were elevated in the spiked group as expected (data not shown). Of the remaining three peaks (arrows), two were located in the region of the blank urine sample and one was located in the spiked samples area. Univariate comparison revealed no significant difference between peak areas (P > 0.5)indicating that these peaks were selected by chance. The higher number of selected peaks relative to the number of spiked peptides is due to the fact that the original data were not deconvoluted with respect to charge state or isotopic distribution. However, this did not affect the classification of samples. The PCA biplot, based on the 17 selected peaks, showed clear discrimination between the blank and the spiked samples, which was confirmed by an MD of 9.91 (significant). Urine samples spiked at nanomolar concentrations with peptides can thus be discriminated using the developed LC-MS method



Figure 5. Comparison of urine samples from proteinuric and nonproteinuric patients with renal disorders. The biplot (A) shows the urine samples from 6 patients with proteinuria (green \blacksquare ; ≥ 1 a/L total protein concentration in a random portion of urine) and from 6 patients without proteinuria (blue \bullet ; \leq 0.1 g/L) but with a medical history of renal morbidity. Ninety-two peaks (red v) out of 11 867 were selected for their discriminatory properties by the nearest shrunken centroid classification method at a leave-oneout cross-validation error of 0. The arrow indicates an upregulated peak in the nonproteinuria group. When this model was used, classification of 24 samples from apparently healthy individuals (purple ♦) resulted in a dense cluster (purple ellipse) that was separated from the nonproteinuric patients (B) implying large differences in the composition of urine between healthy individuals and hospitalized patients with or without proteinuria. The enlarged area in panel B shows that some patients without overt proteinuria show a trend in PC 1 placing them at various levels toward overt proteinuria.

followed by supervised peak selection with cross-validation and visualization of the first two principal components.

3.2.3. Multivariate Statistical Comparison of Urine Samples from Different Individuals To Assess the Effect of Biological Variation. LC–MS data obtained from the analysis of blank and spiked (2–10 times LOD) urine samples (6.6–19.8 μ L injected; AUC₂₁₄ = 1.02 × 10⁵ AU) of six healthy individuals (male/female 3:3; median age [range] 27 years [25–30]) yielded a matrix containing 14 234 peaks. To relate analytical variation to biological variation, we generated a common matrix of



Figure 6. Univariate comparison of peak area and intensity of 6 peaks discriminating proteinuria from nonproteinuria. Represented are box and whisker plots and the corresponding *P*-values for univariate comparisons of peak area (A) and intensity (B) of smoothed and baseline-subtracted extracted ion chromatograms (EIC) from raw LC–MS data of 6 discriminating peaks selected by the nearest shrunken centroid (NSC) classification method. Comparison was performed with the Mann–Whitney U test (nonparametric) and the Student's *t*-test (parametric) dependent on the normality of the distribution (Shapiro-Wilk's test). Panel C shows the EIC of two discriminatory peaks selected by the NSC algorithm for the proteinuric (blue) and nonproteinuric (red) samples.

14 234 peaks including the samples described under Section 3.2.2, which reflect analytical variation only.

Figure 3A shows a PC score plot generated with the entire peak matrix. Blank (blue \bullet) and spiked (green \blacksquare) samples of the same individual colocalized in 5 out of 6 cases. This indicates that the analytical variation is much smaller than the biological variation between different healthy individuals. Colocalization of the data points from the repetitive analyses of blank (purple \bullet) and spiked (gray \star) pooled urine samples (analytical variation only) emphasizes the importance of biological variation relative to analytical variation further. Moreover, pooling the urine of these six healthy individuals averaged the explained variation represented by PC 1 and PC 2 (both around 0) out.

It was, thus, of importance to assess whether spiking of the seven standard peptides at 2-10 times the LOD could still be discriminated despite the observed biological variation. The

NSC algorithm yielded 16 discriminatory peaks (red \checkmark) from the original peak matrix at a shrinkage value of 1.85 with an LOOCV error of 0. Fourteen (88%) of these 16 peaks could again be related to 6 of the 7 added peptides based on m/z values and retention times (Figure 3B). Peak areas and intensities, derived from the EIC of the first two isotopic peaks of the NSCselected peptide peaks, were compared by univariate statistics (Figure 4). P-values were less than 0.001 for area (Figure 4A) and intensity (Figure 4B) for 5 out of the 7 spiked peptides, whereas no significant difference was found for the two other peaks that the NSC algorithm had selected as being discriminatory. One of these peaks (peak X in Figure 3B; 694.6 m/z) coeluted with one of the spiked peptides (YPFPGPI; [M+ H]⁺ = 790.4 m/z). The nonsignificant decrease in peak area and intensity of this signal after spiking suggests an ion-suppression effect. We could not clarify the reason why the peak at 80.85 min (peak Y in Figure 3B; m/z = 356.5) was selected as being

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Table 4. Proteins in a Proteinuric Urine Sample Identified byLC-MS/MS and Subsequent UniProt Database Search by theMASCOT Algorithm

protein	accession no.	score ^a	cov. (%) ^b	no, peptides ^c
α -1-antitrypsin precursor ^d	P01009	1090	43	16/35
serum albumin precursor ^d	P02768	445	11	4/19
haptoglobin precursor ^d	P00738	173	14	1/9
serotransferrin precursor ^d	P02787	153	7	2/7
α -1-acid glycoprotein 1 precursor ^d	P02763	117	12	1/7
apolipoprotein A-I precursor ^d	P12270	103	9	1/3
hemoglobin β -subunit ^d	P68871	102	21	2/3
α -1-antichymotrypsin precursor ^d	P01011	99	10	1/5
transthyretin precursor (prealbumin) ^d	P02766	96	28	2/5
collagen α -1 (XIV) chain precursor	P02766	73	1	1/5

^{*a*} Listed are proteins associated with the 10 highest probability-based Mowse scores (remaining identified proteins with a Mowse score \geq 61 (*P* < 0.001) are listed in Supporting Information). An in-house version of the Mascot search engine (v1.9.05) was used to search the UniProt (release 7.7) database. Enzyme 'none' was specified for the search. ^{*b*} Percent ratio of all amino acids from valid peptide matches to the total number of amino acids in the protein. ^{*c*} Number of unique peptide matches (ion score > 34 [*P* < 0.05] was considered significant) followed by the total number of peptide matches found for the given protein. ^{*d*} Proteins were also identified by Phenyx (Table 5). Further detailed information is available in Supporting Information.

discriminatory by the NSC algorithm. The NSC algorithm missed the added peptide GYYPT as being discriminatory probably because it eluted early in the gradient and was added to the urine at a concentration equivalent to 2.2 times the LOD. Despite this fact, it was clearly possible to discriminate the two groups of samples based on a PCA score plot, explaining 89.6% of the variation between blank and spiked samples, using the 16 selected peaks (MD of 9.10) in the presence of the observed biological variation.

Classification of the repetitions of the blank and spiked pooled urine samples, using the 16 peaks selected by the NSC algorithm, resulted also in assignment of the samples to the appropriate classes (MD of 8.04; not shown).

3.2.4. Application of the Developed Methodology to Urine from Patients with and without Proteinuria. To evaluate the applicability of the described methodology, we studied urine of hospitalized patients with established proteinuria (P; male/ female 5:1; median age, 60 years [60–75]; median (range) total protein concentration in a random portion of urine, 7.6 g/L [6.5–10]) due to primary or secondary renal morbidity, and we

studied urine of hospitalized patients with a medical history of renal morbidity but without proteinuria at the time of sampling (NP; male/female 5:1; median age, 63 years [56–72]; total protein concentration in portion urine, 0.1 g/L [0.0–0.2]). Proteinuria was defined as having a total urinary protein concentration above 1 g/L in a random portion. The total peak matrix contained 11 867 peaks and did not allow discrimination between the proteinuric and nonproteinuric samples (MD = 1.84) without supervised classification.

The NSC classifier yielded 92 discriminatory peaks at a shrinkage value of 1.88 with a LOOCV error of 0. The urine samples with and without proteinuria were discriminated (MD = 6.95) in the PC 1 dimension of the biplot (Figure 5A). Ninetyone out of 92 discriminatory peaks (red ▼) increased in intensity in proteinuria (green I), whereas one peak seemed to be increased (arrow) in the samples without proteinuria (blue \bullet). Classification of the urine samples from six healthy individuals described in Sections 3.2.2 and 3.2.3 (spiked or blank; total of 24 samples; purple \blacklozenge) using the model generated with the variables selected from the 12 chromatograms described in this section resulted in the formation of a dense cluster close to the nonproteinuric samples (Figure 5B), discriminating them even more strongly from the proteinuric samples (MD = 15.40) notwithstanding the fact that some of them contained spiked peptides. Most remarkable was the difference (MD = 3.39) between the urine samples of apparently healthy individuals without known renal morbidity used for spiking experiments and the patients with (a history of) renal morbidity but without proteinuria. A trend of progressing renal disease may be implied, as patients shift to the right along the PC 1-axis.

3.2.5. Selection of Discriminatory Peaks and Identification by Tandem Mass Spectrometry. The 92 discriminatory peaks obtained by NSC from the total peak matrix were curated by manual de-isotoping and charge deconvolution. From the resulting 54 curated peaks, we selected 6 peaks for further univariate statistical comparison, based on their elevated levels in the majority of the proteinuric samples. Figure 6 shows the box and whisker plots and corresponding *P*-values for univariate comparison of the peak areas (Figure 6A) and intensities (Figure 6B) of these 6 peaks for the two patient groups. The overlaid EIC of 2 of the 6 peaks (Figure 6C) showed that both of these peaks were increased in 3 out of 6 proteinuric urine samples, but not in the nonproteinuric samples. This indicates

 Table 5.
 Proteins in a Proteinuric Urine Sample Identified by LC-MS/MS and Subsequent UniProt/Swiss-Prot Databases Search by

 the Phenyx Algorithm
 Phenyx Algorithm

protein	accession no.	score ^a	cov. (%) ^b	no. peptides ^c
α -1-antitrypsin precursor ^d	P01009_WOSIG0	187.8	35	39/57
serum albumin precursor ^d	P02768_WOSIG0	47.43	4	9/21
haptoglobin-related protein precursor ^d	P00739_WOSIG0	40.49	12	8/10
α -1-acid glycoprotein 1 precursor ^d	P02763_WOSIG0	28.28	14	6/10
hemoglobin β -subunit ^d	P68871	25.33	16	4/6
serotransferrin precursor ^d	P02787_WOSIG0	24	3	4/9
α -1-acid glycoprotein 2 precursor	P19652_WOSIG0	14.95	9	3/3
apolipoprotein A-I precursor ^d	P02647_WOSIG0	13.93	8	2/5
α-1b-glycoprotein precursor	P04217_WOSIG0	9.33	2	1/3
zinc-α-2-glycoprotein precursor	P25311_WOSIG0	8.87	3	3/3
transthyretin precursor (prealbumin) ^{d}	P02766_WOSIG0	8.82	8	1/4
α -1-antichymotrypsin precursor ^d	P01011_WOSIG0	8.37	3	2/7
angiotensinogen precursor ^e	P01019_PEPT0	8.29	80	1/1

^{*a*} Listed are the proteins with AC-scores \geq 8 (remaining identified proteins with an AC-score \geq 5 are listed in Supporting Information). A Web-based version of the Phenyx search engine (v2.1) was used to search the UniProt_Sprot (r. 48.8 of 10-Jan-2006) at AC-scores \geq 5. Enzyme 'Chymotrypsin (FYL)' was specified for the search. ^{*b*} Percent ratio of all amino acids from valid peptide matches to the total number of amino acids in the protein. ^{*c*} Number of valid peptide matches followed by the total number of peptide matches found for the given protein. Further detailed information is available in Supporting Information. ^{*d*} Proteins were also identified by MASCOT (Table 4). ^{*e*} Angiotensin II was spiked into the urine sample at a concentration of 0.5 μ M (2.5 pmol).

А



Figure 7. Serum albumin peptides identified by LC-MS/MS. (A) The amino acid sequence of serum albumin precursor (Uniprot_Sprot AC no. P02768-CHAIN0) is shown. Peptide matches are listed and highlighted in colored boxes (green for valid peptides, red for invalid peptides, and orange for valid half-cleaved peptides). Two of the serum albumin precursor peptides (boxed amino acids in italic and bold) were selected by nearest shrunken centroid (NSC) classification to be discriminatory between proteinuria and nonproteinuria, whereas two (boxed amino acids in bold) of the other albumin peptides were not selected by NSC. Panel B depicts the extracted ion chromatograms of these two nondiscriminatory albumin peptides for the proteinuric (blue) and nonproteinuric (red) samples indicating that these peptides are also elevated in some proteinuric patients (see Figure 6C for discriminatory peptides).

a considerable variability in the proteinuric patient group, which is also evident from the PCA score plot (see Figure 5), relative to patients with kidney disease but no proteinuria. Interestingly, there was no correlation (P > 0.05, $R^2 \le 0.0276$) between total protein content in urine and peak area or intensity of these 6 peaks in patients with proteinuria, suggesting that the selected peaks discriminate patients not simply based on the total protein concentration in urine. Remarkably, most of the selected peaks did not reach statistical significance (P < 0.05) on their own when comparing proteinuric and

nonproteinuric patient samples due to the large variation in the proteinuric patient group. This adds further support to the value of a multivariate statistical comparison for discrimination between complex samples.

The proteinuric urine sample containing the highest level of these six peaks was analyzed in duplicate by nanoLC ESI-Q-TOF MS/MS to identify the parent protein(s) from which the discriminating peaks were derived. LC-MS data were processed as described under Experimental Procedures. Tables 4 and 5 list the most significant hits of identified proteins for MASCOT-

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Table 6. Peaks Identified by LC-MS/MS as Serum Albumin-Derived Peptides

sequence	cleavage specificity	modifications	precursor mass (Da)	charge	mass error (Da)	score	position	
Selected by NSC ^a								
L.VRYTKKVPQVSTPTL.V ^b	1 MC^{c}	none	1715.99	3+	0.02	40^d	415-429	
L.IAFAQY.L ^e	1 MC	none	711.359	1 +	0.036	4.99^{f}	31-36	
Not Selected by NSC								
L.GEENFKALVL.I ^e	2 MC	none	1118.597	2+	0.014	5.1^{f}	21-30	
L.PSLAADF.V ^e	half	none	719.349	1 +	0.03	4.73^{f}	303-309	

^{*a*} Peptides selected by nearest shrunken centroid (NSC) classifier. ^{*b*} Peptide identified using MASCOT. ^{*c*} MC, missed cleavage. ^{*d*} Individual ion score > 33 indicates identity or extensive homology (p < 0.05). ^{*e*} Peptides identified using Phenyx. ^{*f*} Peptides with *z*-scores \geq 4 and p < 0.0001 were considered significant. Further detailed information is available in Supporting Information.

and Phenyx-algorithm based Uniprot/Swiss-Prot database searches, respectively. Most of the identified proteins are derived from blood.

Two of the peaks selected by the NSC algorithm to be elevated in proteinuric urine samples (Ion-Trap 573.0 m/z, 3+ and 712.3 m/z, 1+; Figure 6C) were identified as possible breakdown products of human serum albumin by both algorithms (Figure 7A: serum albumin precursor protein, P02768-CHAIN0 in uniprot_sprot, L.VRYTKKVPQVSTPTL.V, and L.IAFAQY.L position 31-36; Table 6), a protein that is known to be increased in concentration in glomerular proteinuria.5 Manual conversion of the monoisotopic multiply charged fragment ions to monoisotopic singly charged fragment ions confirmed the identity of one peptide with a higher score (Table 6). To evaluate peak selection by the NSC algorithm, we compared the respective EIC of two other albumin-derived peptides that were not selected by the NSC algorithm (Table 6 and Figure 7B). The levels of the two discriminatory peptides were elevated in at least 3 out of 6 proteinuric patients (for both peptides; P > 0.05), while the other two nondiscriminatory peptides showed increased intensities in only 2 out of 6 patients (for both peptides; P > 0.05). The fact that the NSC algorithm did not select these two peptides indicates that it was not able to detect them as discriminatory due to the large biological variability in the proteinuric patient group. The observation that there are proteolytic fragments of albumin with a higher discriminatory value than others points toward the existence of 'disease-specific' proteolytic degradation of albumin and urinary excretion of its fragments. This idea is supported by recent data of Osicka and Comper⁴⁵ and Villanueva et al.⁴⁶

4. Discussion and Conclusion

The aim of this work was to develop a platform for the comparative profiling of urine by microbore, reversed-phase HPLC coupled on-line with ESI-Ion Trap MS. Dedicated data preprocessing followed by statistical classification (with cross-validation) and PCA were used to assess the relative contributions of analytical and biological variation to the obtained results as well as to apply the methodology to a sample set of kidney patients with and without proteinuria.

4.1. Method Development and Evaluation. Urine has been the biofluid of choice in many clinical and pharmacological studies focusing on diseases of the genitourinary tract.^{47–50} Profiling urinary (trypsin-digested) proteins and peptides usually starts with prefractionation or (affinity-) enrichment followed by separation based on physicochemical properties (e.g., by HPLC and/or 1D/2D electrophoresis [1DE/2DE]) and detection/identification by mass spectrometry.^{18,51–53} While 1DE/2DE are often used in urinary proteomics, their application is limited by poor coverage of proteins with extreme properties (e.g., low

molecular weight; high isoelectric point) or low concentration, a relatively low sample throughput, and the difficulty to automate handling of large numbers of clinical samples.51 Profiling by LC-MS provides an alternative with advantages in areas where 1DE/2DE is weak, notably, the low-molecular weight region of the proteome also termed the peptidome. The urinary peptidome can be considered complementary to the urinary proteome, since most peptides are derived from higher molecular weight proteins through proteolytic cleavage.¹⁸ Since there is evidence that the "degradome" of high molecular weight proteins may give insights into disease mechanisms and provide new diagnostic biomarkers,^{46,54} we have developed a simple, rapid, and robust on-line reversed-phase LC ESI Ion Trap MS-based profiling method with minimal sample pretreatment directed at low-molecular weight compounds in urine.

Validation parameters such as the LOD, linearity, within- and between-day analytical variation, and standard deviation for retention time and peak area were used to characterize the method. Sensitivity for peptides (3-8 amino acids) is comparable to what can be routinely attained with a microbore-LC ESI-Ion Trap MS configuration, that is, detection of low nanomolar (<25 nmol/L) concentrations and femtomole quantities (<250 fmole) of peptides.⁵⁵ However, it must be remarked that small peptides, such as one of the standard peptides VYV, that elute at the beginning of the gradient are prone to higher analytical variation in terms of peak area and retention time, especially at low concentrations. This is likely due to some chromatographic migration under the isocratic sample loading conditions affecting retention time and possibly small losses during loading of the trap column. Very small peptides, however, tend to be less disease-specific, as they may be derived from a wide range of precursor proteins. Since our method does not rely on stable isotope-labeled internal standards, it was critical to evaluate its linearity, which was larger than $R^2 = 0.9$ for each of the 7 added peptides tested over a concentration range from 10 to 500 nM, supporting the presumed linear relationship between peptide concentration and MS response.28 A linear correlation between peptide concentration and detected signal allows compositional analyses not only from a qualitative but also from a quantitative point of view enabling us to address changes in concentrations of the detected compounds.56 Randomization of the order of analysis was used to level out systematic errors due to unavoidable between-day and within-day analytical variation, which might otherwise confound the ensuing statistical analysis. Together, these performance characteristics can be used for power calculations when setting up biomarker discovery projects.¹⁸ Guidelines for power calculations in LC-MS-driven biomarker research are lacking. However, experimental data from microarray studies provide some guidelines with respect to power calculations, that is, sample size, variability of the population, desired detectable differences, acceptable error rate, experimental design, technical variability, and data preprocessing.^{57,58}

In contrast to most published studies, we opted for preanalytical normalization of the amount of injected sample as compared to postanalytical data normalization, since postanalysis normalization cannot account for nonlinear effects such as ion suppression. There are still many issues to address in postanalysis data normalization, such as global versus local normalization, replicate filtering, and averaging.^{34,38,59} The urinary creatinine concentration is generally used as normalization factor in comparative studies.^{17,18} Normalization based on creatinine was compared with a multicompound normalization strategy based on the AUC₂₁₄ of the chromatographic region of interest (excluding amino acids, small hydrophilic peptides, and other early eluting UV-absorbing compounds). The latter approach seems better adapted to normalizing the amount of urine over a wide range of compounds, as was supported by the lower CV of the AUC_{TIC} and the peak areas of the added peptides. The drawback of this approach is that duplicate analyses are required and that the relatively large contribution of one peak/compound to the total UV-area suggests, in essence, normalization to only a few highly abundant UV-absorbing compounds. Development and evaluation of other preanalytical multicompound normalization strategies for the comparative profiling of urine remains, therefore, an issue.

4.2. Data (Pre-)Processing and Multivariate Statistical Comparison of LC–MS Profiles of Urinary Compounds in Different Experimental Settings. The performance of the developed analytical platform was evaluated (i) by assessing the effect of analytical and biological variation on the comparison of urine samples spiked with exogenous peptides and (ii) by a preliminary study comparing urine samples of hospitalized patients with and without proteinuria due to various causes.

Unsupervised multivariate statistical analysis by PCA using the complete peak matrix of about 14 000 peaks was unable to discriminate blank from spiked pooled urine samples. The observed variation between the samples is, in this case, composed of analytical variation and variation introduced by the added peptides (low nanomolar concentrations). Our results suggest that spiking affects the linear combination of all elements composing the data vectors in an unsupervised setting only minimally. It is therefore necessary to apply a supervised classification algorithm (the NSC with permutationbased cross-validation in our case) to select discriminating elements prior to PCA, which resulted in clear separation between spiked and blank samples in the PC 1 dimension. We employed the NSC classifier, because of its simplicity and the fact that Tibshirani et al.³⁹ found it to perform as well or even better than several other classifiers. The validity of the described approach was shown by the fact that the NSC algorithm was capable of extracting the added peptides as discriminatory peaks out of the 14 000 feature-containing peak matrix. However, 3 peaks selected by the classifier could not be related to the added peptides through possible ion-suppression or insource fragmentation products of the added peptides. Analytical artifacts and suboptimal data processing issues, for example, binning,⁵⁶ might have played a role. These issues are presently being addressed.

Comparing blank and spiked urine samples of different healthy individuals by unsupervised PCA proved that variation in urinary peptide profiles between-subjects (i.e., biological variation) was much larger than within-subjects (i.e., analytical variation), irrespective of spiking. However, the correct classification of blank versus spiked urine samples from different individuals using the NSC-selected peaks (14 out of 16 were related to the spiked peptides) emphasizes the applicability of the platform also in the presence of considerable biological variation. Nevertheless, some issues, for example, the selection of false-positive discriminatory peaks, remain to be addressed.

Our preliminary study comparing urine samples from hospitalized patients with a normal and high protein content resembled a study of Jurgens et al. 18 Using LC combined with off-line MALDI-TOF MS detection and differential peptide display to compare pathological with healthy urines, they observed a substantial number of peptides in post-renal disease, which are absent in normal urine. Ninety-two peaks were selected from the LC-MS data to obtain a zero classification error. The larger portion (75%) of these peaks eluted late in the gradient, which suggests that they are rather hydrophobic. Clustering of the nonproteinuric samples from healthy individuals apart from the nonproteinuric samples from hospitalized patients, indicates that there is a trend of increased renal dysfunction in these patients as visualized by a rightshift in PC 1. Our current platform appears to be suitable for the study of early stage renal disease, but larger sets of samples need to be analyzed to substantiate these findings. The identified precursor proteins in the proteinuric urine samples are in good agreement with other studies profiling the urinary proteome^{3,10,16,51} and peptidome.¹⁸ The identification of two discriminatory peaks as serum albumin-derived peptides is in accordance with the expected rise of albumin during glomerular proteinuria. Albumin is the first protein to rise in proteinuria and is degraded intracellularly in the lysosomes after which the fragments are exocytosed to the apical and basolateral sides of the renal tubular cells.⁶⁰ Recently, it has also been suggested that significant amounts of albumin fragments are excreted in urine, possibly resulting from tubular degradation of filtered albumin, followed by luminal secretion of its fragments.^{61,62} Disease-specific proteolytic degradation products of tissue or biofluid proteins are possible biomarker candidates.⁴⁶ However, most discriminatory peaks, including the albumin-derived peptide peak, were not significantly elevated in proteinuria when analyzed by univariate statistics due to a very large biological variation among the proteinuric patient group. The use of higher-order interactions between features in multivariate statistical analysis, thus, provides more discriminative power than univariate statistics. Manual univariate comparison of selected peaks remains, however, imperative for biomarker selection and further identification.

In summary, we have developed an analytical platform for the comparative analysis of urine samples by LC–MS followed by dedicated data preprocessing and multivariate statistical analysis of the obtained profiles. The analytical and biological variation did not adversely affect the performance of the method with respect to the classification of blank and spiked samples of different origin. The preliminary study of pathological and healthy urine samples emphasizes the potential of the platform not only for patient classification but also for detecting trends. However, some issues remain to be addressed and improved to enhance the performance of the platform. Currently, we are implementing more advanced data processing

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and multivariate statistical analysis approaches such as data meshing instead of binning, optimized M-N rule filtering for peak detection, and improved peak matching methods together with de-isotoping and charge-deconvolution. Advanced retention time alignment and possibly m/z alignment algorithms are also being considered, as well as other classifying algorithms and methods. Combined with larger and better defined urine sample sets, this will allow us to do advanced proof-of-principle studies toward the goal of defining reliable biomarkers suitable for clinical validation.

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Supporting Information Available: Detailed experimental description of the urine analysis method and MS/MS database queries for protein and peptide identification. This material is available free of charge via the Internet at http:// pubs.acs.org.

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