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Optimization of analytical and pre-analytical conditions for MALDI-TOF-MS human urine protein profiles

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ABSTRACT

Protein analysis in biological fluids, such as urine, by means of mass spectrometry (MS) still suffers for insufficient standardization in protocols for sample collection, storage and preparation. In this work, the influence of these variables on healthy donors human urine protein profiling performed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was studied.

A screening of various urine sample pre-treatment procedures and different sample deposition approaches on the MALDI target was performed. The influence of urine samples storage time and temperature on spectral profiles was evaluated by means of principal component analysis (PCA). The whole optimized procedure was eventually applied to the MALDI-TOF-MS analysis of human urine samples taken from prostate cancer patients.

The best results in terms of detected ions number and abundance in the MS spectra were obtained by using home-made microcolumns packed with hydrophilic–lipophilic balance (HLB) resin as sample pre-treatment method; this procedure was also less expensive and suitable for high throughput analyses. Afterwards, the spin coating approach for sample deposition on the MALDI target plate was optimized, obtaining homogenous and reproducible spots. Then, PCA indicated that low storage temperatures of acidified and centrifuged samples, together with short handling time, allowed to obtain reproducible profiles without artifacts contribution due to experimental conditions. Finally, interesting differences were found by comparing the MALDI-TOF-MS protein profiles of pooled urine samples of healthy donors and prostate cancer patients.

The results showed that analytical and pre-analytical variables are crucial for the success of urine analysis, to obtain meaningful and reproducible data, even if the intra-patient variability is very difficult to avoid. It has been proven how pooled urine samples can be an interesting way to make easier the comparison between healthy and pathological samples and to individuate possible differences in the protein expression between the two sets of samples.

1. Introduction

Cellular metabolism generates numerous waste compounds that require elimination from the bloodstream. This waste is eventually expelled from the body with urine, a liquid waste product secreted by the kidneys through a filtration process from blood. Serum proteins are filtered based on their sizes and charges at the glomerular filtration barrier and can be subsequently reabsorbed in proximal renal tubules. Thus, normal urine contains a very low protein concentration (150 mg/24 h), constituted of those filtered from the plasma, as well as those arising from kidney and urogenital tract. An excess of serum proteins in urine is defined as proteinuria and is indicative of glomerular or reabsorption dysfunction.

Urine can be collected in a non-invasive fashion and in large amounts, thus being an ideal protein research substrate, permitting the recovery of adequate amounts of material after protein preconcentration. For instance, more than 1500 proteins have been recently identified by Adachi et al. [1] from an in-depth study in urine samples from healthy individuals.

Normal urinary proteins generally reflect normal kidney tubular physiology due to the presence of kidney proteins [2,3–7]. Thus, urine is a good material for the analysis of kidney failure resulting from high blood pressure and diabetic nephropathy, which is the most frequent cause of renal failure in the Western world [8]. Urine is also an important source of information for bladder and prostate cancers. The levels of several proteins in urine are mea-

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sured as markers for bladder cancers as well as those in blood, such as bladder tumor antigen (BTA) [9], nuclear matrix protein 22 [10], urinary fibronectin (FN) fragments [11,12], and fibrinogen degradation products [13].

In view of the above considerations, the main goal of urinary proteomics is the individuation of a disease and/or of biological markers; the comparison of a normal proteome with the proteome from patients with a defined disease can detect proteins expressed differentially from one another. A recent review presented [14] the situation of urinary proteomics, putting special emphasis on its application in the diagnosis of glomerular diseases and urological cancers.

Urinary proteomic is usually conducted by means of different mass spectrometry (MS) techniques coupled to various protein concentration and separation approaches and is far from being an easy task. The state-of-the-art and the analytical challenges of mass spectrometry based urine proteomics has been recently discussed [15] in a review paper. The first issue that must be taken into account concerns the pre-analytical variables; sample collection, handling and storage can deeply influence the quality of the whole analysis, resulting in meaningless data. However, works dealing with the standardization of the above mentioned parameters are very limited [14,15] at the present. Recently, Papale et al. [16] investigated the influence of a number of pre-analytical and analytical variables on the urinary proteome by SELDI-TOF/MS in healthy donors, while Gika et al. [17] deeply studied urine samples stability under different handling and storage conditions by ultra-performance liquid chromatography–mass spectrometry and principal component analysis, even if in view of metabonomics studies. Furthermore, urine has a very diluted protein concentration with a high salt content which interferes with analysis; thus sample pre-treatment is another crucial step in urine proteomic studies. The proteins/peptides eventually observed are strongly dependent on the sample pre-treatment method adopted, indicating that it has to be selected and optimized for the specific disease under investigation.

The first objective of the present work was to unravel optimal strategies for the analysis of urinary protein profiles by MALDI-TOF-MS. To reach this aim, a systematic study on the influence of pre-analytical variables (i.e., sample storage time and temperature, pre-processing and preparation), sample preparation methods (i.e., ultrafiltration, solid-phase extraction, desalting, MALDI matrix choice and deposition mode) on the protein profiles of healthy subjects urine samples, obtained by MALDI-TOF-MS, was accomplished. As far as the contribution of storage time and temperature to the overall variability is concerned, principal component analysis (PCA) was applied to MS data in order to estimate the reproducibility and applicability of each storage strategy. The optimized procedure was eventually used to compare the MALDI-TOF-MS protein profiles of urine samples of healthy donors and prostate cancer patients for a differential proteomic study.

2. Experimental

2.1. Materials

All chemicals and solvents used were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents were HPLC grade and were used without further purification. Alpha-cyano-hydroxycinnamic acid (CHCA) and sinapinic acid (SA) were from Sigma–Aldrich.

Poros R1 and R3 reversed phase resins were from Applied Biosystems (Framingham, MA). Empore extraction disks (C8, chelating (IMAC), SDB-XC (strong cation-exchange) and ANION SR) were from 3 M Bioanalytical Technologies (St. Paul, MN, USA). ZipTip® pipette tips (C18) and Amicon ultrafiltration devices were from Millipore Corporation (Billerica, MA, USA). OASIS HLB extraction cartridges were from Waters Corporation (Milford, Massachusetts).

2.2. Urine samples collection

All urine specimens collected were from individuals with similar age, body weight, diet, anamnesis and pharmacological therapies. Urine samples used for protocol optimization and as control group were collected from healthy non-smoking individuals. Prostate cancer urine samples were collected at the Dipartimento dell'Emergenza e dei Trapianti di Organi of the local University. Middle stream early morning urine samples were immediately acidified (pH 3) with phosphoric acid (0.5 M) and centrifuged for 10 min at 5000 × *g*. The supernatant was immediately used or divided in 100 μL aliquots and stored (at the temperature of 4 or −20 °C).

2.3. Urine samples pre-treatment

Urine samples were subjected to ZipTip® (according to the producer specifications), ultrafiltration followed by ZipTip®, or SPE using home-made microcolumns prepared into tips packed with either selected Poros resins (R1, R3) and OASIS HLB disassembled cartridges or Empore extraction disks (C8, IMAC, SDB-XC and ANION SR).

2.3.1. Ultrafiltration followed by ZipTip®

Columns with polyethersulfone membranes (Vivaspin 500, 10 and 30 kDa MWCO) were activated loading 50 μL of 0.1% TFA followed by centrifugation at 15,000 × *g* for 10 min. Then, 400 μL of human urine (diluted 1:5 with TFA 0.5%) were loaded and centrifuged at 5000 × *g* for 30 min. The eluate was collected and subjected to ZipTip® as described in the specification sheet.

2.3.2. OASIS HLB and reverse phase

Waters OASIS HLB extraction cartridges contain a copolymer (N-vinylpyrrolidone and divinylbenzene) designed to have a hydrophilic–lipophilic balance (HLB), giving reproducible recoveries for a wide range of compounds (basic, acidic and neutral). The resin was obtained from a disassembled cartridge. Aliquots of 10 mg were weighted, suspended in 100 μL of 70% acetonitrile, 0.1% TFA, and used to pack each microcolumn, consisting of a P200 syringe tip previously plugged with inert material used as a frit to retain the resin.

Urine samples acidified with phosphoric acid (100 μL) were loaded onto the microcolumn. Salts and weakly bound analytes were removed by washing two times with water (total volume 250 μL). Peptides and small proteins were eluted with acetonitrile/water (6:4, v/v; 100 μL) with 0.02% TFA. Eluted samples were dried under nitrogen, then dissolved in 20 μL of 0.1% TFA, mixed with matrix (both CHCA and SA, 1 μL) and loaded on the MALDI target.

For Poros R3 and R1 material a similar protocol was adopted except for the washing step where water was substituted by 0.1% TFA.

2.3.3. Anion-exchange chromatography

The SPE microcolumn (anion-exchange disc placed above a C8 disc) was washed with 7% acetonitrile/0.01% TFA (30 μL). The SPE microcolumn was equilibrated with 0.1% TFA (40 μL). Urine samples were dried and reconstituted with 40 μL of 0.1% TFA and subsequently loaded onto the microcolumn. Samples were first washed with 30 μL of 5% formic acid containing 0.3 M NaCl and then with 30 μL of 5% formic acid to remove salt. Proteins

were directly eluted with the MALDI matrix (2 μL) onto the target plate.

2.3.4. Weak cation-exchange chromatography (chelating discs without metal)

The SPE microcolumn was wetted with water (40 μL), washed with 3 M HCl (40 μL) and then twice with water (2 × 30 μL). The SPE microcolumn was equilibrated with ammonium acetate buffer (0.1 M, pH 5.3, 30 μL) and then washed with water (30 μL). Urine samples were dried, dissolved in 20 μL of 0.1% TFA and loaded. Bound analytes were eluted with 3 M HCl (1 μL) directly onto an equilibrated C8 disc and desalted as described above for HLB resins.

2.3.5. Strong cation-exchange chromatography (SDB-XC discs)

The SPE microcolumn was washed with 70% acetonitrile/5% formic acid (30 μL) and then equilibrated with 5% formic acid (40 μL). Urine samples were dried and dissolved in 20 μL of 5% formic acid and subsequently loaded onto the microcolumn. Salts were removed by washing with 7% ACN/5% FA (30 μL). The analytes were directly eluted with the matrix (2 μL) onto the MALDI target.

2.4. MALDI-TOF-MS

Alpha-cyano-hydroxycinnamic acid (CHCA), sinapinic acid (SA) and CHCA/SA mixtures were tested as matrices. All matrix solutions were prepared in ACN/0.1% TFA (7:3, v/v) at the concentration level of 10 mg/mL. Different matrix/analyte volume ratios (1:1, 3:1, 5:1) were tested. The dried droplet and spin coating (the sample was allowed to dry into a spin-coater under vacuum at 1000 rpm for 5 min) matrix deposition methods were compared.

MS experiments were performed using a Micromass M@LDI™-LR (Waters MS Technologies, Manchester, UK) time-of-flight mass spectrometer equipped with a nitrogen UV laser (337 nm wavelength), a precision flat target plate sample introduction system bearing a micro-titer target plate, reflectron optics with effective path length of 2.3 m, a fast dual micro-channel plate (MCP) detector, and a high magnification (70×) camera system.

Positive ion spectra were acquired in linear mode in the *m*/*z* range 1000–12,000. The following voltages were applied: pulse, 1400 V; source, 15,000 V; MCP 1850 V; matrix suppression delay: 500 amu. The laser power densities were in the range of 10^7 to 10^8 W cm^{-2} with a laser pulse duration of 4 ns. The laser firing rate was 5 Hz, and 60 laser shots were used for each well. The 60 resulting spectra were averaged, background subtracted and smoothed with a mean algorithm.

Calibration was performed using a protein mixture composed of insulin beta chain (3497.0 Da), insulin (5735.0 Da) and cytochrome C (12361.1 Da).

Spectra were converted into ASCII file format using the MassLynx software (Micromass, Waters). The Statistica 7.0 program (Tulsa, OK, USA) was used for principal components analysis (PCA).

3. Results and discussion

3.1. Sample pre-treatment

The first step of the work was the optimization of a sample pre-treatment method able to detect the higher number of protein ions and the higher relative abundances. Thus, different approaches were attempted. At first, ZipTip® pipette tips, known to be the simplest sample preparation method for MALDI-TOF-MS analysis, were tested. Fig. 1A shows the MALDI-TOF-MS spectrum acquired in linear mode (1000–12,000 Da) relevant to a healthy donor urine sample simply eluted from a C18 ZipTip®. As apparent, few peptides were detected in the explored range, likely due to a suppression effect [18,19] on the ionization of low molecular weight (LMW) peptides that could be ascribed to the presence of high molecular weight (HMW) proteins or to limits in the loading capacity of the column.

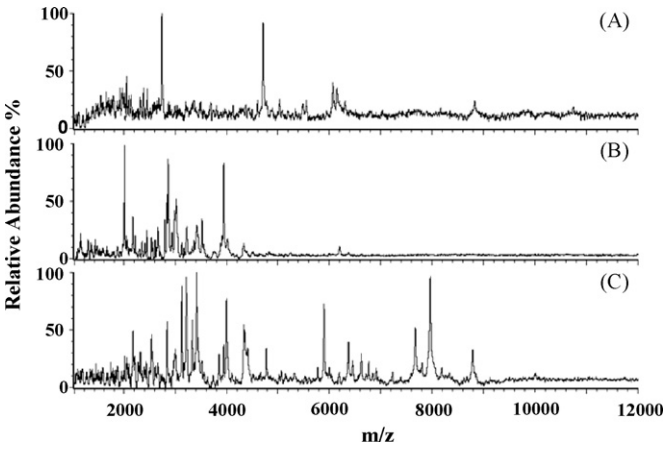

Fig. 1. MALDI-TOF-MS spectra (linear acquisition mode, 1000–12,000 Da) relevant to a healthy donor urine sample eluted from (A) ZipTip®, (B) ultrafiltration (50 kDa molecular weight cut off membranes) followed by ZipTip® columns, and (C) ultrafiltration (10 kDa molecular weight cut off membranes) followed by ZipTip® columns.

Then, in order to deplete proteins with higher molecular weight and to enrich the lower molecular weight fraction, an ultrafiltration step on 10 or 50 kDa molecular weight cut off membranes was performed before ZipTip® extraction. Fig. 1B and C, respectively, report the MALDI-TOF-MS spectra relevant to the same urine sample of Fig. 1A, subjected to the combined ultrafiltration (10 and 50 kDa, respectively) – ZipTip® approach.

The first observation one can made considering the whole spectra shown in Fig. 1 is that the profiles obtained for the three purification procedures are very different, clearly indicating how variable can be the information that can be drawn by the same sample varying few steps in the pre-treatment procedure. As already mentioned, unsatisfying results were obtained (see Fig. 1A) using only the ZipTip® procedure. Better results were observed after the 50 kDa cut off membrane (Fig. 1B), even if the number of detected peaks still remained low, probably because the residual high molecular weight proteins can still interfere with the analysis (see above). Definitely, the most satisfactory data were obtained (see Fig. 1C) in the case of ultrafiltration with the 10 kDa cut off membrane followed by ZipTip®, with the higher number of protein ions and the higher relative abundances.

It has been recently demonstrated [20,21] in the case of MALDI-TOF-MS analysis of breast cancer women serum samples, that an emerging technique for optimal sample pre-treatment of complex biological matrices for proteomic analysis purposes can be represented by home-made miniaturized solid-phase extraction (μ-SPE), that adopts microcolumns assembled using capillary tips and resins taken from SPE commercial cartridges. Moreover, a great number of columns can be prepared disassembling only one SPE cartridge and reproducible results can be obtained carefully weighting the packing material. Thus, the mentioned approach was tested also for urine pre-treatment. In particular, columns were packed with different resins as described in Section 2, and urine samples were directly applied onto the home-made μ-SPE columns and eventually subjected to MALDI-TOF-MS analysis. The relevant results are shown in Fig. 2A–F. Of course, different results were obtained by varying the column packing material, even if better results in terms of observed ions number and relative abundance were obtained using microcolumns packed with the HLB resin (see Fig. 2A). The latter approach permitted to observe a number of peaks relevant to the LMW fraction even without a previous deple-

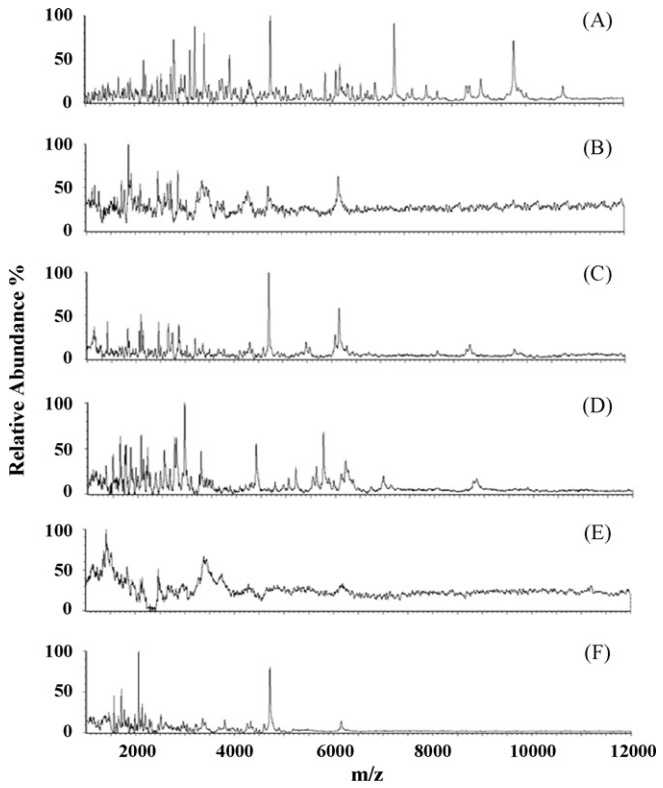

Fig. 2. MALDI-TOF-MS spectra (linear acquisition mode, 1000–12,000 Da) obtained in the analysis of urine sample. Clean-up methods: (A) HLB, (B) R1 resin, (C) R3 resin, (D) ANION SR, (E) SDB-XC, and (F) chelating.

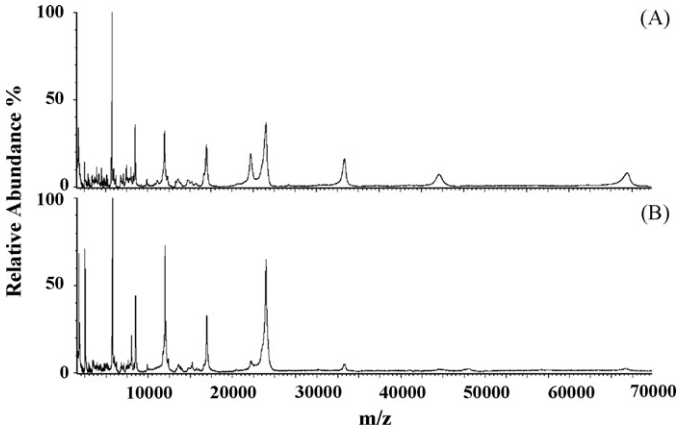

Fig. 3. MALDI-TOF-MS spectra (linear acquisition mode, 1000–70,000 Da) obtained in the analysis of a standard protein mixture (A) before and (B) after HLB μ-SPE.

tion of HMW proteins (probably, the resin itself operates a cut off into the high MW fraction). In order to verify this hypothesis, a mixture of standard proteins having very different molecular weights (in the range 5.000–70.000 Da) was analyzed by MALDI-TOF-MS before and after the elution on a μ-SPE HLB column, and the relevant spectra are reported in Fig. 3A and B, respectively. As expected, the simultaneous depletion of the HMW and enrichment of LMW proteins was observed. Thus, microcolumns packed with the HLB resin were chosen for further analyses.

3.2. Analytical variables

The successive step of the work was the optimization of MALDI-TOF-MS analytical variables, i.e., MALDI matrix (tested matrices: CHCA, SA, and various mixtures of them), analyte:matrix ratio (tested ratios: 1:1; 1:3; 1:5, v/v) and sample deposition method. The best performances were shown by CHCA as matrix and by an analyte:matrix ratio of 1:1 (v/v). As far as the choice of the sample deposition method is concerned, a comparison between the dried droplet (DD) and the spin coating (SC) approaches was accomplished (see Section 2 for the detailed procedures). The same urine sample was pre-treated using the optimized procedure, the resulting extract mixed with CHCA (1:1, v/v), and finally deposited on the target plate using both the DD and SC methods. Each procedure was repeated five times. In order to determinate the optimal deposition technique, within-spot and spot-to-spot repeatability were calculated. In the case of within-spot repeatability, the spot was virtually divided in five sections, then the peaks intensities obtained for each section for both the techniques were considered. In the case of spot-to-spot repeatability, peaks intensities of five spots were registered using both the DD and SC approaches. The relevant results are shown in Table 1 ("within-spot") and Table 2 ("spot-to-spot"), respectively. Higher peaks intensities and lower standard deviation and coefficient of variation, indicating a more homogenous deposition, were obtained by using the SC technique, that was then chosen for the prosecution of the work.

3.3. Pre-analytical variables

As already mentioned in Section 1, the pre-analytical variables can deeply influence the quality of the whole analysis. Thus, the following step of the work was their standardization, in particular of storage time and temperature, using principal component analysis (PCA). PCA was used to reduce the dimensionality of a data set in which there are a large number of correlated variables, while retaining as much as possible of the data set variance. The data sets consist of the mass spectra expressed as the intensities of individual m/z ratios (i.e., variables) acquired at different days (i.e., cases). The aim was to find new co-ordinates, linear combination of the original variables (mass-to-charge ratios, m/z), so that the main trends in the data are simply visualised. Moreover this method does not require information for classification as it clusters individual samples on the basis of similarity among their data.

Urine samples of a healthy subject were acidified, centrifuged, and different aliquots were stored at 4 and −20 °C, respectively (see Section 2 for details), for variable periods of time. Samples were then progressively pre-treated using the optimized procedure and subjected to MALDI-TOF-MS analysis. Observations at time 0 and after 2, 6, 11, 14, 21, 24 days were made in the case of samples kept at 4 °C, since the total disappearance of significant peaks was observed after 24 days. As to the samples stored at −20 °C, the study was carried out for 55 days.

3.3.1. Storage at 4 °C

From the mass spectrum of the fresh urine sample, the 70 most abundant ions in the range 1000–12,000 Da were first selected, those with a relative intensity higher than 3% of the full scale and with a signal/noise ratio not less than 3. These m/z were compiled in a peak list with no redundancy. Each mass spectrum was compared with this list to generate the data set whose values are the intensities of the selected peaks. The matrix was then autoscaled (each data column scaled to zero mean and unit variance) before statistical analysis.

PCA on all samples describes the variation observed in the data set. In particular, 93.62% of variance was explained in the first two components and the weight of each variable on these two principal components was deduced by loading values. Fig. 4 shows the score plot and, in particular, two groups were characterized by PC1: the first relevant to sample replicates at day 0, while the second far away from the first, relevant to the other days. Along the second

Table 1
Within sample ($N = 5$) precision obtained for peak intensities values on urine samples. Sample deposition: (A) dried droplet, (B) spin coating. Acquisition mode: linear.

Mean m/z (monoisotopic)	Intensity							
	10:20	20:30	30:40	40:50	50:60	Media	SD	CV (%)
(A) Dried droplet within-spot								
10,762	30	37	26	17	19	25.8	8.2	31.7
9753	14	23	0	0	0	7.4	10.6	143.52
8779	34	37	0	20	0	18.2	17.8	97.85
5399	0	19	0	0	0	3.8	8.49	223.65
4773	44	43	38	25	26	35.2	9.14	25.99
4181	22	22	21	0	19	16.8	9.47	56.37
2950	46	34	32	24	22	31.6	9.52	30.15
2800	63	46	41	26	23	39.8	16.20	40.72
2469	40	40	33	21	19	30.6	10.11	33.05
2222	39	27	0	19	24	21.8	14.23	65.30
(B) Spin coating within-spot								
10,763	21	21	19	18	16	19.0	2.12	11.2
9752	41	55	38	37	36	41.4	7.83	18.9
8781	59	85	60	53	53	62.0	13.3	21.4
5399	27	34	21	24	23	25.8	5.07	19.6
4775	246	280	248	231	192	239.4	31.9	13.3
4183	31	37	28	25	24	29.0	5.24	18.1
2952	176	200	178	186	133	174.6	25.1	14.4
2803	172	250	170	187	125	180.8	45.1	24.9
2468	302	432	288	295	222	307.8	76.4	24.8
2223	438	605	433	510	295	456.2	114.0	25.0

principal component, explaining only the 4% of variance, it is possible to distinguish the samples analyzed after 2 days from all the remaining ones. The loading plot (not shown) explains this data pattern: it reveals that the variation on PC1 is homogenous along all the mass range analyzed even if a larger variation is due to high molecular weight masses. This result indicates that after storage an alteration of the sample takes place, then PC1 explains the differences between fresh and stored samples. The variance explained by PC2 is due to peptides at m/z 4181 and at m/z 8782 whose intensities went to zero after 2 and 14 days of storage.

The variation in the protein profile already observed after 2 days indicates that sample degradation occurs mainly in the high molecular weight range, where the loss of peaks was first detected. The careful observation of the spectra relevant to the stored samples supported this evidence as they showed new peaks having low m/z values and whose intensities increased with the storage time. As a result, the storage at 4 °C should be avoided even for short time.

Table 2
Between samples ($N = 5$) precision obtained for both mass and peak intensities values on urine samples. Sample deposition: (A) dried droplet and (B) spin coating. Acquisition mode: linear.

m/z			Intensity		
Mean m/z (monoisotopic)	SD	CV(%)	Media	SD	CV(%)
(A) Dried droplet spot-to-spot					
10,762	1.6	0.015	184	85	46
9753	1.2	0.012	69	34	50
8779	1.0	0.012	189	91	48
5399	1.6	0.030	69	30	44
4773	0.60	0.013	177	69	39
4181	2.0	0.032	70	21	30
2949	0.40	0.014	130	31	24
2800	1.1	0.040	114	40	35
2469	2.0	0.079	137	45	33
2222	1.1	0.050	83	14	17
(B) Spin-coater spot-to-spot					
10,764	1.0	0.0095	59	0.50	0.84
9755	0.64	0.0066	164	5.7	3.5
8781	0.22	0.0025	247	28	11
5399	0.53	0.0098	95	12	13
4778	0.21	0.0044	1092	101	9.3
4186	0.74	0.018	97	6.1	6.4
2953	0.16	0.0055	814	57	7.0
2804	0.13	0.0045	755	57	7.5
2469	0.38	0.015	1299	97	7.5
2223	0.19	0.0086	1965	77	3.9

3.3.2. Storage at −20 °C

Some aliquots were analyzed after storage at −20 °C with the same criteria. As expected, the lower temperature assured a better preservation of samples and the protein profile could be monitored for a longer time period. The data set was compiled following the same procedure above explained and the PCA was again performed on the autoscaled data.

The first two principal components, explaining, respectively 65.03% and 16.47% of variance, were selected and the relevant score plot is shown in Fig. 5. The changes along the first PC are associated to the different relative intensities of the peaks. Looking closer at the loading plot (not shown), it is evident that the lower region

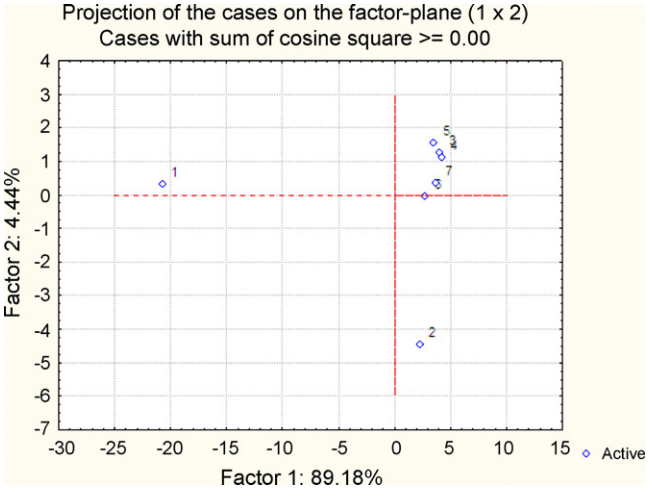

Fig. 4. Principal component analysis (PCA) of the mass intensities of a urine sample after different time storage. Each spot on the plot represents the mass spectrum average of at least three replicates. The numbers labeling each point indicate the storage time at 4 °C and, in particular, 1 corresponds to the fresh urine sample analyses, whereas the labels from 2 to 7 individuate samples stored 2, 6, 11, 14, 21, 24 days, respectively.

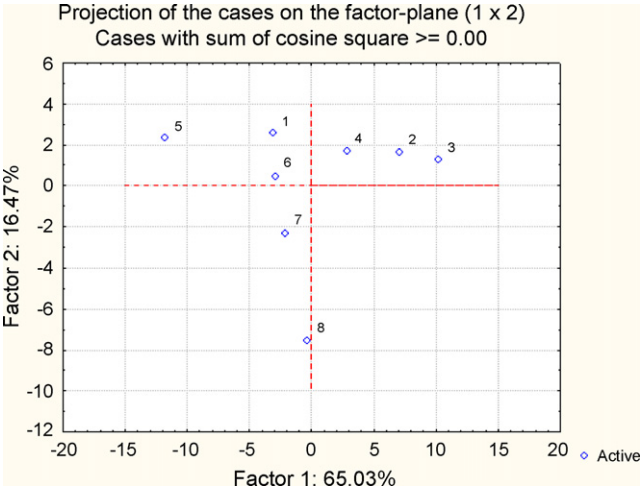

Fig. 5. Principal component analysis (PCA) of the mass intensities of a urine sample after different time storage. Each spot on the plot represents the mass spectrum average of at least three replicates. The numbers labeling each point indicate the storage time at −20 °C and, in particular, 1 corresponds to the fresh urine sample analyses, whereas the labels from 2 to 8 individuate samples analyzed after 2, 6, 11, 14, 35, 45 and 55 days of storage, respectively.

of the spectrum (those peaks having <5300 Da) is responsible for most of the variance on PC1 as the other portion of the spectrum (>5300 Da) is more reproducible. The variance explained by the second PC is more interesting: it is possible, indeed, to distinguish data along PC2 in two groups, one formed by analyses done in the first 35 days and the other by those done later (i.e., after 45 and 55 days). As to the *m*/*z* characterizing PC2, it is possible to identify those peaks whose intensities definitely decrease after 35 days. The degradation of samples stored at −20 °C is again confirmed by the recording of new peaks after these longer sample storage times.

As a result, it should not be under-stressed that the quite common storage temperature of −20 °C, often considered completely safe to the stability of urine samples, also deserves some threats, and for longer storages, liquid nitrogen is desirable.

To confirm these results and to exclude a variability due to the preparation technique rather than the sample protein profiling alteration in time, samples have been re-analyzed adding an internal standard as control. The internal standard chosen for this purpose was the bovine insulin (5734 Da) for many reasons: it is not present in human fluid; its *m*/*z* value is in a region were few or no peaks of sample are present; the moderately low molecular weight allowed the formation of a singly charged ion avoiding the possibility of fragmentation or multi-charged ions formation and then the dispersion of signal in different forms.

The new matrix of data was obtained considering the ratio of intensity values between sample peaks and internal standard. In this case, the same cluster previously observed was obtained confirming the preceding findings about storage time.

The optimized protocol with controlled analytical and pre-analytical conditions was eventually used to compare urinary protein profiling of two different classes of samples (healthy–pathological) to look for characteristic fingerprint.

3.4. Pathological samples

As previously shown (see Table 1), good repeatability data were obtained for the MALDI-TOF-MS protein profiles relevant to the same urine sample, indicating the reliability of the optimized protocol. Then, urine samples taken from 30 healthy individuals and 30 prostate cancer patients were processed and analyzed by MALDI-TOF-MS using the conditions optimized in the present work. Table 3 reports the main observable ions in the two sets of samples, together with their percentage of presence in the MS spectra. As apparent, significant differences in the urinary protein profiles were found even across individuals of the same group. As urine is a specific filtrate of blood, the variation of the urinary proteins across and within individuals seems to be inevitable. In the human population, the food consumed, stress, physiological conditions, environment, genetic background, and psychological status vary from individual to individual. In contrast, in cases of lab animals or live stock animals, the above conditions are almost uniform so that their urine proteins show very similar 2D gel patterns [22,23]. The variations of MALDI-TOF-MS patterns of urinary proteins have made it difficult to localize new biomarkers. It might require painstaking efforts to select and identify useful biomarkers from human urine related to diseases if MALDI-TOF-MS patterns of individual urine is analyzed singularly. Instead, to achieve a goal of identifying disease-associated protein biomarkers in urine, the establishment of a representative urinary protein profile can simplify the task and make the comparison easier [24]. To satisfy this requirement, the pooled urine of 30 healthy individuals and 30 prostate cancer patients were treated as described previously and analyzed by MALDI-TOF-MS. The relevant results are shown in Fig. 6A (healthy) and Fig. 6B (cancer), respectively; no significant differences between the MALDI protein patterns of the two pooled samples were found, even if not negligible variations in a number of peaks intensities were clearly observable, that could likely be ascribed to a different protein expression in the two categories and, then, to the pathological state. In particular, the higher variability was observed at lower *m*/*z* values; thus,

Table 3
Main observable ions in urine samples taken from 30 healthy individuals and 30 prostate cancer patients (processed and analyzed by MALDI-TOF-MS using the conditions optimized in the present work) together with their percentage of presence in the MS spectra.

m/*z* value	Healthy (%)	Pathologic (%)
10,772	66.7	50
9,878	36.7	56.7
9,759	63.3	66.7
9,075	33.3	40
8,846	53.3	70
8,774	80	76.6
8,184	66.7	70
6,428	30	43.3
6,290	53.3	93.3
6,172	96.5	96.7
6,129	53.3	70
6,075	36.6	33.3
5,578	56.6	50
5,496	43.3	73.3
5,426	70	43.3
5,168	90	100
5,021	96.5	96.7
4,796	80	73.3
4,744	86.7	100
4,614	43.3	50
4,359	53.3	90
3,835	33.3	46.7
2,931	80	100
2,910	76.7	20
2,778	63.3	70
2,720	86.5	53.3
2,703	86.6	80
2,432	90	96.7
2,182	96.5	76.7
2,161	46.7	93.3
2,141	90	80
1,900	86.7	40
1,895	53.3	83.3
1,671	30	73.3
1,662	36.7	50
1,296	63.4	66.7

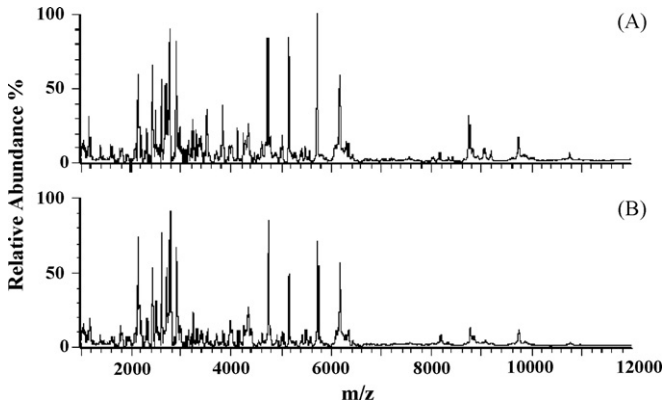

Fig. 6. MALDI-TOF-MS spectra (linear acquisition mode, 1000–12,000 Da) relevant to pooled urine samples of (A) 30 healthy donors and (B) 30 prostate cancer patients.

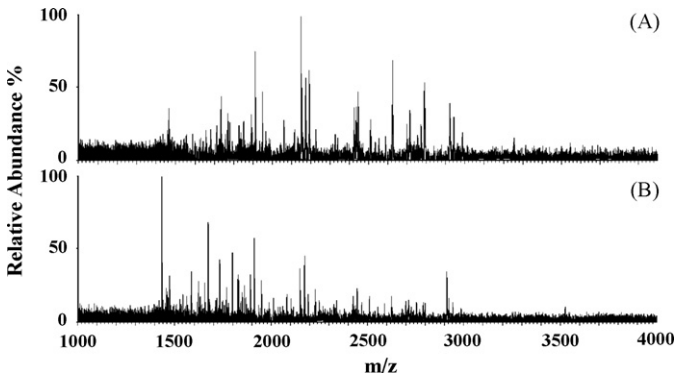

Fig. 7. MALDI-TOF-MS spectra (reflectron acquisition mode, 500–4000 Da), relevant to pooled urine samples of (A) 30 healthy donors and (B) 30 prostate cancer patients.

the same samples were analyzed by MALDI-TOF-MS in reflectron acquisition mode; Fig. 7A and B, reports the results obtained in the case of the pooled healthy and cancer urine samples, respectively, while Table 4 lists the *m*/*z* values of the peaks observable in the spectra reported in Fig. 7 and the relevant presence (+) or absence (−). As apparent, some peaks were present in both spectra, some others were observable only in the healthy donors pooled sample while brand new peaks, that could be, at least some of them, potentially related to the disease, appeared in the cancer patients pooled sample. However, since in serum are active different proteases [25], the peptides eventually present in the urine arise from unspecific cleavage of proteins, and this did not allow to perform a peptide mass fingerprint. Further investigation should be carried out by MS^n techniques to determine the sequence of these peptides and, consequently, to individuate their possible origin. For instance, one of the peptides observed in the present paper, the *m*/*z* ion 1912, that seems to remain constant in the two sets of samples analyzed in the present work, was very recently found to be down regulated in nephropathic urine samples by Lapolla et al. [26]; it was sequenced by MALDI-TOF-TOF MS and found to arise from uromodulin. M'Koma et al. [27] tentatively attributed by LC-MS/MS the *m*/*z* ion 2484 to a fragment from uromodulin, founding it down regulated in prostate cancer patients. Another mass peak observed and sequenced by M'Koma et al., i.e., the *m*/*z* ion 1433 (probably attributable to semenogelin I isoform b preprotein), was found to increase in prostate cancer patients, in accordance with our findings (see peak 1432.91 in Table 4). Other higher masses (in particular the *m*/*z* ion 10,788) were found increased in prostate cancer patients by Okamoto et al. [28] using SELDI-TOF in linear mode. A consideration should be done considering the results mentioned above: it is very difficult to compare different studies on this topic, even if performed on the same matrix and on similar patients, since the sample pre-treatment approaches are always

Table 4
List of the *m*/*z* values of the peaks observable in the spectra reported in Fig. 7 (obtained from both healthy and pathological samples and acquired in reflectron mode), and the relevant presence (+) or absence (−).

m/*z* value	Healthy (%)	Pathologic (%)	*m*/*z* value	Healthy (%)	Pathologic (%)
931.64	–	+	1989.17	–	+
1161.73	–	+	2011.08	–	+
1195.70	–	+	2060.15	+	–
1218.64	+	+	2082.01	–	+
1238.73	–	+	2102.26	–	+
1308.81	–	+	2115.31	+	+
1432.91	–	+	2148.13	+	+
1456.78	–	+	2173.19	+	+
1462.83	+	+	2190.91	+	+
1473.87	–	+	2225.09	+	–
1542.90	+	+	2229.16	–	+
1561.94	–	+	2250.11	–	+
1585.92	+	+	2325.10	+	–
1586.93	+	+	2339.20	+	+
1622.99	–	+	2424.21	+	+
1628.94	–	+	2435.10	+	–
1631.95	–	+	2445.21	+	+
1656.01	+	+	2469.24	–	+
1674.04	–	+	2509.22	+	+
1734.02	+	+	2553.43	+	+
1770.09	+	+	2587.24	+	+
1799.00	–	+	2623.26	+	+
1827.03	+	+	2696.21	+	+
1830.16	–	+	2713.33	+	+
1850.03	+	+	2753.50	+	+
1861.10	–	+	2770.60	+	–
1869.09	–	+	2789.16	+	+
1892.11	+	+	2910.42	+	+
1912.13	+	+	2921.56	+	+
1949.06	+	+	2942.48	+	+
1964.96	+	–			

different. For instance, in this work, the proposed optimized sample pre-treatment approach (HLB resins SPE microcolumn) is new and different by those already proposed (for example, a mixed bed of C8 and C18 resins by M'Koma et al., three different ProteinChip arrays by Okamoto et al.). Thus, it is normal to extract and observe different m/z values in the resulting mass spectra, even if sometimes it can happen to obtain common peaks. One thing that could be very useful in the next future is the collection of the m/z values found in the existing literature in order to start to create urine peptide databases for each pathology.

3.5. Conclusions

An optimized protocol for the analysis of low molecular weight proteins/peptides in human urine has been developed. For the pre-analytical conditions useful information about collection and time storage has been obtained by means of statistical analysis. In particular, it has been proved that urine, if not immediately analyzed, should be stored not more than 35 days at −20 °C to avoid sample degradation and then the risk to occur in a false positive marker for diagnostic purposes.

For analytical variables, different procedures of sample pre-treatment were tested and the best results were obtained using HLB micro-solid-phase extraction (μ-SPE) as sample purification, CHCA as matrix and spin coating for spot deposition. The procedure optimized achieved a satisfactory repeatability (on both m/z values and peak intensity) and the higher number and abundance of ions in MALDI-TOF-MS spectra. The method was finally used to compare urine samples of healthy donors with those of prostate cancer patients, and it was possible to individuate some differences between the two sets of samples by analyzing pooled urine.

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