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# Urine profiling by SELDI-TOF/MS: Monitoring of the critical steps in sample collection, handling and analysis

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**Abstract**

The topic of this study is the impact of several pre-analytical and analytical variables on proteomic profiling of human urine by surface enhanced laser desorption/ionization time of flight–mass spectrometry (SELDI-TOF–MS) in healthy subjects. Urine storage at room temperature caused a progressive degradation of proteins, which was prevented by the addition of protease inhibitors only up to 2 h from the collection. The timing of collection over the day had only a minor impact on protein profile, although influencing the intensity of peaks. Repeated freeze/thaw cycles (up to five) did not affect either the number or the intensity of the peaks. A comparison of the protein profile from eight different healthy individuals showed fairly consistent inter-subject similarities, along with between-subject differences, which were markedly dependent on the sex and the type of ProteinChip® array used. The addition of a variety of denaturing agents improved the quality of the spectra with all the chips tested (CM10, Q10 and H50), but not with the copper-coated IMAC-30 chip. Finally, SPA matrix allowed to achieve a better performance of SELDI-TOF/MS spectrum, as compared with CHCA, regardless of the ProteinChip® array used and even in the low *m/z* range (2500–10,000). In conclusion, we suggest that a careful choice of a number of pre-analytical and analytical conditions is required to accomplish and define a unifying protocol for the analysis of human urine by SELDI-TOF/MS, in physiological and in pathological states.


*Keywords:* Urine; Sample collection; SELDI-TOF/MS; Standardization

## 1. Introduction

Human urine can be collected in large amounts in a non-invasive fashion and has been extensively used over the centuries mainly for the study and monitoring of renal physiology and pathology. Normal urine contains up to 150 mg/24 h of proteins and peptides which are derived from a variety of sources including glomerular filtration of blood plasma, cell sloughing, apoptosis, proteolytic cleavage of cell surface glycosylphosphatidylinositol-linked proteins and secretion of exosomes by epithelial cells [1–3]. The soluble proteins in urine are derived largely from glomerular filtration and represent around 50% of total urinary proteins excreted by healthy individuals [4]. The glomerular filter effectively retards passage of high molecular weight proteins. However, even with very low sieving coefficients, proteins that are abundant in the blood plasma such as albumin and various globulins can pass the glomerular filter in substantial amounts to enter the lumen of the nephron. Beyond this, peptides and small proteins (<10 kDa) are freely filtered by the glomerulus. Most of the proteins and peptides that pass the glomerular filter are scavenged and proteolyzed in

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the proximal tubule [5,6]. Thus, a change in the amount of a given soluble protein that reaches the final urine can result from a change in its concentration in the blood plasma, a change in the function of the glomerular filter, or an alteration in the proximal tubule scavenging system. Based on these mechanisms, changes in excretion rate of specific urinary proteins can be indicative of systemic disease, glomerular disease, or diseases affecting the proximal tubule, respectively [7–12]. Finally, some of the soluble proteins in urine originate as membrane-bound proteins that are proteolytically cleaved from their membrane attachments, for instance Tamm–Horsfall protein (uromodulin) [13,14].

The use of urinary biomarkers to diagnose disease is a long-standing practice: the presence of albumin in the urine has been measured as an indicator of renal disease for centuries. The advent of protein mass spectrometry has enabled a new approach to the identification of putative urine biomarkers, for early detection of disease, as a means of differential diagnosis, or as a means of guiding therapy. Then, the recent development of high-throughput proteomic approaches has facilitated progresses in the cataloguing of the protein composition of several biological fluids such as plasma [15,16], serum [17,18], urine [19,20] and saliva [21,22].

The classical proteomic approach for the identification and quantification of proteins in complex media is based on two-dimensional electrophoresis coupled to matrix assisted laser desorption/ionization-time of flight–mass spectrometry (MALDI-TOF/MS). This approach, however, suffers from some limitations: it is rather time consuming, costly, and, more importantly, some concerns have been raised regarding the reproducibility of the results. This has led to the search of alternative and/or complementary strategies [23]. In the last few years, several "gel-free" methods have been explored, namely liquid chromatography coupled to electrospray mass spectrometry (LC-ESI/MS), capillary electrophoresis (CE) and surface enhanced laser desorption/ionization-time of flight–mass spectrometry (SELDI-TOF/MS), as complementary methods to better elucidate the protein composition of biological samples [24]. Among the different proteomic approaches, SELDI-TOF/MS is particularly appropriate for the investigation of low molecular weight proteins (<20 kDa) with femtomole sensitivity and the ability to examine native proteins without preliminary treatment of biological samples. SELDI-TOF–MS is a high throughput technique based on the chromatographic separation of proteins according to their physical characteristics (i.e. hydrophobic, hydrophilic, acidic, basic, metal affinity). The coupling of these chromatographic surfaces to a laser desorption time of flight mass spectrometer allows to generate an accurate protein profile of a biological sample requiring minimal amounts of sample. This approach has two main advantages over other protein separation methods: it is very rapid and does not require prior digestion of native proteins and peptides. Further, it allows to preferentially direct the analysis towards a selected range of proteins and peptides, by using specific chip types, thereby reducing the complexity of the sample analysis.

In spite of the growing interest in the analysis and characterization of urinary proteome in physiological as well as in different pathological conditions, data concerning the standardization of the urine analysis by SELDI-TOF/MS are presently rather limited [25–28]. The lack of standardization of sample collection and of the entire analytical process strongly impinges on the discovery of reliable urinary biomarkers.

Inclusion of large numbers of diverse subjects from multiple centers is crucial to sufficiently power translational proteomics investigations. In this context, pre-analytical variables, concerning sample processing and preparation, handling and storage, although often overlooked, may markedly influence the general quality and the degree of variability of the results obtained, along with crucial analytical variables, such as the choice of ProteinChip® array and of the matrix. Therefore, the aim of this study was to expand the analysis of the influence of a number of pre-analytical and analytical variables on urine proteomic profiling by SELDI-TOF/MS in healthy subjects.

## 2. Materials and methods

### 2.1. Reagents

Milli-Q deionized water (Millipore, Molsheim, France) was used for all homemade solutions. Bioprocessors, H50, CM10, Q10 and IMAC-30 chip arrays, All in 1 protein standard II, sinapinic acid (SPA) and α-cyano-hydroxycinnamic acid (CHCA) were purchased from Ciphergen (Ciphergen Biosystems, Fremont, California, USA). Protease inhibitors cocktail (AEBSF 104 mM, aprotinin 0.08 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin A 1.5 mM, E-64 1.4 mM), TFA and DTT were purchased from Sigma (Sigma–Aldrich St. Louis, MO, USA), urea and CHAPS were purchased from PlusOne Amersham Biosciences (Uppsala, Sweden), sodium acetate was from Fluka (Buchs, Switzerland), ethanol from Merck (Darmastadt, Germany) and Tris from USB Corporation (Cleveland, OH, USA). All solvents used were Ultra-Resi-Analyzed grade.

### 2.2. Urine collection and preparation

Urine samples were obtained from eight non-smoking healthy subjects (four males and four females) and were tested for standard parameters (pH, glucose, blood content, specific weight, etc.) using Multistix reactive stripes (Bayer Diagnostics, Munich, Germany). Urine samples were then either used fresh or aliquoted and stored at −80 °C until used.

For storage evaluation experiments, 3 ml of fresh urine were collected. The samples were divided into two sets of 10 aliquots each, with or without the addition of protease inhibitors, and left at room temperature (RT) from 0 to 48 h, and finally stored at −80 °C until analysis. For freeze and thaw experiments, urine was aliquoted into five fractions, frozen at −80 °C and subsequently thawed at room temperature. The above procedure was repeated up to five times.

For the evaluation of the difference in urine composition within the day, urine (2 ml) was randomly collected during the day, twice in the morning and once in the afternoon, and then stored at −80 °C until use.

To examine the impact of denaturing agents on the quality of spectral profiles, urine samples were either diluted with the bind-

ing buffer specific for the ProteinChip® array tested or mixed (2:3, v/v) to several denaturing buffer (DB) solutions prior to the loading. The DB used were: DB1 (9 M urea and 2% CHAPS); DB2 (100 mM DTT) and DB3 (9 M urea and 2% CHAPS and 100 mM DTT).

The mixture of urine and binding buffer was incubated at 4 °C for 30 min under continuous shaking before loading onto ProteinChip® arrays.

### 2.3. ProteinChip® arrays

Four chip types were tested: (1) H50, which binds proteins by hydrophobic interaction, was used to test the effect of sample centrifugation on the quality of subsequent protein analysis, and to evaluate the effect of freeze–thaw cycles on the stability of the sample; (2) CM10, which binds proteins by cationic exchange, was used mainly to explore intra-individual variations in the protein profile over the day and the impact of storage conditions; (3) copper-coated IMAC 30, which binds metal binding proteins and (4) Q10, which binds proteins through strong anionic exchange as well as H50 and CM10.

All of them were used to determine the effect of denaturing buffers on the quality of the spectra. All the chips allowed to evaluate the influence of SPA and CHCA matrix on the spectral profile, and to explore inter-subject differences in the protein profile.

### 2.4. Chip preparation and analysis

If not otherwise stated, frozen urine samples were thawed on ice and centrifuged for 10 min at 13,000 × *g* at 4 °C to remove insoluble particles before analysis. The supernatant was then transferred to a clean tube and 30 μl was analyzed by the bioprocessor. All the chip types were prepared according to the manufacturer's instructions. Each chip was first washed twice with its specific binding buffer (10% acetonitrile/0.1% trifluoroacetic acid (TFA) for H50; 100 mM sodium acetate pH 4 for CM10; 100 mM Tris–HCl pH 8.8 for Q10; 0.1 M Tris–HCl, pH 7.4, for IMAC-30, which required preliminary loading with $Cu^{2+}$ and neutralization by sodium acetate pH 4, before the exposure to its specific binding buffer), and then loaded with the sample. The chip was then washed thrice with 150 μl washing buffer and finally with 200 μl deionized Millipore water and air dried for 20 min. A saturated solution of sinapinic acid (SPA, Ciphergen) was prepared in 50% acetonitrile/0.1% trifluoroacetic acid. SPA was then diluted by 50% in 50% acetonitrile/1% trifluoroacetic acid solution and 2 μl were applied to each spot. α-cyano-hydroxycinnamic acid (CHCA, Ciphergen) was prepared in 50% acetonitrile/0.5% trifluoroacetic acid and 2 μl directly applied to each spot. All the chips were read by adopting the same protocol (laser energy 15,000 nJ, matrix attenuation 2500, focus mass 10,000, sample rate 800, partition 1 of 4, acquired mass range from 2500 to 25,000), unless otherwise specified. The software was externally calibrated by using the all in 1 protein standard II kit (Ciphergen, Fermont, USA) and all the spectra were normalized by means of total ion current. The analysis was finally performed in a *m/z* range from 2500 to 25,000 with the software Ciphergen express 3.0 considering as real peaks those having S/N ratio greater than 4 and peak height greater than 6.

## 3. Results

### 3.1. Reproducibility of the spectral analysis

To evaluate the intra-assay variability, we applied 60 μl of whole human urine from the same subject to four different spots of H50 ProteinChip® array. We recorded a mean of 32 peaks (min. 30 and max. 34) in the four spectra. The coefficient of variation (CV) in the number of peaks, as identified by means of Ciphergen express 3.0 software, was around 6%. Then, we selected the 24 peaks which were common to all the spectra and calculated the CV in the intensity of each of the peaks. It ranged from 6 to 40% with a median value of ∼19%. Of note, the CV in peak intensity did not show any correlation with the molecular weight of the peaks [26].

Thereafter, we defined the inter-assay CV by analysing the number and the intensity of the peaks recorded with the same sample in three independent experiments. We recorded 9% CV in the number of peaks and 23% CV in the intensity of peaks: thus, inter-assay CV resulted closely similar to intra-assay CV.

### 3.2. Sample collection and handling

#### 3.2.1. Changes in urine protein profiling over the day

To explore the possible impact of the timing of urine collection on the quality of spectra, three urine samples were randomly collected from each subject during the daytime, twice in the morning and once in the afternoon. SELDI-TOF/MS protein profiling showed only minor differences among samples: 37 peaks were identified in the first sample, 45 in the second sample and 37 in the third one, with an inter-assay coefficient of variation superimposable with intra-assay CV (11% versus 8%, respectively). The peaks were then clustered according to their *m/z* and the resulting analysis confirmed the overlap of the spectral profiles from the three samples: 30 peaks were common to all the spectra, 32 were common to the first two samples, 30 were common to the first and the last sample and, finally, 32 peaks were common to the second and the third sample. In contrast, the intensity of the peaks resulted to largely vary among samples (Fig. 1).

#### 3.2.2. Between-subject differences

Urine samples from eight healthy subjects (four males and four females) were collected early in the morning, centrifuged and the supernatants were analyzed by H50, CM10, IMAC-30 and Q10 ProteinChip® array. The number of peaks detected largely varied, depending on the chip used: CM10 separated a mean of 42 peaks (min. 29 and max. 50) for males and 51 peaks (min. 43 and max. 62) for females, respectively; IMAC-Cu separated a mean of 55 (min. 40 and max. 66) and 54 (min. 48 and max. 59) peaks; H50 separated a mean of 31 (min. 22 and max. 48) versus 35 (min. 25 and max. 47) peaks, while Q10 identified a mean of 33 (min. 24 and max. 34) and 30 (min. 29

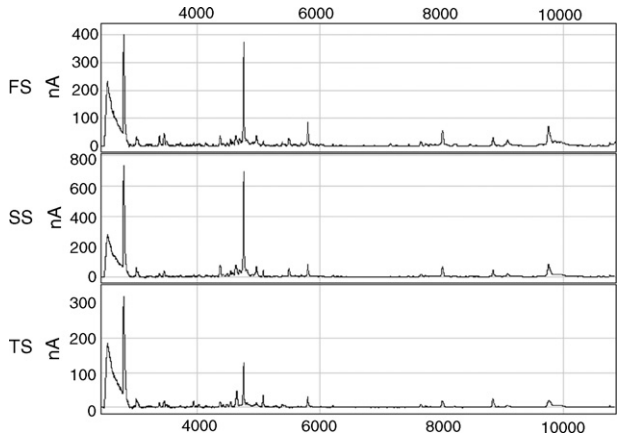


Fig. 1. Protein profile of three urine samples randomly collected from the same subject during the daytime, as analyzed by CM10 ProteinChip® array. Peak analysis showed a good reproducibility of the spectra [37 peaks in the first sample (FS) vs. 45 in the second sample (SS) and 37 in the third sample (TS)], with a between-sample coefficient of variation around 11%. In contrast, the intensity of the peaks largely varied among samples.

and max. 40) peaks (Fig. 2). For each group (males and females), we then identified the peaks shared by all the subjects on a given ProteinChip® array. For CM10, we found 43% matching in the male group (17/42 peaks in common) and 27% matching in the female group (14/51 peaks). For Q10, there were 15/30 peaks in common in the male group (50% match) and 13/33 peaks in common in the female group (39% match). For H50, we identified 15/31 shared peaks in the male group (48% matching) and 7/35 peaks shared in the female group (20% matching). Finally, we found 28/55 (51%) peaks in common in the male group and 27/54 peaks in common in the female group (50%) by using IMAC-Cu. In summary, there was a rather large inter-subject variation in urine proteomic profiling, which was seemingly slightly less relevant among male subjects. When all the samples, males and females, were analyzed and compared together, the percentage of peaks in common among all the spectral profiles was similar to that recorded within the female group (see table in Fig. 2).

Finally, it is worth noting that the protein profiles of the same sample, as obtained by using different chips, shared only a few, if any, peaks (4 of 165 in sample 1, 6 of 145 in sample 2, 9 of 165 in sample 3, 4 of 165 in sample 4, 2 of 135 in sample 5, 2 of 149 in sample 6, 4 of 131 in sample 7 and 3 of 168 in sample 8) (spectra in Fig. 2), indicating the need for the analysis by several ProteinChip® array to deepen and enlarge the study of urinary proteome.

#### 3.2.3. Storage conditions

Sixteen milliliters urine was collected from a healthy man to determine the impact of sample storage at room temperature. Urine was divided into 1 ml aliquots, which were left at RT from 0 to 48 h before the analysis, with or without the addition of a mixture of protease inhibitors (1 μl for each ml of urine). Sixty microliters of each sample were finally loaded, in duplicate, on a CM10 ProteinChip® array and analyzed by SELDI-TOF/MS. Overall, we recorded a time-dependent decrease in the number of protein peaks, which was antagonized by the addition of protease inhibitors only up to 2 h of storage at RT (Fig. 3). This suggested a scanty, if any, intrinsic protease activity of human urine and, on the other hand, the appearance of a progressive degradation of urinary proteins over time, which turned out to be largely insensitive to the addition of protease inhibitors (Fig. 3).

#### 3.2.4. Freeze–thaw cycles

Repeated freeze–thaw cycles (up to five) had a meager influence on the protein profile by SELDI-TOF–MS analysis, as evidenced by 9% CV in the number of peaks (Fig. 4). Moreover, to examine the impact of freeze–thaw cycles on the intensity of signals, we chose 10 peaks and calculated the CV of their intensity. It ranged between 10 and 30% (mean 20%) and was comparable to the mean intra-assay CV of peak intensity (∼21%). In conclusion, freeze and thaw cycles do not appear to produce a significant modification in either the number or the intensity of the peaks.

### 3.3. Sample preparation for the analysis

#### 3.3.1. Centrifugation

To investigate whether the presence of cell residues and other insoluble particles could interfere with the SELDI-TOF/MS analysis, a urine sample was loaded on H50 ProteinChip® array with or without prior centrifugation for 10 min at 13,000 × *g*. Urine centrifugation resulted in an increase in the number of protein peaks (from 29 to 38) and with a two- to three-fold increase in the median intensity of the peaks (data not shown).

#### 3.3.2. Addition of denaturing solutions

To ascertain whether the addition of denaturing agents would influence the quality of the analysis, we compared the protein profiles of centrifuged urine, in the presence or in the absence of three different denaturing buffer solutions (Table 1). The analysis was performed with all the chip types tested, in the attempt to identify the best buffer for each ProteinChip® array. The addition of DB3 to the sample before the analysis increased the number of peaks when CM10 and Q10 were used, while H50 resulted to yield the highest number of peaks in the presence of DB1. At variance, the addition of denaturing solutions seemed to interfere with the capture of metal binding proteins by IMAC 30: with this chip, the best results (39 peaks) were achieved using the standard IMAC-30 buffer.

Then, we compared the protein profiles obtained with the different chips using their respective optimal buffers (see above) and calculated the percentage of peak matching between chips. Specifically, only those peaks which shared the same mass and the same shape were considered to match. This criterion is generally used to identify redundant peaks among different ProteinChip® arrays. The number and the percentage of matching peaks among the different ProteinChip® arrays tested are summarized in Table 2. H50 had 15 peaks in common with IMAC 30 (32% matching), 9 peaks in common with Q10 (19.5% matching) and 17 peaks in common with CM10 (37% matching). IMAC 30 showed 15 (38.4%), 8 (20.5%) and 11 (28.2%) peaks matching with H50, Q10 and CM10, respectively. Q10

| Chip type | Mean number of Peaks: Males (4) | Mean number of Peaks: Females (4) | Number of shared peaks (%): Males | Number of shared peaks (%): Females | Number of shared peaks (%): Males + Females |
|---|---|---|---|---|---|
| CM10 | 42 | 51 | 17/42 **(43%)** | 14/51 **(27%)** | **13/46 (28%)** |
| Q10 | 30 | 33 | 15/30 **(50%)** | 13/33 **(39%)** | **14/32 (43%)** |
| H50 | 31 | 35 | 15/31 **(48%)** | 7/35 **(20%)** | **9/32 (28%)** |
| IMAC-Cu | 55 | 54 | 28/55 **(51%)** | 27/44 **(50%)** | **(46%)** |

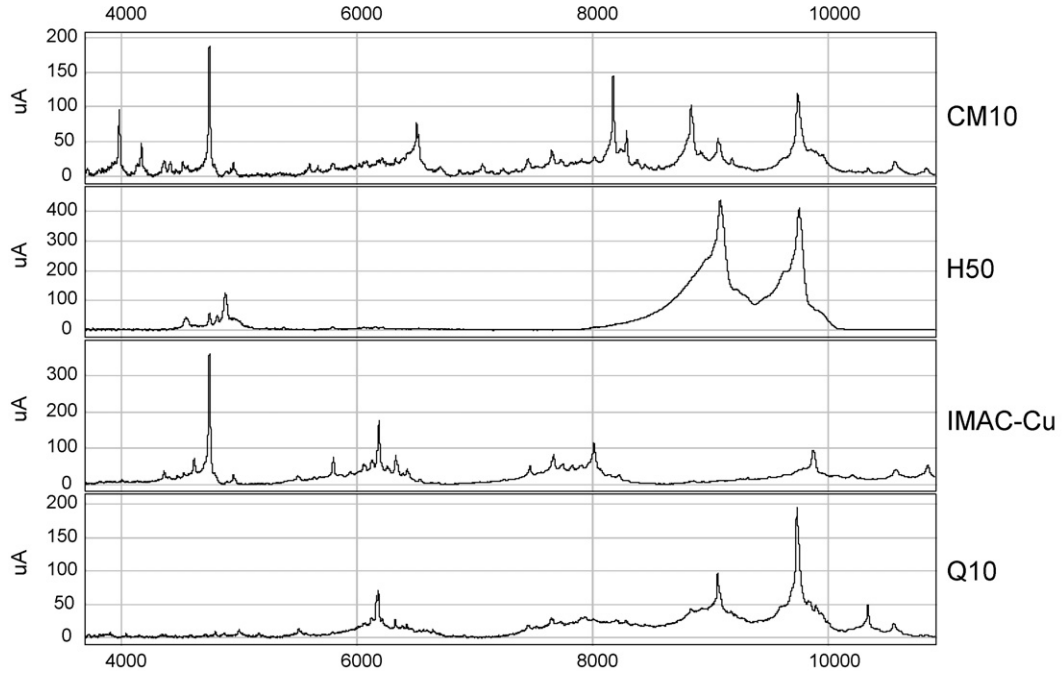


Fig. 2. Inter-individual variations in the urine proteome by SELDI-TOF–MS analysis. Upper part: inter-individual variability among the eight subjects tested, regardless of the type of ProteinChip used. It is reported the mean number of peaks recognized by each ProteinChip in male and in female healthy controls, and the number (percentage) of peaks in common among those subjects with each ProteinChip. The number and the percentage of shared peaks among all the samples (males + females) are also shown. Lower part: protein profiles of the same sample, analyzed by four different ProteinChips, to illustrate the large difference in the proteomic profiling of the same subject, according to the ProteinChip used.

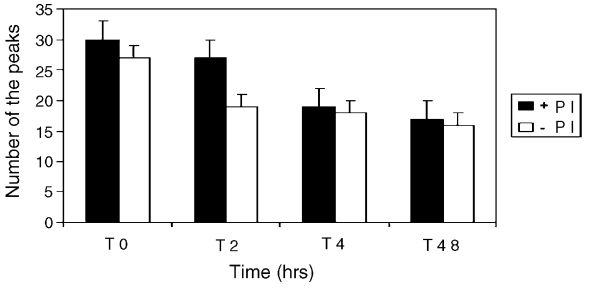


Fig. 3. Effect of the time of storage at RT, and of the presence/absence of protease inhibitors, on urine protein profiling by SELDI-TOF/MS analysis, PI: protease inhibitors.

had 9 (18.3%), 8 (16.3%) and 7 (14.5%) peaks in common with H50, IMAC 30 and CM10, respectively. Finally, CM10 shared 17 (34.7%), 11 (22.4%) and 7 (14.3%) peaks with H50, IMAC30 and Q10.

### 3.3.3. *Influence of the matrix type*

We further examined which matrix would produce the best results in terms of number and average intensity of peaks, by comparing the protein profiles obtained using SPA or CHCA. Thirty micoliters were analyzed in duplicate with all the chips tested using either SPA or CHCA. First, we compared the number of peaks in the range 2500–25,000 *m/z*. SPA showed a better performance with all the ProteinChip® arrays tested, when compared to CHCA (Table 3). Then, the comparison was extended to

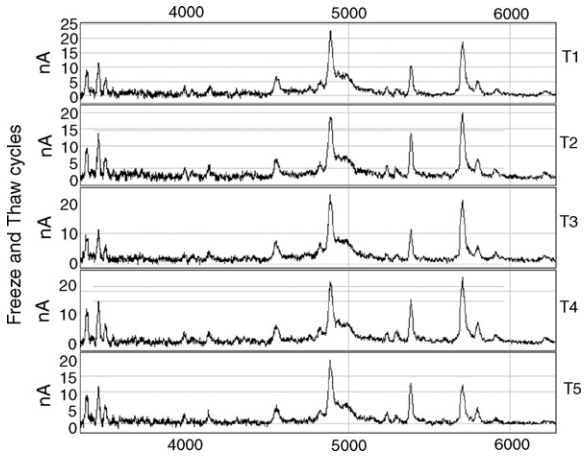


Fig. 4. Effect of freeze and thawing on urine proteomic analysis by SELDI-TOF/MS. Urine samples underwent 1–5 freeze and thaw cycles (T1–T5) and the corresponding spectral profiles were compared. The figure shows the lack of significant effect of the procedure on both the number and the intensity of the peaks identified by the analysis, performed using H50 ProteinChip® array.

Table 1
The peaks counted with the all ProteinChip® arrays tested in presence (DB1, DB2 and DB3) and in absence (No DB) of several buffers in the samples are summarized in the table

| ProteinChip | Buffers | | | |
|---|---|---|---|---|
| | No DB | DB1 | DB2 | DB3 |
| | Peaks counted | | | |
| H50 | 38 | **46** | 32 | 34 |
| IMAC 30 | **30** | 22 | 18 | 24 |
| Q10 | 42 | 42 | 47 | **48** |
| CM10 | 32 | 39 | 35 | **49** |

The best matching between ProteinChip® array and buffer is shown in bold font.

Table 3
Evaluation of the impact of two matrices (SPA and CHCA) on the number of the peaks obtained by four ProteinChip® arrays

| Range (*m/z*) | Number of peaks | | Clusters |
|---|---|---|---|
| | SPA | CHCA | |
| IMAC-30 | | | |
| 2500–25,000 | 44 | 40 | 27 |
| 1500–10,000 | 38 | 35 | 22 |
| CM10 | | | |
| 2500–25,000 | 42 | 30 | 12 |
| 1500–10,000 | 36 | 42 | 20 |
| H50 | | | |
| 2500–25,000 | 44 | 26 | 13 |
| 1500–10,000 | 39 | 29 | 10 |
| Q10 | | | |
| 2500–25,000 | 38 | 21 | 14 |
| 1500–10,000 | 35 | 23 | 13 |

The best performances are highlighted in bold. Clusters identify the peaks recognized by both types of matrices.

the low molecular weight mass range (1500–10,000 *m/z*). Again, we found a higher number of peaks with SPA using all types of chips, except CM10 (Table 3). Finally, we quantitated the number of peaks that were recognized by both types of matrices: overall, the overlap of detected peaks was rather limited, and this was true with any of the chips tested (Table 3).

## 4. Discussion

SELDI-TOF/MS is a relatively new and expanding proteomic technology that, thanks to the selective capture of specific subgroups of proteins by different ProteinChip® surfaces and the ability to provide high-throughput analysis, is becoming one of the most promising tools for the discovery of novel clinical

Table 2
Peaks matching among the different ProteinChip® arrays tested (both as number of peaks and percentage)

| | Peaks matching | | | |
|---|---|---|---|---|
| ProteinChip | H50 | IMAC 30 | Q10 | CM10 |
| H50 | | 15 ( 32%) | 9 (19.5%) | 17 (37%) |
| IMAC 30 | 15 (38.4%) | | 8 (20.5%) | 11 (28.2%) |
| Q10 | 9 (18.3%) | 8 (16.3%) | | 7 (14.5%) |
| CM10 | 17 (34.7%) | 11 (22.4%) | 7 (14.3%) | |

Matching peaks were considered those peaks which had the same *m/z* and the same shape with the different ProteinChip® arrays. Results are expressed as number of matching peaks (and percentage of total peaks). The analysis was performed the optimal binding buffer for each ProteinChip® array, as reported in Table 1.

biomarkers. Underpinning the process of biomarker discovery in urine by SELDI-TOF–MS, and presumably by any proteomic approach, is the need for adequate qualification/validation of the analytical approach used at the various stages (urine sampling and storage, sample preparation and handling, and analytical platforms), in order to improve the analytical reproducibility. Although several SELDI-based studies have been published so far to investigate the changes in the urine protein profile associated with the onset and progression of kidney and systemic diseases, the complexity of this biological fluid requires to explore in depth pre-analytical and analytical conditions that can significantly affect urine proteomic profiling, especially in the setting of clinically-oriented multicenter studies. However the information concerning the standardization of urine analysis by SELDI-TOF/MS are rather limited [26,28].

Schaub et al. [26] examined the impact of a number of extrinsic factors, such as urine storage and freeze and thaw cycles, matrix dilution and instruments setting, and of intrinsic factors, namely presence of blood in urine, relevance of urine dilution and difference between first void and midstream void, on the quality of the SELDI-TOF/MS spectra. They observed that the spectra of human urine stored at RT changed with the gender and the modality of urine collection (first void versus mid-stream urine samples) [26]. Specifically, midstream urine of males showed only minor changes even after 3 days at RT, whereas first-void urine from both males and females showed considerable changes in the spectral profile following 3-day storage at RT or at 4 °C, due the appearance of a series of new peaks in the 2–6 kD range [26]. At variance, Traum et al. [27] were unable to find any modification of SELDI-TOF/MS spectrum in the urine from a male subject, stored at 4 °C for up to 24 h. Our data seemingly agree with those reported by Schaub et al. [26]. Moreover, we could demonstrate that urine samples could be stored at RT for only 2 h, the protein degradation within the biological sample being seemingly independent from an intrinsic protease activity.

We confirmed that the urine proteome profiling was not significantly modified by repeated freeze and thaw cycles. Further, we reported that the addition of denaturing agents, like urea and CHAPS, could improve the quality of the spectra. With respect to previous studies [26–28], the present investigation could identify the most appropriate denaturing solution for each of the ProteinChip® arrays tested. Very recently, Roelosfen et al. [28] have compared the performance of seven ProteinChip® arrays in the presence of either SPA or CHCA. They concluded that CHCA was the best matrix for H50, SPA ensured the best performance with IMAC-Cu, and both matrices did well with CM10. We found that SPA detected a higher number of peaks than CHCA, regardless of the ProteinChip® array tested. It is worth noting that, in our experiments, the two matrices allowed to identify largely different protein peaks: in other words, the overlap between the proteomic profile yielded by CHCA or SPA is rather limited. This implies that the combined use of both matrices increases the number of identified peaks for each ProteinChip® array.

In the present investigation, we used four ProteinChip® arrays, namely H50, Q10, IMAC-Cu and CM10. Q10 and CM10 bind proteins by ionic exchange that ensures the reversible binding of the peptides and proteins to the surface according to their net surface charge. Since surface charge is the result of weak acidic and basic amino acids within the protein, the pH of the binding buffer can deeply affect the binding of the proteins to the arrays. For CM10 ProteinChip® array, that incorporates carboxylate chemistry and acts as a weak cationic exchanger, we used 0.1 M sodium acetate as binding buffer. The low pH of this buffer imparted an overall positive charge to a greater number of proteins within the sample, thus favoring their binding to the array. Similarly for Q10, that incorporates quaternized ammonium groups and acts as a strong anion exchanger, we used a binding buffer (100 mM Tris–HCl pH 8.8) that confers an overall negative charge on a greater number of proteins and favors their binding to Q10 ProteinChip® surface. Hydrophobic proteins were recruited by H50 ProteinChip® array that has binding characteristics similar to that of a C6–C12 alkyl chromatographic resin. Also in this case, the binding of the proteins to the surface is influenced by the nature of the binding buffer. An increase in the concentration of organic solvent in the binding buffer determines an increase of the selectivity of the surface, thus restricting the binding to only those proteins which are more hydrophobic than the buffer. We used 10% ACN/0.1%TFA to capture a larger amount of hydrophobic proteins. Finally, we used IMAC-Cu ProteinChip® array to visualize metal binding proteins. IMAC30 incorporates nitrilotriacetic Acid groups that are able to form stable complexes with polyvalent metals including $Cu^{2+}$, $Ni^{2+}$, $Fe^{3+}$ and $Ga^{3+}$. IMAC-Cu is generally used to achieve an overall protein profile of a biological sample, while the coupling of IMAC30 with other metals can be used for the investigation of specific subsets of proteins (i.e. phosphorilated proteins and peptides with $Fe^{3+}$ or $Ga^{3+}$). We analyzed the proteome of the eight individuals by using all the above ProteinChip® arrays. Software analysis revealed that for each subject only a few peaks were common to all the ProteinChips®, reinforcing the concept that their simultaneous use does improve the analysis of the human urinary proteome. We also tested both SPA and CHCA with all the ProteinChips®. In fact, we were interested to explore the best match between a given ProteinChip® array and a specific matrix, beyond the general assertion that SPA preferentially visualizes the protein profile, while CHCA allows to appreciate the peptidic profile. Furthermore, we could note that for each ProteinChip® array only a few peaks were common between the two matrices, thus indicating that their combined use with the same ProteinChip® array can help to further increase the number of peaks detected. Finally, all the ProteinChip® arrays were used to test the impact of three denaturing solutions (DB1, DB2 and DB3) on SELDI-TOF profiling, in the attempt to define an optimal match between chip and denaturing buffer.

Instead, we tested only one chip type to explore the possible changes in the urine protein profile over the day, and to evaluate the changes in the profile of the same sample following specific procedures (i.e. centrifugation, repeated freeze and thaw cycles, and storage at RT), since we reasoned that the impact of above pre-analytical variables, whenever demonstrated, would be definitely independent from the specific ProteinChip® array adopted.

As for the timing of sample collection, the individual protein profile showed only minor variations in the number and distribution of the peaks, whereas the intensity of the peaks had relevant variations over the day. This trend may reflect the different dilution of urinary proteins. On the other hand, we cannot rule out the possibility of a circadian rhythm in the excretion of some proteins.

We then focused on sample storage conditions, and particularly we wondered how long human urine remained stable at RT. We demonstrated that urinary proteins undergo rapid degradation when left at RT, regardless of the presence of protease inhibitors, which exert a protective effect only within 2 h from the collection. The latter finding would imply the lack of relevant protease activity in human urine, and suggests that time-dependent protein degradation is seemingly independent from enzymatic lysis of the biological sample.

We analyzed the effect of 1–5 freeze and thaw cycles on the quality of the spectral profile to determine whether a frozen aliquot could be reused more that one time without any significant quantitative and qualitative modification of its protein integrity. Within the above experimental conditions, we could not appreciate any significant modification of the protein profile, which ensures a relative flexibility in the setting of standard protocols. Our findings are similar to those reported by Schaub et al. [26], though these authors found some loss of peak intensity already starting from the fifth cycle of freeze–thaw.

The definition of a standard urine protein profile of healthy human subjects by SELDI-TOF/MS analysis was beyond the aims of the present study. Nevertheless, a comparative analysis, though limited to only eight subjects, revealed the presence of rather large inter-individual differences. All subjects were studied by using all the ProteinChips and revealed a large inter-individual variability, ranging from 49 to 80% according to the chip tested, mostly among females.

After examining the impact of some critical pre-analytical variables, chosen among those of practical relevance for the definition of standardization protocols, mainly in the setting of clinically oriented studies, we focused our interest on the standardization of a series of analytical variables which would greatly affect the reproducibility as well as the quality of the results by SELDI-TOF–MS analysis. We showed that sample centrifugation and the addition of denaturing agents improved the quality and reproducibility of data. We tested two types of denaturing solutions, those containing caotropic agents, which loosen protein–protein interactions, and the solutions comprising reducing agents, which are able to break disulphide bonds. We found that the addition of a solution containing both caotropic and reducing agents (DB3) significantly improved the spectral quality and the number of the peak attained with CM10 and Q10, while H50 had a better performance with caotropic agents (DB1) and IMAC buffer was the most appropriate to favor metal binding proteins capture by IMAC-Cu (Table 1). We would observe that both sample centrifugation and the addition of denaturing solution are rapid, simple and reproducible manipulations, which might be easily included in any analytical protocol thereby likely improving the quality of SELDI spectra.

Finally, we looked at the influence of matrix on the number and quality of peaks and found that SPA was the best matrix for all the ProteinChip® arrays tested, both in the high (2500–25,000 *m/z*) and in the low (1500–10,000 *m/z*) mass range. The latter finding was unexpected, since CHCA has been previously reported to offer the best performance in the low *m/z* range, below 8–10 kDa, at least using NP20 ProteinChip® array [26]. We would like to point out that only few peaks were common to SPA and CHCA analysis, in other words the use of each specific matrix highlights some peaks and excludes others. In the clinical setting, this may lead to the preferential use of a given matrix, according to the target protein, or protein set, chosen.

In conclusion, the present study allows to define the following points, concerning the collection and handling of human urine for subsequent proteomic analysis by SELDI-TOF/MS: (1) the timing of the collection can significantly affect the proteomic profile. Thus, for inter-subject comparisons, for sequential analysis of the same subject in follow-up studies, or generically for quantitative analysis, it is advisable to use a fixed timing for the collection of the sample. (2) Urine appears to remain stable at RT for no more than two hours, regardless of the presence of protease inhibitors, which dictates the need for relatively rapid procedures of sample freezing. (3) Repeated cycles of freezing and thawing do not significantly alter the protein profile, and this in a way simplifies the storage and reduces the need for multiple aliquoting. (4) The wide inter-individual variability of urine proteome represents one of the greatest hurdles to the identification of reliable biomarkers of disease, and asks for the examination of very large numbers of subjects to depict a reference profile of healthy controls. (5) The addition of denaturing agents to the sample improves the quality of the peaks with some ProteinChip® arrays, i.e. CM10, Q10 and H50, while it should be avoided with others, namely with IMAC-30. (6) SPA generally ensured the best performance, in terms of number of peaks detected, with all the ProteinChip® arrays used. Regardless, each matrix identified some peaks which were not recognized by the other, which leads to conclude that the combined analysis of the same sample by using both matrices enlarges the pool of information on the urine proteome. Finally, we would suggest that this study, together with previous investigations examining similar issues, may represent a valuable premise to accomplish and define a unifying protocol for the analysis of human urine by SELDI-TOF/MS, in physiological as well as in pathological states.

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