Luís André Botelho de Carvalho

Licenciando em Ciências Biomédicas

Stabilizor-T1 Technology for Urine Proteome Preservation at Room Temperature

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Prof. Doutor José Luís Capelo, PhD, FCT-UNL

Coorientador: Doutor Hugo Miguel Santos, PhD, FCT-UNL

Júri:

Presidente: Prof. Doutor Ricardo Franco

Arguente: Prof. Doutora Ana Coelho

Vogal: Prof. Doutor José Luís Capelo

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2018

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Universidade Nova de Lisboa
Faculdade de Ciências e Tecnologia
Departamento de Química
Monte da Caparica
Setembro, 2018

Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito.

Fernando Pessoa

On-going manuscripts

- **“Freeze-Free preservation of urine proteome”**

(manuscript in progress).

Luís Carvalho; Hugo Santos; Carlos Lodeiro; José Luis Capelo-Martínez.

Acknowledgments

I am about to end a chapter of my life and at the same time starting a new one. Over this last two years, I felt that I had accomplished many objectives, but I also gained new ones as I was able to solidify my goal. I had the privileged to make new friends and meet professionals who led me through these years.

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To my star that has been taking care of me, a warm thank you full of saudade …

Resumo

A urina é uma amostra biológica com grandes quantidades de informação e alto valor prognóstico. O método não invasivo e fácil de colher urina permite a sua obtenção em grandes quantidades e em diferentes momentos. É uma amostra fulcral no diagnóstico médico e é amplamente utilizada na descoberta de novos biomarcadores. Em estudos proteómicos, a grande diversidade entre as amostras de urina de diferentes indivíduos, representa uma das principais desvantagens no tratamento e armazenamento de urina. Assim sendo, é necessário o desenvolvimento de uma nova metodologia padrão e otimizada para o seu processamento. Atualmente, o método mais comum para o armazenamento de urina é o congelamento a -20 ou -80 ˚C. No entanto, este método requer refrigeradores dedicados e específicos. Associado este método, os efeitos dos ciclos de congelação e descongelação na urina demonstraram ter um impacto negativo, induzindo alterações no proteoma da urina e não sendo ideal para a descoberta e validação de novos biomarcadores. Neste trabalho, pretendemos superar essa limitação, desenvolvendo um novo método para preservação e armazenamento de urina à temperatura ambiente. A proteína presente na urina foi inserida numa membrana de ultracentrifugação na qual foi preservada pela inibição das protéases, usando um tratamento de choque térmico aplicado pelo *Stabilizor T1*. As amostras preservadas foram armazenadas à temperatura ambiente e -60˚C. Um armazenamento padrão de urina foi realizado a -20˚C para comparação e validação dos métodos. As amostras foram armazenadas por 6 meses e a preservação de urina foi avaliada por espectrometria de massa, usando quantificação *label-free*. Foi possível comprovar que ambos os métodos de armazenamento testados foram eficientes na preservação do proteoma da urina por 6 meses, não se verificando alterações significativas na quantidade de proteoma. Nós validamos um novo método de armazenamento e preservação para amostras de urina à temperatura ambiente.

Palavras-chave: Armazenamento de urina; Preservação de urina; Proteómica na urina; Quantificação *label-free*; Stabilizor T1; Tratamento de amostras de urina.

Abstract

Urine is a valuable biological sample that contains huge amounts of information with high diagnostic and prognostic value. The non-invasive and easy method underlying urine collection allows the repetitive assemblage of urine in large quantities and in different time points. Urine samples have become a standard sample on medical diagnosis and widely applied in proteomics cohort studies for biomarker discovery. In proteomics studies, the variety present in urine samples from different specimens represents one of the major drawbacks in urine sample handling and storage. Furthermore, the development of a new standard and optimized methodology for urine sample handling is required. Currently, the common urine storage method is by freezing at - 20 or - 80 ˚C. This method requires specific dedicated refrigerators, and the effect of freeze-thaw cycles have been showed to induce proteome alterations which is not ideal for urine biomarker discovery. In this work, we aim to overcome this limitation by developing a new method for urine preservation and storage at room temperature. Urine proteomes were loaded onto an ultrafiltration membrane in which were preserved by protease inhibition using a heat shock treatment applied by the Stabilizor T1. The preserved samples were stored at room temperature and -60˚C. A standard urine storage was proceeded at -20 for comparison and validation of the methods. Samples were storage for 6 months and compared using mass spectrometry label-free quantification. We were able to prove that both storage methods could effetely storage the urine proteome for at least 6 months without significant alterations in the preserved proteome. We have validated a new storage and preservation method for urine preservation at room temperature.

Key Words: Label-free quantification; Stabilizor T1; Urine preservation; Urine proteomics; Urine sample handling; Urine storage.

General Index

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Abbreviations

[#]

2D-PAGE – two-dimensional polyacrylamide gel

[A]

ACN – acetonitrile

AKI – acute kidney injury

Ambic – ammonium bicarbonate

[C]

CHCA – α-cyano-4-hydroxycinnamic acid

CyC – Cystatin-C

[D]

DHB – dihydrobenzoic acid

[E]

ESI – electrospray ionization

ESI-MS – electrospray ionization - mass spectrometry

[F]

FA – formic acid

FASP – filter-aided sample filtration

FT-MS – fourier transform ion cyclotron

[H]

HBD – Human Body Fluids

HPLC – High Performance Liquid Chromatography

[L]

LC – Liquid chromatography

LC-MS – liquid chromatography - mass spectrometry

L-FABP – liver fatty acid binding protein

[M]

MALDI – matrix-assisted laser desorption ionization

MS – mass spectrometry

MWCO – molecular weight cut-off

[N]

nano-LC-MS – nano - liquid chromatography - mass spectrometry

NGAL – neutrophil gelatinase-associated lipocalin

[P]

PSMs – peptide spectrum matches

PTMs – post-translational modifications

[Q]

Q – quadrupole

Q-TOF – quadrupole time-of-flight

[R]

RP-LC – Reverse-phase Liquid Chromatography

[T]

TOF – time-of-flight

I. Introduction

I.1. Proteomics

The discover and decoding of the human genome thought DNA sequencing had a high impact on the scientific community. Such breakthrough led to the accumulation of an extensive amount of DNA sequences in databases which still under assessment. Genes and transcripts can only evaluate protein function and activities. Moreover, the complete sequencing of genomes is not enough to elucidate biological function. To overcome this problem, the analysis of expressed proteins in an organism can offer a more realistic understanding of biological functions. Furthermore, proteomic studies may provide a more comprehensive view about biological and molecular processes [1,2]. In the next sections, a comprehensive explanation about what is proteomics is to be given.

I.1.1. What is Proteomics?

The biological mechanisms can be unlocked with the aid of (**I**) genomics, (**II**) transcriptomics, and (**III**) proteomics (**Figure I.1 A**) [3]. Proteomics, by definition, is the large-scale study of proteins. It consists of an extensive and precise analysis of proteomes in an organism at a particular time. Additionally, a proteome can be characterized and defined by a group of proteins that are being expressed in an organism that can change over time [4].

The concept of Proteomics started with the term *protein* that was first introduced into the scientific community in 1938 by Jöns Jakob Berzelius to describe a class of macromolecules made up of linear chains of amino acids that were present in living organisms. The word *proteomics* was created by Wilkins in 1995, however proteomics studies started earlier in 1975 with the analysis of large numbers of proteins by poly two-dimensional polyacrylamide gels (2D-PAGE). This method has some limitations in terms of protein quantification and identification which were later exceeded in the mid-1990s by the emerging of mass spectrometry (MS) in the proteomics field. Nowadays mass spectrometry has turn into the core method in proteomics with strategies that allow the accurate identification and quantification of proteins from complex samples [4,5].

I.1.2. Proteomics Complexity

Proteins are macromolecules present in every living being with a wide range of functions. They have a major role in biochemical reactions, structure, communication and regulation [4]. Through the **Figure I.1 B** it is possible to understand the proteome landscape complexity in a single cell since the abundance of a given transcript may not reflect the abundance of the corresponding protein.

The cellular proteome is highly complex however the high numbers of proteins showed in the figure cannot demonstrate his true complexity. Proteins can exist in several modified forms due to post-translational modifications (PTMs) and alternative splicing which can generate distinct protein isoforms. Proteome imbalance also contributes to the proteome complexity, the protein biogenesis and degradation determine the abundance of each protein within the proteome [6].

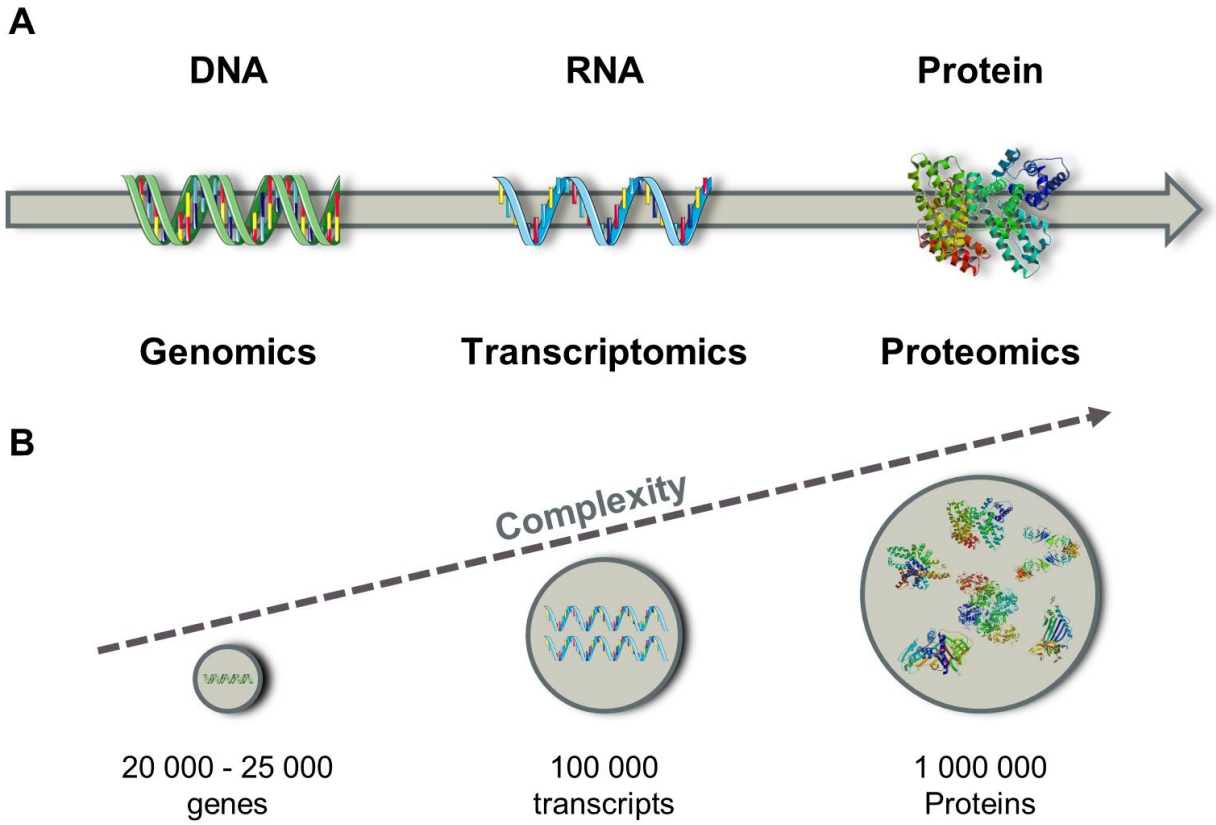

Figure I.1 – Biological Complexity in the Omics. The human genome is comprised by 20,000 to 25,000 genes while transcripts increase in sample size (~100,000 transcripts) due to alternative splicing. Human proteome is highly complex and increases exponential in size compared to the total transcripts as a result of dynamic changes and PTMs underlaying proteome balance. Adapted from ThermoFisher Scientific web page [7].

I.1.3. Proteomics Applications and Workflow

Proteomics research has multiple applications; it can be used to explore when and where the proteome is being expressed. It is of great importance to characterize the expressed proteins in a specific disease or medical condition. In more detail, it is possible to characterize (i) protein production and degradation, (ii) PTMs, (iii) protein interaction with others proteins and (iv) metabolic pathways [8].

In proteomics studies, there are different approaches to identify and characterize proteins. A proteomics workflow is established during sample preparation in which proteins are either enzymatically digested into peptides (bottom-up analysis) or analyzed intact (top-down analysis). Shotgun proteomics workflow present in the **Figure I.2** is a common methodology in proteomics that uses bottom-up analysis. It is one of the major methods for protein identification and quantification, and it can deal with complex proteome samples [9].

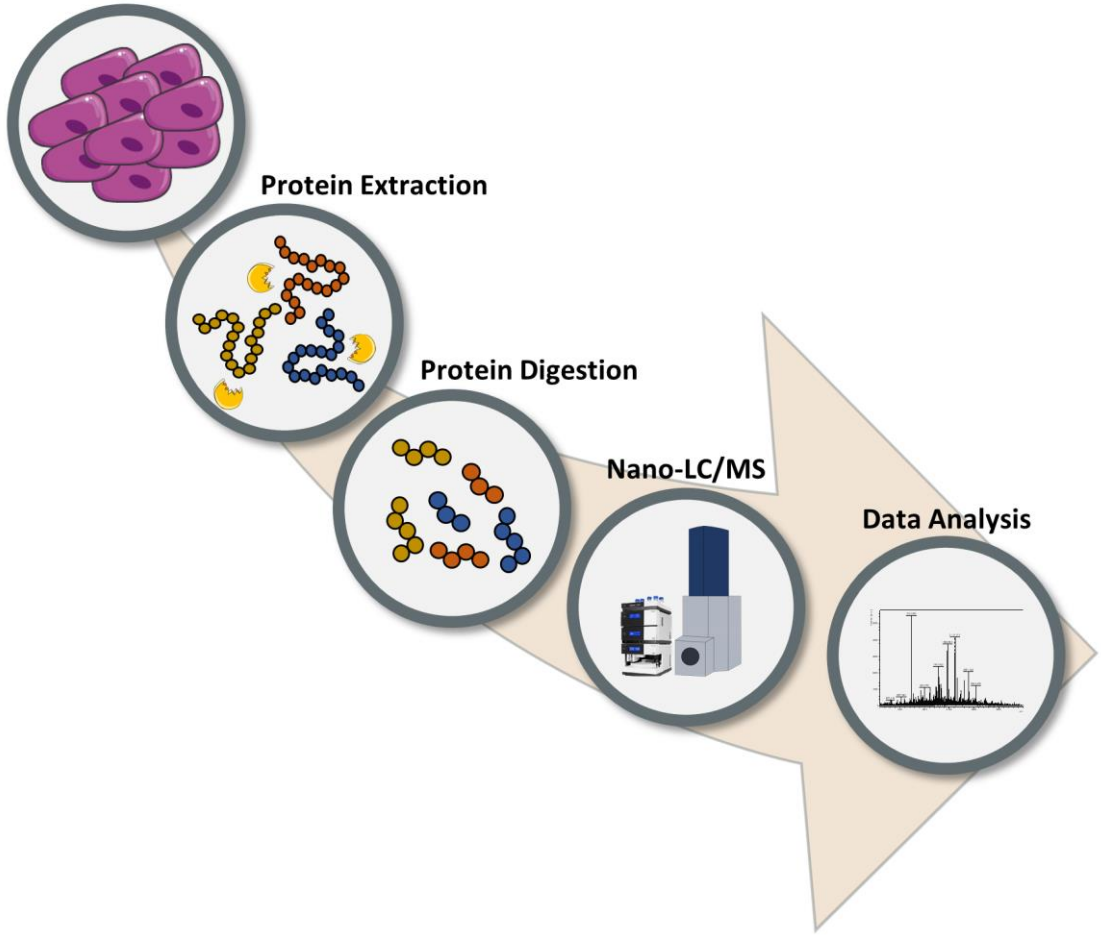

Figure I.2 – Shotgun proteomics workflow: from complex proteome to data analysis. The main steps that characterize shotgun proteomics are represented, Shotgun uses bottom-up workflows to identify proteins in complex samples.

Proteins are proteolytically digested into peptides prior to mass spectrometric analysis. The resulting peptides are separated by liquid chromatography and then sequenced using a tandem mass spectrometer. With up-to-date technology, shotgun proteomics has a higher sensitivity and provides a better coverage of the proteome than the top-down method [10]. However, the main drawback of the shotgun approach is the high number of missing values that leads to a limited protein sequence coverage, due to sample complexity. Protein digests are extremely complex and measuring all the peptides is not possible with the current software available [11]. Both, top-down and bottom-up are complementary approaches.

I.2. Mass Spectrometry - Based Proteomics

I.2.1. Mass Spectrometer

As mention before, mass spectrometry has become an indispensable tool for modern research in living sciences. Mass spectrometry (MS) measures the mass-to-charge ratio (m/z) of analyte ions in a gas-phase and relies on 3 major topics: (**I**) ion generation, (**II**) ion separation and (**III**) quantitative and qualitative ion detection by their m/z and abundance. A mass spectrometer follows typically the scheme shown below (**Figure I.3**) that consists of an ion source, a mass analyzer, and a detector [12].

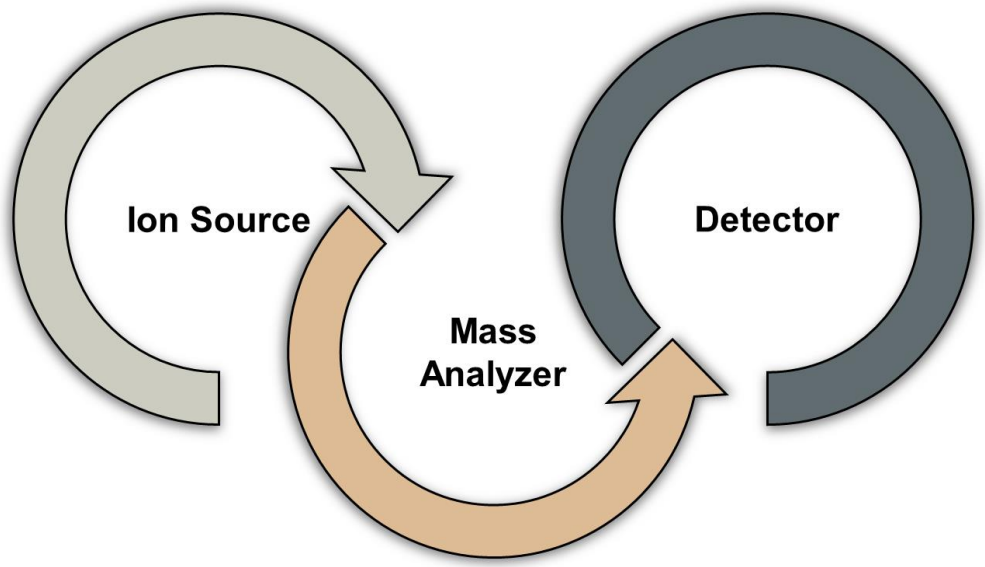

Figure I.3 – Main components present in a mass spectrometer. Mass spectrometer instrument consists in 3 components: (i) Ion source to produced phase gas ions, (ii) Mass analyzer that separates ions according to their mass-to-charge ratio (m/z), (iii) Detector that allows the detection of ions and records their abundance.

I.2.2. Ionization Sources

There is a large variety of ionization techniques that can be classified by their hardness or softness concerning the quantity of energy used. Soft ionization methods are a key step in protein mass spectrometry, allowing the analyte transfer into the gas phase without degradation. Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are the two major soft ionization techniques used to volatilize and ionize proteins or peptides [9].

In MALDI, a laser sublimates and ionizes the analyte out of a crystalline matrix that is in a metal plate (**Figure I.4 A**). This is done in a controlled vacuum. The generation of the gas phase, protonated ions is possible by the formation of a crystalline structure on the plate that is irradiated by laser pulses.

The crystalline structure is formed by pipetting a small volume of sample into a matrix that is usually a small organic molecule (**Figure I.4 B**). This molecule forms a crystal after drying. In such crystal, the sample is embedded. The organic molecule can absorb energy in the ultraviolet wavelength range with a maximum near the wavelength of the laser used in the MALDI, which typically is a nitrogen laser with a wavelength of 337 nm [13]. MALDI ionization is a fast mass spectrometer, with a spectrum being obtained in a few seconds. The information retrieved using a MALDI system is lower than the one obtained with an ESI, however for profiling purposes in tissues and bacteria MALDI offers the unique advantages of speed and easiness. Currently, is becoming the standard method of analysis in hospitals for clinical microbiology [14].

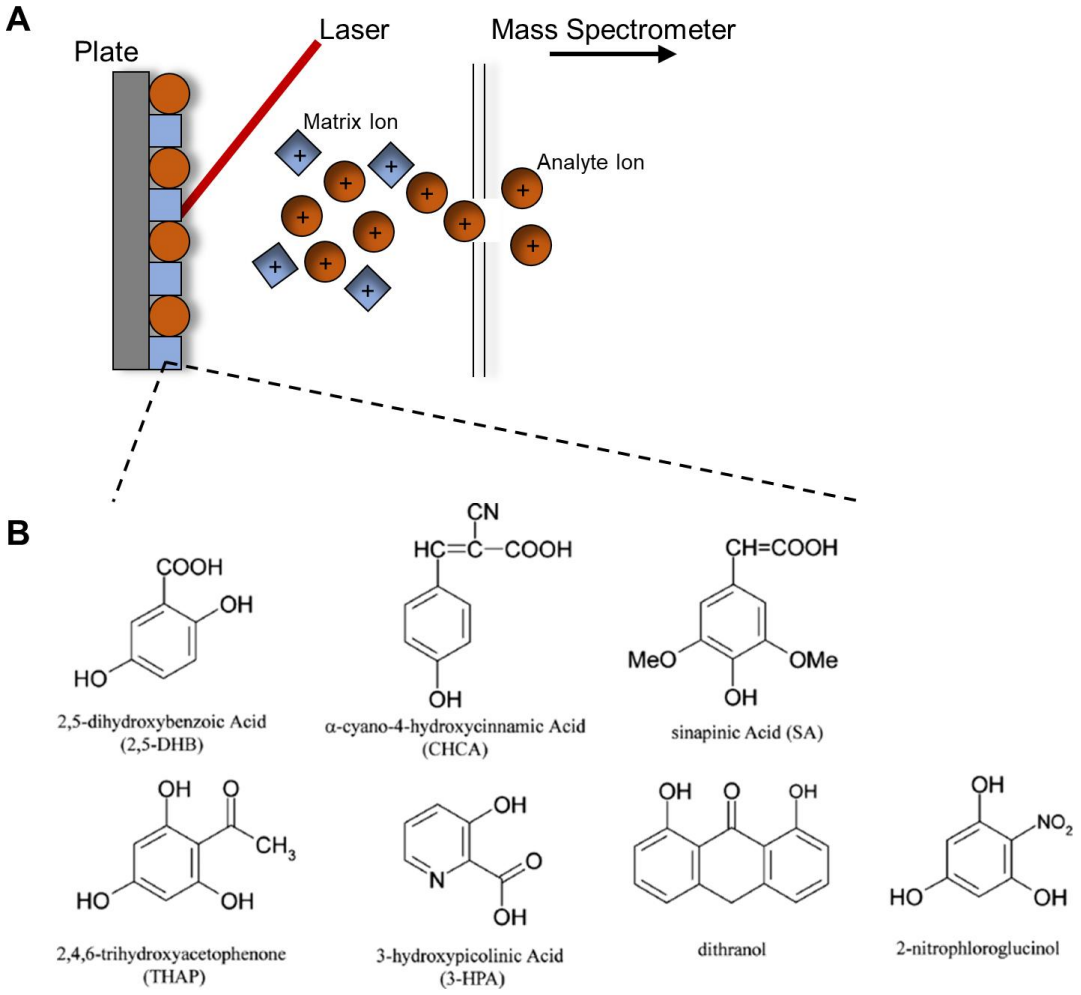

Figure I.4 – Matrix-assisted laser desorption ionization. A - Principal of MALDI ionization. It shows the formation of a plume of matrix and analyte ions as a consequence of the impact of the laser beam. **B** - Type of organic molecules that can form a MALDI matrix. Normally for biomolecules studies and investigations the main matrices used are α-cyano-4-hydroxycinnamic acid (CHCA) or dihydroxybenzoic acid (DHB) [15].

Figure I.5 shows the ion formation underlaying electrospray ionization that takes place in an atmospheric pressure. Ions are produced from a solution in which is applied a high voltage (2 - 6 kV). The analyte solution is injected at a low flow rate by a needle with a high voltage in its tip that generates a strong electric field causing sample charge and dispersion. The resulting droplets from sample dispersion go into an aerosol and generate charged electrospray droplets. In addition, a dry gas flow (nitrogen) helps with the sample nebulization and assists in the charged droplets desolvation. The droplets diminish in size by solvent evaporation and originate charged analytes in gas phase that are transferred into the mass spectrometer with high efficiency and no fragmentation [16].

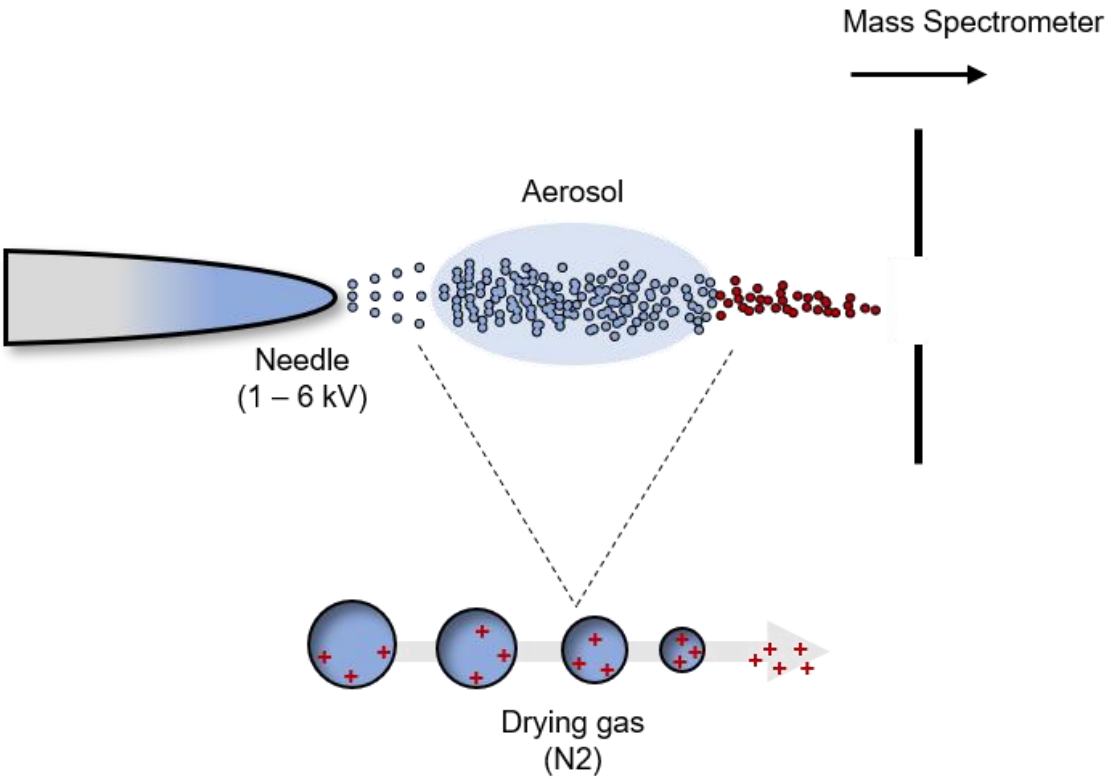

Figure I.5 – Electrospray ionization. The Sample is injected by a charged needle that disperses charged sample droplets into an aerosol and a flow of dry gas (N2). The sample droplets previous dispersed goes by a process of desolvation resulting in charged ions.

Protein mass spectrometry his highly dependent on (i) sample preparation and (ii) peptide separation methodologies. Proteins are identified by m/z of their peptides, and an efficient separation is critical for unambiguous identifications and signal detection of low-abundance proteins.

Electrospray injection is used in combination with high-performance liquid chromatography (HPLC). Electrospray ionization-mass spectrometry (ESI-MS) analysis of the sample analytes takes place as they elute from the chromatography column and allows the analysis of a large range of compounds and complex mixtures.

Reverse-phase Liquid chromatography (RP-LC), is used as the main chromatographic fractionation method in MS proteomics. This combination, called liquid chromatography - mass spectrometry (LC-MS) first separates peptides according to their hydrophobicity, and then they are introduced into the mass spectrometer. Nowadays, the advances in technology have allowed to use sample volumes in the range of the nano-liters, the so called nano-liquid chromatography-mass spectrometry (nano-LC-MS) that functions as LC-MS in a nanoscale allowing the analysis of small amount of biological sample with higher sensitive [17].

I.2.3. Mass Analyzers

In mass spectrometry-based proteomics there are five primary types of mass analyzers which are the ion trap, time-of-flight (TOF), quadrupole (Q), fourier transform ion cyclotron (FT-MS) and orbitrap. Each one of them has its advantages and disadvantages. In any case, they can be combined to take advantage of the individual strengths [18,19].

The TOF is one of the main analyzers used in proteomics, and it has been used for decades. TOF is the simplest mass analyzer with an extremely high scan speed and a wide cover of m/z ranges. The principle of TOF is shown in **Figure I.6 A**, and it relies on the ion acceleration and dispersion during their flight on a tube of known length. The ions travel to the detector as a function of their masses, lighter ions arrive earlier, and the heavier ones reach the detector later [12].

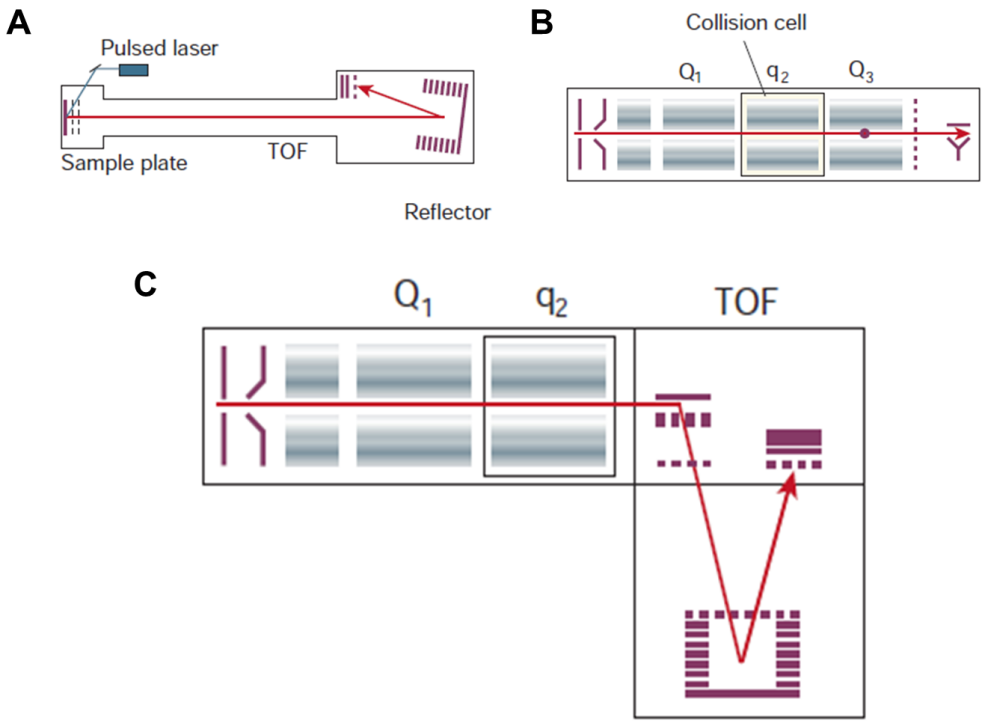

Figure I.6 – Mass spectrometry mass analyzers. A - Time-of-flight (TOF). **B** - Quadrupole (Q). **C** - quadrupole time-of-flight (Q TOF). Figure adapted from Aebersold *et al.* 2003 [19].

Normally, the TOF mass analyzer is combined with a triple quadrupole (Q) (**Figure I.6 B**), originating a quadrupole time-of-flight (Q-TOF) (**Figure I.6 C**) mass spectrometer. Briefly, a Quadrupole is composed by 4 parallel rods with a fixed current and alternative radio-frequency potentials allowing an efficient ion selection and fragmentation in a collision cell. The fragment ions are then detected by a TOF mass analyzer [20]. This combination of both mass analyzers is suited for tandem mass spectrometry and have a high mass accuracy. Furthermore, QTOF represents a workhouse on most proteomic scientific groups because of the high sensitivity and fast scan generation that respectively Q and TOF mass analyzer offer [21].

I.2.4. Quantitative Proteomics

Quantitative measurements of protein concentrations are a fundamental aim of proteomics as it allows to find differences among samples. There are two broad groups of quantitative methods in mass spectrometry-based proteomics: (i) stable isotope labeling and (ii) label-free (**Figure I.7**) [8].

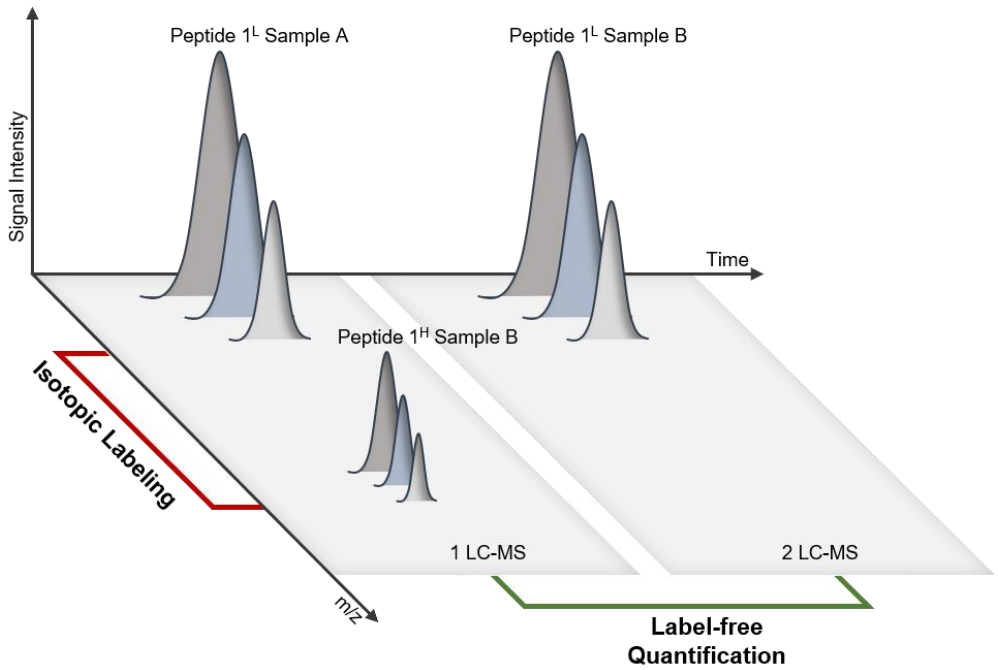

Figure I.7 – Isototopic labeling and label-free quantification. (**i**): Isotopic labeling analize peptides from 2 samples that are mixed together (A and B) in the same LC-MS run. Thus peptides are differentiated by their different masses due to the isotopic labeling. (**ii**): label-free quantification maps peptides across LC-MS runs, the same peptide detected in both runs is then compared by their MS signal intensities. Figure adapted from Mueller *et al.* 2008 [22].

The paradigm is changing in quantitative proteomics. Slowly but constantly, stable isotope labeling is being replaced as the standard method by the free-label approach. Label-free is a new trending quantification method and shows a great potential due to its simplicity and low-cost nature. Label-free quantification relies on the measuring and comparison of mass spectrometric signal intensities of

peptide ions. In a simple way, peptide retention times are aligned, and the peptides identifications between runs are matched. Label-free quantification still has to improve, especially at the level of computation, besides this method is called to replace the stable isotope labeling within the next 10 years [23,24].

I.3. Proteomics of the body fluids

I.3.1. Body Fluids

Recently biological fluids have been a focus on the research science field and medical diagnosis. Human body fluids (HBF) circulating the body and involving tissues often manifests protein profiles that can characterize a specific disease. With the technical advances in mass spectrometry-based proteomics, biomarker discovery is one of the main applications of clinical proteomics [25]. HBF are an attractive biological source for biomarker discover because of its key advantages once compared to other biological samples. Commonly, HBF samples are characterized by their (i) easier, (ii) low cost and (iii) less invasive collection methods [26].

Through **Figure I.8**, it is possible to understand the main landscape of human body fluids in proteomics. There are around fifteen different types of HBF, and from those, the most studied are plasma and blood [27]. Ideally, the best HBF must be easy to obtain, in large quantity and causing no pain to the patient [28]. Urine meets all the aforementioned requirements.

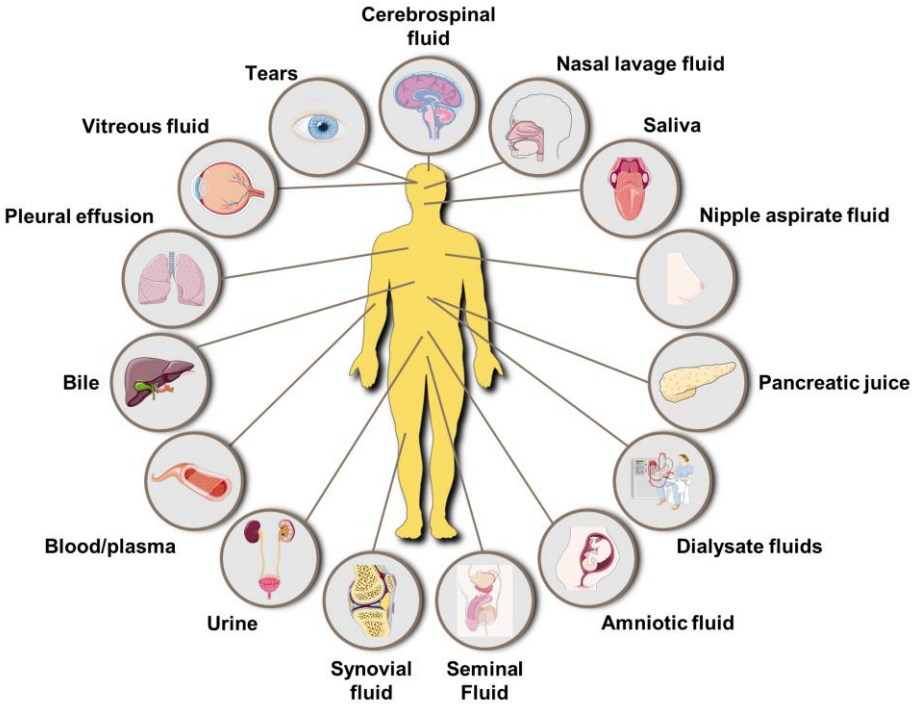

Figure I.8 – Human Body Fluids in Proteomics. Fifteen types of HBF are being applied in clinical proteomics. Invasiveness collection methods in some samples cannot be avoided, and the use of no-invasive types of HBF (such as saliva and urine) have been widly applied in medical diagnosis and biomarker discovery.

Urine is characterized by their non-invasive nature and low sample complexity. Due to its inherent advantages, has become one of the most attractive and feasible alternatives to other invasive HBF such as cerebrospinal fluid, amniotic fluid and serum. Furthermore, it has been widely applied in the medical and clinical proteomics field for the diagnosis of a large panel of diseases.

I.3.2. Urine formation and composition

Urine is produced on the renal system (**Figure I.9**), which is composed of two kidneys, a single midline urinary bladder and two ureters that carry urine from the kidneys to the urinary bladder. Furthermore, urine is expelled out of the body by a single urethra. The kidneys are the major excretory organ in the body, with the main function of filtering the plasma. It has a high role in the regulation of blood volume pressure and in the concentrations of solutes that are present in the blood [29].

Urine formation takes place on the functional unit of the kidney, nephron (**Figure I.9 B**) and consists in three processes: (I) plasma filtration, (II) reabsorption and (III) secretion. Plasma filtration (**Figure I.9 C**) occurs in the glomerulus which is formed by a cluster of capillaries that are encircled by a capsule, Bowman's capsule (beginning part of the nephron) acts as a biological barrier designed for blood ultrafiltration. After the filtration step, most of the water and ions are reabsorbed by an osmolarity gradient. Furthermore, other molecules such as proteins, are secreted to the renal tubule [30].

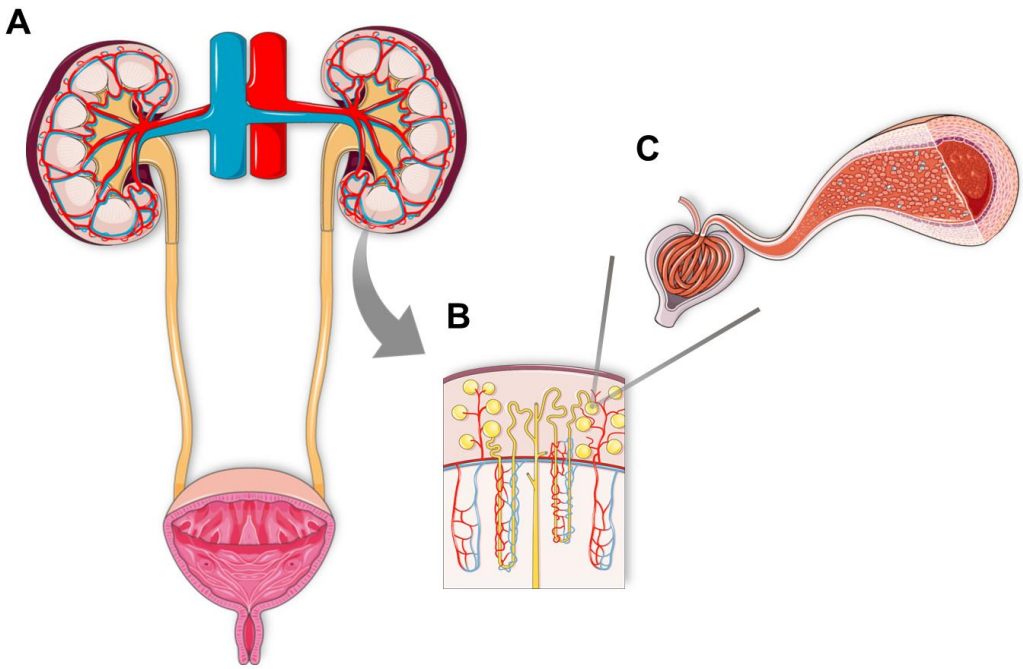

Figure I.9 – Human urinary system. A - Principal components of the urinary system. **B** - Nephron structure. **C** - Blood filtration at Bowman's capsule.

Approximately, 180 liters of plasma are filtered every twenty-four hours in the kidneys and from 600 up to 1600 ml of urine are formed. In healthy individuals, the main principal solutes present in urine are urea, chloride, sodium, potassium, phosphate, sulfate, creatinine, uric acid and a low percentage of proteins [30].

From the urinary proteins found in healthy individuals, 70% are from the urinary tract, and the remaining 30% corresponds to plasma proteins. Urinary proteins can originate from (i) glomerular filtration of plasma proteins, (ii) secretion of proteins by the renal tubular epithelial cells, (iii) shedding of cells along the urinary tract and (iv) exosome secretion [31].

I.3.3. Urine in medical diagnosis and clinical proteomics

Nowadays, clinical proteomics focus mainly on the biomarker discover with the main objective of characterize a protein that can early diagnose a disease or condition. Blood is the main and most used biological sample on biomarker discover, however, in the last decade urine as showed a great potential mainly because urine samples are less complex than plasma and contains proteins and peptides that have not been discovered in the plasma [31,32.] In addition, urine is non-invasive, can be taken in large amount and can be obtained at different times reflecting different biorhythms.

Many reliable biomarkers candidates have already been identified using urine samples. Such biomarkers can detect, diagnose or monitor diseases. From those candidates, most of them are focused on urinary system diseases, and only a few to date have been moved forward to validation. FDA-approved urine protein biomarkers focus on the early detection of acute kidney injury and in the diagnose and monitoring of bladder cancer [33].

In a large scale, the urinary proteomics is moving towards the application phase yet despite technological improvements and discover breakthroughs, pre-analytical variables in urine sample handling are a key step that requires further studies and optimized standard protocols.

I.4. Urine Proteomics

I.4.1. Milestones in urine proteomics

Urine contains significant amounts of proteins, and the number of identified proteins is still increasing. The human urine proteome map has been changing over time, in **Figure I.10** it is outlined the evolution of the urine proteome. As it may be seen, first, in 2001 Spahr *et al*. [34] identified 124 urinary proteins. In 2005, Sun *et al*. [35] identified 226 proteins, and in the next year, Adachi *et al*.[36] discover 1543 proteins, increasing drastically the number of proteins identified in the human urine proteome. With the improvements achieved in the high-resolution mass spectrometry techniques, Marimuthu *et al*. [37] identified 1823 proteins followed up by Farrah *et al*. [38] that have identified in 2014, 2491 proteins. Recently, Zhao *et al.* 2017 [39] in an extensive and comprehensive study with 3 dimensions of separation for the human urine proteome annotation have identified 6085 proteins in normal healthy urine specimens.

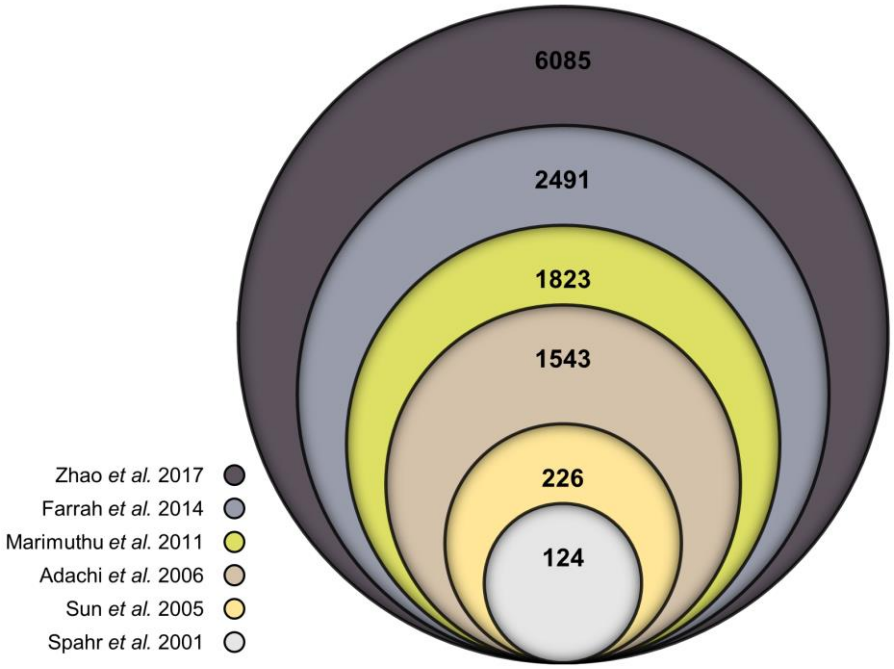

Figure I.10 – Urine Proteome landscape. Evoluition of urine protein discover during the last decade

I.4.2. Urine sample Handling

The handling of a urine sample is a pre-analytical factor that represents a crucial step for the urine proteome analysis. **Figure I.11** depicts the main 4 topics regarding urine sample handling: (i) collection, (ii) preservation, (iii) preparation and (iv) storage [40].

There are 3 major types of urine collection, first-morning urine, second-morning urine and 24h urine. Each type provides different information. Normally, mid-stream urine collection is preferable to avoid high urine contamination with renal tubular epithelial and bladder cells. In proteomics biomarker discovery, second-morning urine is becoming the standard urine collection due to the low probability of protein contamination from overgrown bacteria and epithelial cells [41].

Urine preservation is also important, normally, urine preservation is often done by protease and bacterial growth inhibitors. Proteases inhibitor have been suggested for urine preservation however, Thongboonkerd and Havanapan *et al*. 2009 [42] showed that the presence of protease inhibitors was unnecessary. Urine sample has a low concentration of proteases, and normally the protease inhibitors consist of both peptides and small molecules that can interfere with mass spectra analysis. On the other hand, bacterial growth inhibitors are essential for urine preservation because bacterial growth in urine might affect their proteome. Thongboonkerd *et al*. 2007 also showed that sodium azide or boric acid can delay bacterial overgrowth in pooled urine. The addition of 20mM boric acid is ideal for the urine preservation of a random void urine [43].

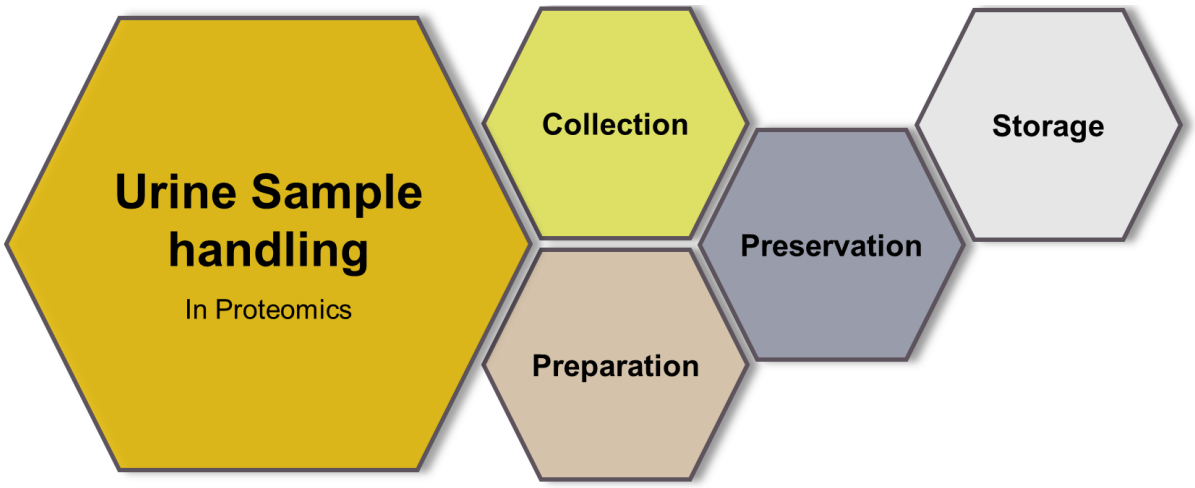

Figure I.11 – Urine Sample handling. Main topics in urine sample treatment for proteomics aproaches.

Stabilization and storage of urine proteomes in the long term is critical for studies dealing with large cohorts of individuals. The in-depth study of the urine proteome by mass spectrometry is time-consuming, and it requires a complicated sample handling. The impossibility of handling/analyzing many urine samples in short periods of time makes mandatory the need of preservation and storage [44].

Currently, the gold standard methodology for urine storage and proteomics application is defined by the Human Kidney and Urine Proteome Project (HUKPP) and European Urine and Kidney Proteomics (EuroKUP) [45] as it follows. Briefly, it is suggested a low centrifugation speed to remove cell debris without cell lysis and then the storage at -20°C or -80°C. This method presents some drawbacks, as there is the need for dedicated refrigerators which must be connected to emergency energy supply systems to prevent the effects of power failures. Repeated freezing and thawing cycles have a detrimental effect in the sample, and it has been shown proteome alterations after just 5 freeze-thaw cycles [46].

Other methods for protein preservation in urine samples have been proposed by the research community. Thus, proteome storage in a polyvinylidene difluoride (PVDF) membrane has been proposed [47]. However, this methodology did not become an accepted practice by the medical and scientific community. In addition, Norgen developed a urine collection and storage kit that can preserve urine for over 2 years at room temperature, but the kit is mainly used for DNA and RNA storage, and the lack of data does not properly support the urine proteome preservation [48].

I.4.3. Urine Sample preparation for mass spectrometry analysis

Protein precipitation and ultracentrifugation are classic methods for protein separation. Different types of organic solvents can be used for urine protein separation, such as methanol, ethanol, acetone, acetonitrile, and mixed solvents such as chloroform/methanol. Ultracentrifugation relies on protein sedimentation by centrifugation at high speeds however, this method has a drawback because some proteins are lost in the overall process, composed of different sedimentation steps.

Ultrafiltration is a popular method for urine protein extraction that uses an ultrafiltration membrane for sample concentration and fractionation, removing of metabolites, salts and other small molecules. Filter-aided sample preparation (FASP) is a type of ultrafiltration that was developed by Wiśniewski *et al*. 2009 [49] to separate proteins from cell culture and tissues extracts. FASP is a method for bottom-up proteomics applications. It consists in the separation of the proteins through a membrane that has a molecular weight cut-off (MWCO), normally within a range comprised between 10,000 or 30,000 Da. Proteins are fixed in the membrane and then submitted to the steps of washing, reduction, alkylation and digestion, all of them are processed through the membrane. Then, the peptides are eluted and collected out of the membrane for further analysis. In the last decade (**Figure I.12**), the number of experimental studies that used FASP has increased. Furthermore, this sample treatment method has been applied to diverse types of liquid biopsies [50,51], including urine. Morever with urine the application of FASP in a 96-well plate has also been shown [52].

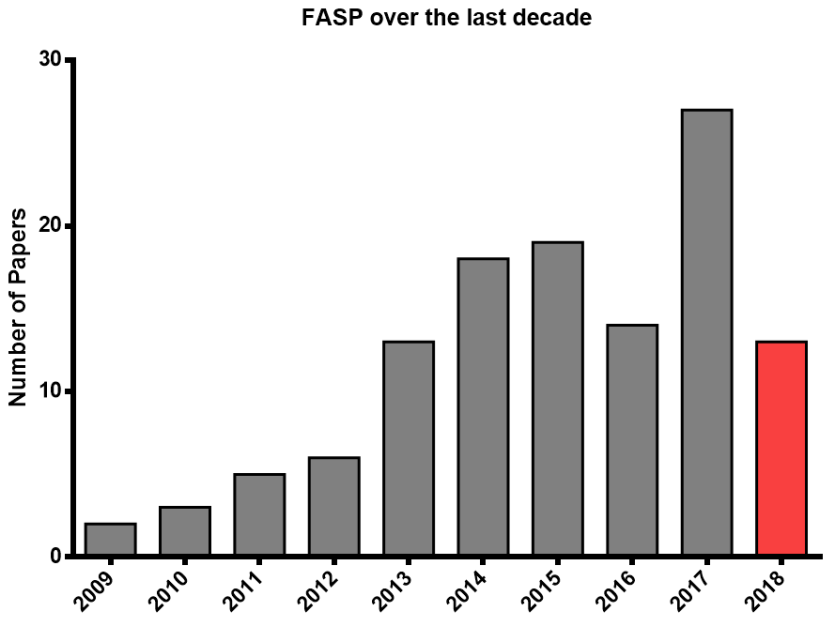

Figure I.12 – Number of papers that used FASP over the last decade. The search was conducted 7^{tth} of september, 2018 in scopus with the term “filter-aided sample preparation” and “FASP”.

II. Objectives and working plan

This work is addressed to overcome the limitations for the preservation of the urine proteome of current standard methods by developing a method based on heat stabilization and the FASP methodology. The system to provide the heating is a stabilization system developed by the Denator Company (http://www.denator.com) in collaboration with the Bioscope Group in 2007 for serum samples. The aims of the work to be developed are:

Objective (i): to avoid the need of large volumes of storage for urine samples.

Objective (ii): to avoid the need for freezing the samples.

Objective (iii): to Snapshotting urine proteomes.

Objective (iv): to implement the FASP methodology as a part of the heating process to simplify further next processing of samples.

III. Materials and Methods

III.1. Experimental Study Design

The experimental study design showed in **Figure III.1**, consisted of a longitudinal study (T0-0 months and T1-6 months) in which three different conditions of urine storage and preservation were evaluated for its application in proteomics. Normal urine proteomes from four healthy donors were preserved and stored in triplicate, accordingly to the conditions highlighted in Figure III. The study encompasses 72 LC-MS/MS runs (3 preservation condition x 3 replicates x 2-time points x 4 biological samples).

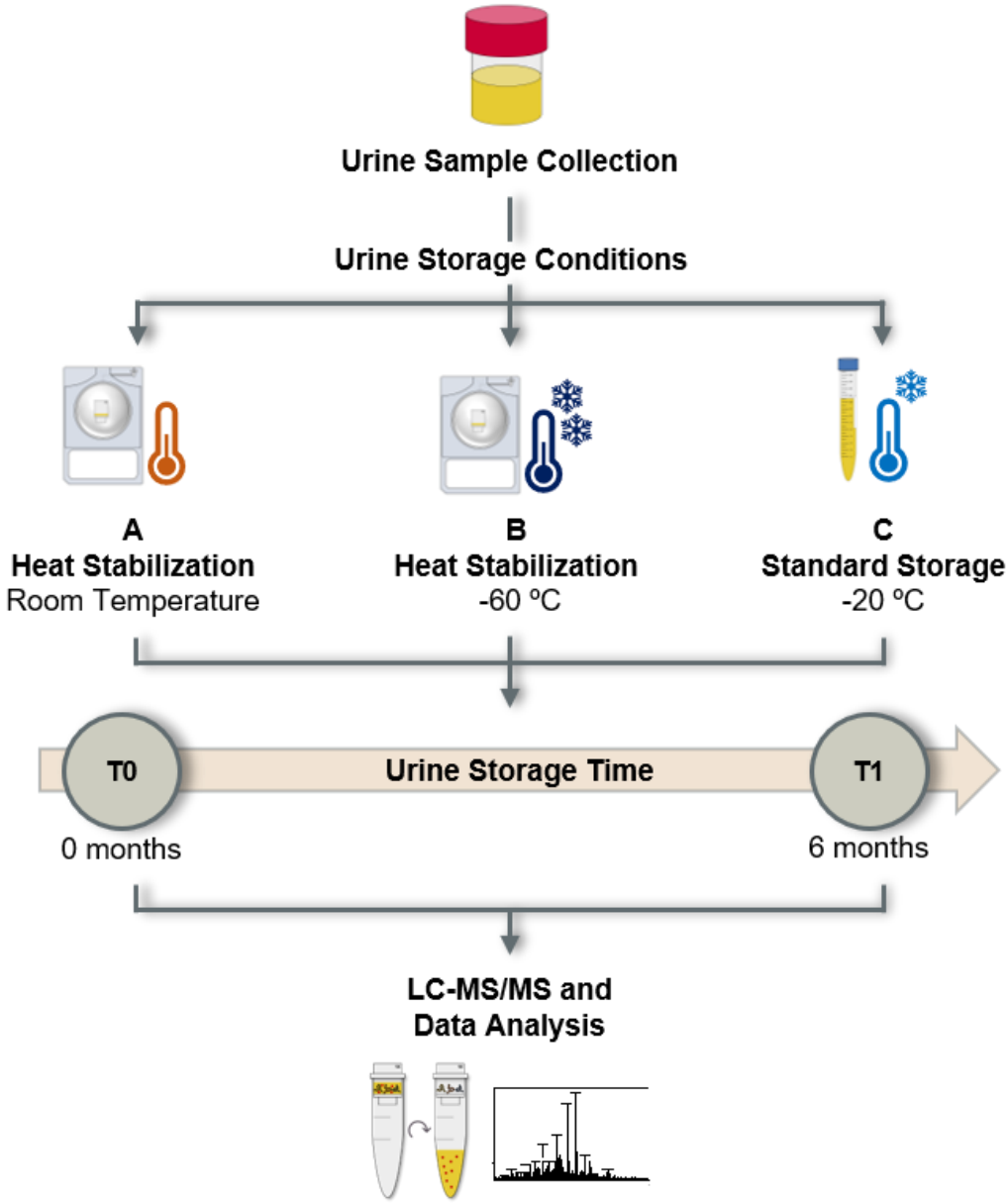

Figure III.1 – Experimental study design. The preservation of urine proteome was evaluated in 3 different storage methods for over 6 months. 4 different urine specimens with 3 different conditions of storages and 3 replicates each was used to evaluate the preservation in two different time points of storage (T0 and T1).

III.2. Sample treatment and processing timeline

Figure III.2 depicts the urine sample collection and treatment timeline. For this study, it is of high importance the collection of fresh urine samples and urine storage time. In the less time possible, urines were collected from donors and storage with their respective condition.

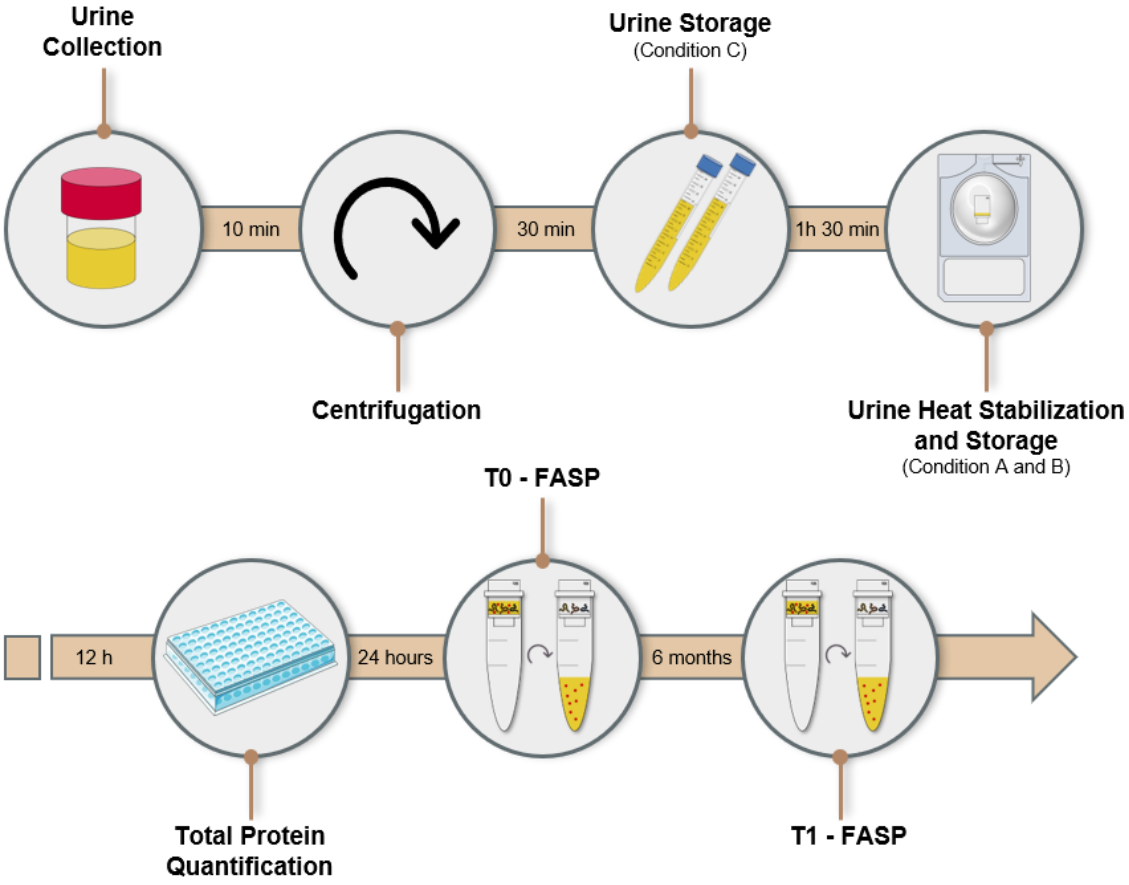

Figure III.2 – **Urine sample treatment timeline.** Urine sample handling was performed in the less time possible. It was required around 40 minutes to storage urine at condition A and approximatly 2 hours and 30 minutes to storage urine at A and B conditions.

III.3. Urine Collection

Reagent: Boric Acid (Panreac AppliChem)

Apparatus: 10 mL serological pipette; 50 mL Falcon (Abdos); 10 mL Falcon (Abdos); Biological Safety Cabinet (Albina); Centrifuge MPW-35 (Rotor 11457); FastPette Plus (Labnet); Urine Collection tube.

Procedure: Fresh second-morning mid-stream urines were collected between from 4 healthy male volunteers (**Table III.1**). Invasive methods were not needed during urine collection, and all the volunteers provided a signed consent for the usage of their urine in this study. In this study, mid-stream urine samples were collected to decrease the number of bacteria and epithelial cells present in the urine samples. The urine samples were collected into a urine collection tube with boric acid 20 mM. The boric acid acts as a urine preservative by preventing bacterial growth [43]. The biological samples were

processed in a Biological Safety Cabinet and all the material used was sterile, DNase / RNase free and non-pyrogenic. Urine samples were split into two 50 mL falcons and centrifuged at 1000x g for 10 min to remove cell debris and prevent sample contamination [45]. After centrifugation, the supernatants were extracted and split into 10mL samples.

Table III.1 – List of urine donors and their characteristics. Urine samples were chosen from specimens with the same characteristics presented. The group was chosen with small differences allowing the comparison of urine proteome preservation with minimal biological differences between the samples.

Specimens	Gender	Age	Healthy	Medicines	Smoker
1	Male	25	Yes	No	No
2	Male	25	Yes	No	No
3	Male	23	Yes	No	No
4	Male	24	Yes	No	No

III.4. Urine Sample Treatment and Storage

Reagents: Ethanol (Sigma-Aldrich); Urea (Sigma); Hydrochloric acid solution 50% (Panreac AppliChem).

Apparatus: Maintainer Card (Denator); Refrigerated Micro-Centrifuge (Labnet Prism); Stabilizor T1 (Denator); Vivacon 500 - 10 000 MWCO Hydrosart (Sartorius).

III.4.1. Protein extraction and concentration

Procedure: Urine protein extraction was done by ultrafiltration as it follows: Sample concentration was processed in a Vivacon 500 that has a Hydrosart membrane with MWCO of 10 000 Daltons (**Figure III.3 A**). The Hydrosart membrane present in the filter device is characterized by their extremely low unspecific binding properties and is ideal for concentration and buffer exchanges of protein/peptide samples [53]. Before sample loading, 100 µL of 70% ethanol solution was pipetted to the vivacon and then centrifugated at 14000x g for 15 minutes. A urine volume of 400 µL was then loaded into the filter device and then was centrifuged for approximately 15 minutes at 14000x g. This step was repeated 3 times, and a total of 1,2 mL urine was passed through the membrane. On the last centrifugation, the time was increased to 25 minutes to ensure that the membrane was dried and there was no urine on the top of the membrane. The proteins remained in the membrane.

III.4.2. Heat Stabilization

Procedure: Using the Stabilizor T1 (**Figure III.3 C**) from denator, the protein present in the membrane was preserved by heat stabilization. This device is a benchtop instrument that uses rapid conductive heating under a control pressure to preserve biological samples. The heat shock generates an irreversible thermal denaturation of proteins resulting in the inactivation of proteins and enzymes [54]. It is widely used in tissue preservation and has been used to preserve liquid biopsies like blood [55,56]. The vivacon-500 with the membrane containing the proteins was stored in the denator maintainer card (**Figure III.3 B**). Then, A custom program was used with the following parameters: 30 seconds of sample treatment time, cavity vacuum pressure set to 5mbar and heater position to 2,1mm with a temperature of 95 ˚C. Briefly, the air present in the card is evacuated, and then the card goes inside the device where is applied the heat treatment.

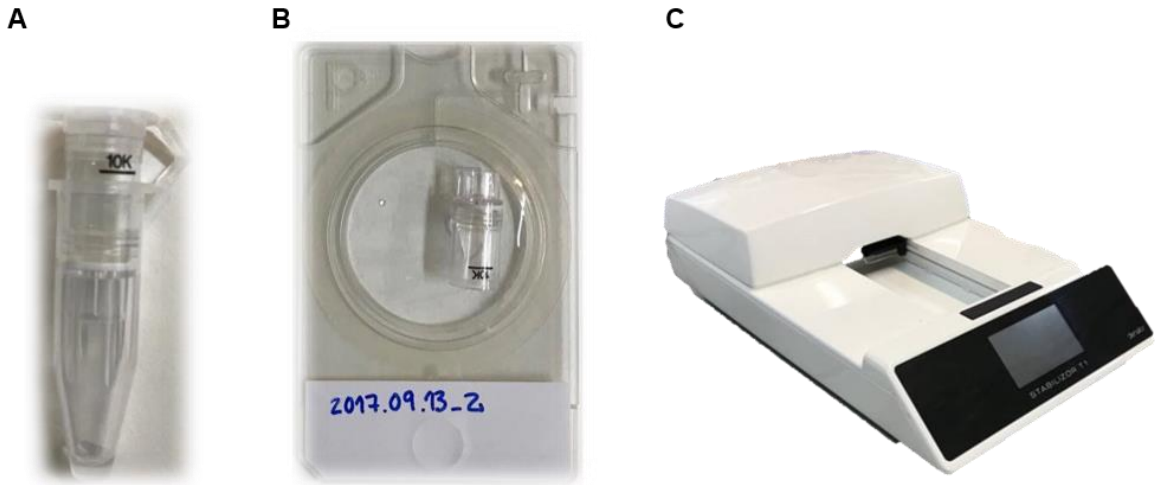

Figure III.3 – Instrumentation used in urine proteome heat stabilization. A - Vivacon 500 from Sartorius with a 10 000 daltons MWCO. **B** - Maintainar Card from denator that goes into the Stabilizor T1. **C -** Stabilizor T1 from Denator company.

III.4.3. Sample Storage

Procedure: Urine sample storage and preservation were processed in 3 different conditions (**Figure III.4**), as it follows: (**A**) Heat stabilization and storage at room temperature (RT), (**B**) Heat stabilization and storage at -60 ˚C and (**C**) normal freezing at -20 ˚C. Storage conditions A and B were processed after urine concentration. The urine proteome was preserved by heat stabilization and then was storage at room temperature (condition A) and -60 ˚C (condition B) until further sample treatment and analysis. Condition C represents a control standard storage in which urine samples were frozen at -20 ˚C right after urine collection. When condition samples in condition C were unfrozen a precipitate was formed, and then the urine sample pH was adjusted to approximately 8 with 1M Tris-HCl to resolubilized the protein precipitate.

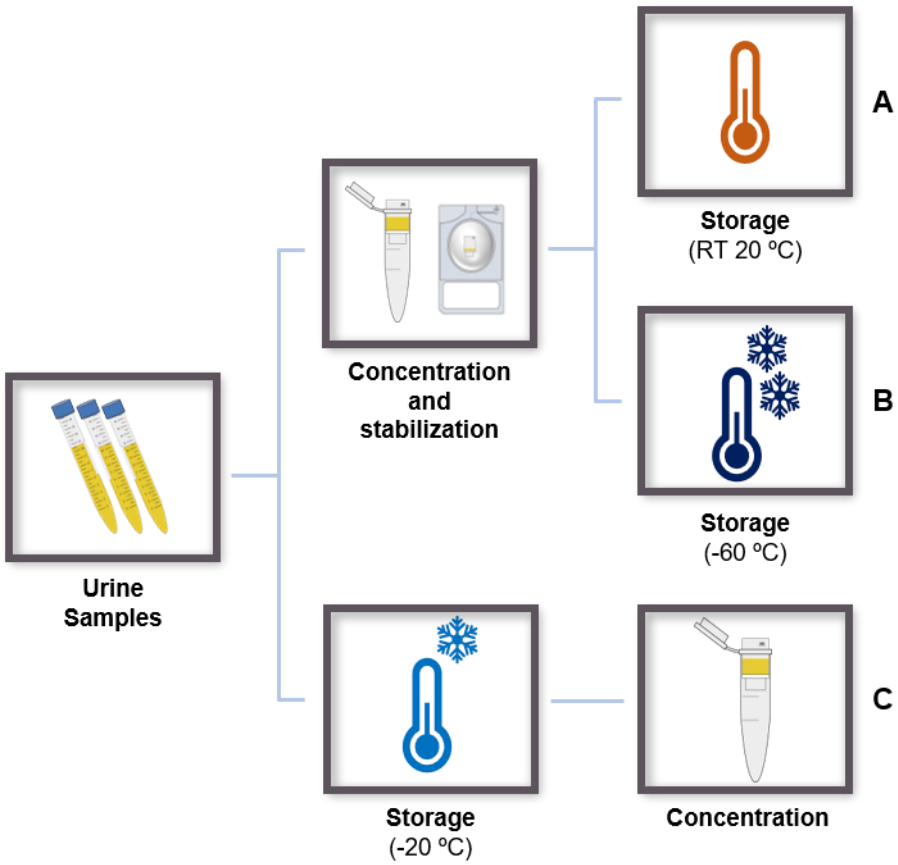

Figure III.4 – Urine storage conditions. **A** - Heat stabilization with storage at room temperature (approximatly 20 ˚C). **B** - Heat stabilization with storage at -60˚C. **C** - Standart control urine storage at -20 ˚C

III.5. Protein Quantification (Bradford Assay)

Reagents: Bradford Reagent (Sigma-Aldrich); BSA (Sigma-Aldrich); MQH2O.

Apparatus: ClarioStar Spectrophotometer (BMG LABTECH); 96-well plate; Centrifuge and vortex (Sky-Line).

Procedure: A stock standard working solution containing 100 µg/mg of BSA was prepared and then standards of lower concentration were processed as explained in **Table III.2**. Using a 96-well plate, the calibration curve was done in duplicate as explained in **Table III.2**. To each standard 150µL of Bradford solution were added. The urine samples were diluted to ensure that the protein content was within the linear range of the calibration curve (5-20 µg/ml) and they were prepared following the same procedure as the standards. The absorbance was measured at 590nm using a ClarioStar spectrophotometer.

Table III.2 – Preparation of diluted BSA standards. Solutions used to provide the bradford assay standard curve.

Concentration (µg/mL)	Volume of BSA 100 µg/mL (µL)	Volume of H2O (µL)
0	0	400
1	4	396
5	20	380
10	40	360
15	60	340
20	80	320
25	100	300

III.6. Filter-aided Sample Preparation (FASP)

Reagents: Ammonium bicarbonate (AmBic)(Fluka); Urea (Sigma); MQH2O; Ditiotreitol (DTT) (Alfa Aesar); Iodoacetamide (IAA) (Sigma); Trypsin Protease MS-Grade (Thermo Scientific).

Apparatus: Refrigerated Micro-Centrifuge (LAB-NET Prism); Speed Vac with vacuum concentrator centrifuge (Univapo 150 ECH) with a refrigerated aspirator vacuum pump (Unijet II); Vivacon 500-10,000 MWCO Hydrosart; Mini incubator (Labnet).

Procedure: Filter-aided sample preparation (FASP), was used as the protein digestion methodology. FASP was introduced by Wiśniewski *et al*. 2009 [49] as a versatile and efficient approach for protein digestion plus peptide recovery. **Figure III.5** describes the FASP process, and in the first step, urine protein must be loaded into the vivacon membrane. Urine protein loading was previously done for the conditions A and B before storage and heat stabilization by the Stabilizor T1. The same process of urine protein extraction and concentration, described at III.4.1., was repeated for condition C previously to FASP. After protein loading, proteins were washed with 200 µL of 8M Urea 25 mM AmBic solution to remove low-weight material and then centrifuged for 20 minutes at 14000x g. Next, proteins were reduced by adding 200 µL of 50mM DTT 8M Urea 25mM AmBic solution and incubated for 60 minutes at 37 ºC. Then, a centrifugation of 20 minutes at 14000x g was done, and then 200 µL of 25mM AmBic were added. Then the FASP was centrifugated again for 20 minutes at 14000x g to remove the excess of DTT. Next, the sample was alkylated during 45 min in the dark by the addition of 100 µL 50mM IAA 8M Urea 25mM AmBic. Solution. Then, the FASP was centrifuged for 20 minutes at 14000x g. Then the sample was washed with 200 µL 25mM AmBic and then centrifugated for 20 minutes at 14000x g to remove any remaining reagents. This step was repeated twice. Finally, protein digestion was done by the addition of 100 µL 1:30 trypsin in 12.5 mM AmBic solution. Protein digestion proceeded overnight (approximately 14 hours) at 37 ºC. After digestion, the sample was centrifugated for 20 minutes at 14000x g, and then 100 µL of 12.5 mM AmBic were added followed by centrifugation for 20 minutes at 14000x g. This step was done twice. The solution of peptides obtained was collected to a new sample tube, and such sample was lyophilized with the help of the speed vac. The lyophilized peptide samples were then storage at -20 ˚C until further LC-MS analysis.

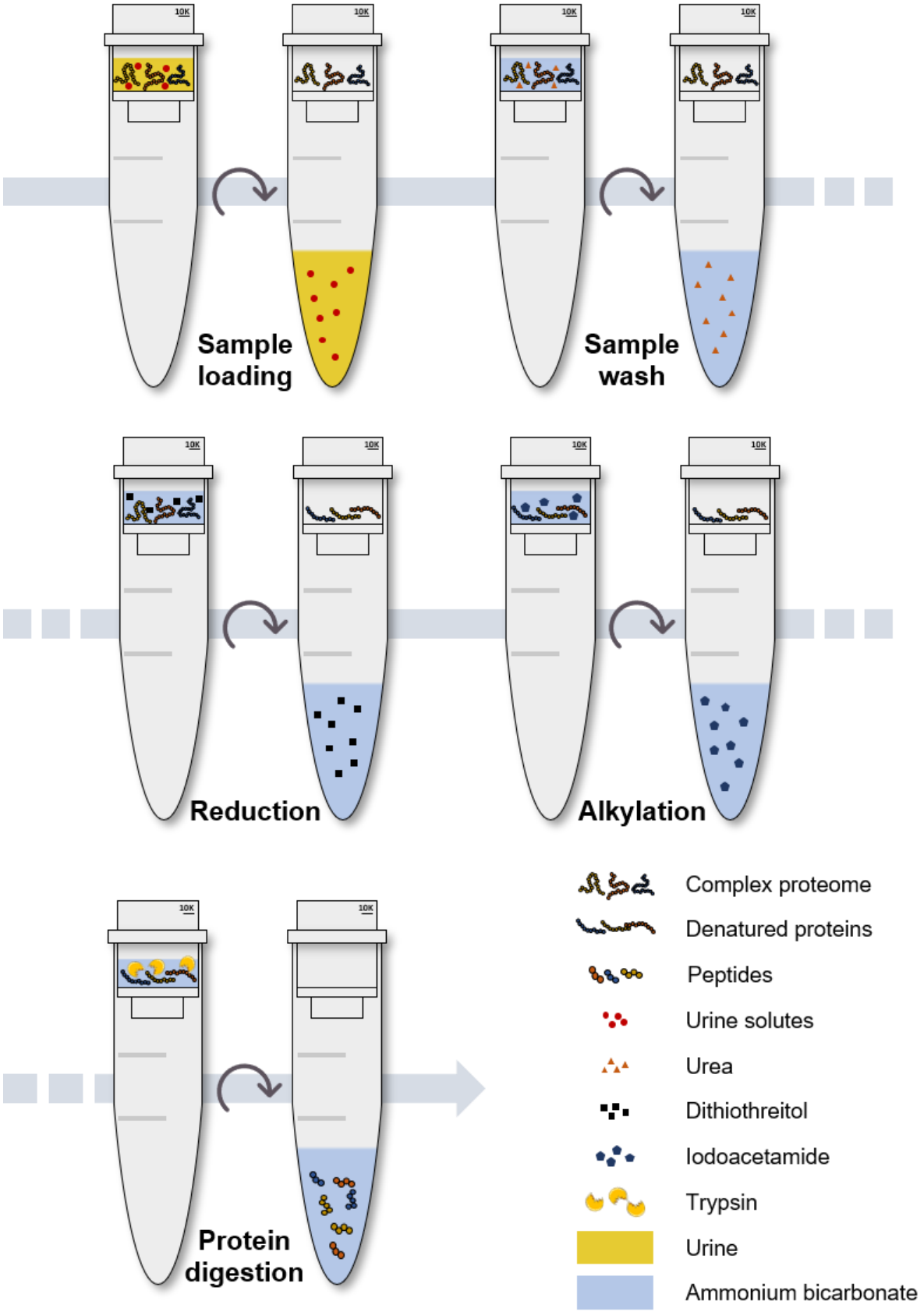

Figure III.5 – Main steps of filter-aided sample preparation (FASP) for urine. FASP methodology is used to filtrate and digest proteins samples on a membrane. It is comprised mainly by 5 steps: (i) loading, (ii) washes, (iii) reduction, (iv) alkylation and (v) protein digestion.

III.7. Peptide Quantification

Reagents: Quantitative Colorimetric Peptide Assay (Thermo Scientific); MQH2O; MS-Grade acetonitrile (Carlo Erba Reagents); MS-Grade formic acid (Fluka); 96-well plate.

Apparatus: Centrifuge and vortex (Sky-Line); ClarioStar Spectrophotometer (BMG LABTECH); 96-well plate; Centrifuge and vortex (Sky-Line); Ultrasonic bath (Elma).

Procedure: Before peptide quantification, peptides samples were resuspended in 100 µL of 3% (v/v) acetonitrile (ACN) containing 0.1% (v/v) aqueous formic acid (FA). Then, samples were homogenised by 5 minutes on vortex followed by 10 minutes on an ultrasonic bath at 100%. Afterwards, samples were quantified using a quantitative colorimetric peptide assay [57]. Briefly, the standard curve was prepared by serial dilution of a peptide pattern contained in the kit (**Table III.3**), and then the samples were prepared with the same procedure as well. Finally, 20 µL of both solutions were loaded by duplicate onto the 96-well plate. Then, the working reagent provided with the kit was added to each of the wells, and the plate was incubated for 15 minutes at 37 ˚C. Next, the absorbance was measured at 480nm by a ClarioStar spectrophotometer and the unknown peptide concentrations were calculated with the aid of the standard curve.

Table III.3 – Preparation of diluted Peptide Digest Assay standards.

Tube	Concentration (µg/mL)	Peptide Digest Assay Standard (1mg/mL)	Volume of H2O (µL)
A	0	0	150
B	15.6	75 µL of tube **C** dilution	75
C	31.3	75 µL of tube **D** dilution	75
D	62.5	75 µL of tube **E** dilution	75
E	125	75 µL of tube **F** dilution	75
F	250	75 µL of tube **G** dilution	75
G	500	75	75

III.8. LC-MS Analysis

Reagents: MS-Grade acetonitrile (Carlo Erba Reagents); MS-Grade formic acid (Fluka).

Apparatus: Analytical column Acclaim™ PepMap™ (Thermo Fisher Scientific); UHR-QqTOF IMPACT HD (Bruker); trap-column Acclaim PepMap100 (Thermo Fisher Scientific).

Procedure: 540 ng of peptides were loaded onto a Trap column Acclaim PepMap100, 5 µm, 100 Å, 300 µm i.d. × 5 mm and desalted for 5 min from 3% to 5% B (B: 90% acetonitrile 0.08% FA) at a flow rate of 15 µL min^{-1}. Then the peptides were separated using an analytical column Acclaim™ PepMap™ 100 C18, 2 µm, 0.075 mm i.d x 150 mm with a linear gradient at 300 nL min^{-1} (mobile phase A: aqueous FA 0.1% (vol/vol); mobile phase B 90% (vol/vol) acetonitrile and 0.08% (vol/vol) FA) 5-90 min from 5% to 35% of mobile phase B, 90-100 min linear gradient from 35% to 95% of mobile phase B, 100-110 95% B. Chromatographic separation was carried out at 35 ºC. MS acquisition was set to cycles of MS (2 Hz), followed by MS/MS (8–32Hz), cycle time 3.0 seconds, active exclusion, exclude after one spectrum, release after 0.5 min. Reconsider precursor if current intensity, previous intensity 3.0 an intensity threshold for fragmentation of 2500 counts. All spectra were acquired in the range 150-2200 m/z.

III.9. Bioinformatic Analysis

LC-MS/MS data were analysed using Data Analysis 4.2 software (Bruker). Proteins were identified using Mascot (Matrix Science, UK). MS/MS spectra were searched against the SwissProt database S_Prot Human (73,045,382 sequences; 24,698,382 residues. Tandem MS data were searched with MASCOT search engine with the following parameters: precursor mass tolerance of 20 ppm, fragment tolerance of 0.05 Da, trypsin specificity with a maximum of 2 missed cleavages, cysteine carbamidomethylation set as fixed modification and methionine oxidation, as variable modification. False discovery rate (FDR) was estimated by running the searches against a randomized decoy database. Results of the identification step were filtered to proteins with a FDR below 1%. Label-free quantification was carried out using MaxQuant software V1.6.0.16. All raw files were processed in a single run with default parameters as recommended by the manufacturer[58]. Database searches are performed using the Andromeda search engine with the UniProt-SwissProt Human Uniprot Proteome database as a reference and a contaminants database of common contaminants. Data processing was performed using Perseus (version 1.5.0.31) [59]. In brief, protein group LFQ intensities were log2-transformed to reduce the effect of outliers. To overcome the obstacle of missing LFQ values, missing values were imputed before fitting the models. Log ratios were calculated as the difference in average log2 LFQ intensity values between the two digestion methods tested (two-tailed, Student's t test). A protein was considered statistically significant if its fold change was ≥ 1.5 and FDR ≤ 0.05 [60].

IV. Results and Discussion

IV.1. Urine protein quantification

Protein digestion is a crucial step for bottom-up proteomic approaches. Before digestion, the total amount of protein in each sample must be quantified, allowing to correctly set the amount of trypsin that is needed to digest the proteome.

Total urine protein was quantified using the Bradford assay, that is a standard procedure already optimized in our laboratory for the determination of microgram quantities of protein. Briefly, it is a colorimetric protein assay based on the absorbance shift of the Bradford reagent used, that was measured at their maximum absorbance at a wavelength of 595 nm. This shift results because of the bond of the protein's carboxyl group to the dye creating a noncovalent complex.

To quantify the urine protein samples (U1, U2, U3 and U4), four different assays were made using different standard curves (**Figure VII.SM. 1**). The urine sample was conveniently diluted in order to make its absorbance fitting the calibration curve at its medium point. Total protein quantification (**Table IV.1)**, revealed a total protein content within the range 25 (protein1) to 350 (protein 4) µg/mL.

For all the urine specimens, a total of 1.2 mL were loaded onto the vivacon 500 membrane (3 times 400µL). All the protein loaded into the membrane was used for digestion (**Table IV.1**). The entire protocol for sample preparation takes 2.5 h.

Table IV.1 – Urine Protein Quantification by Bradford Assay. Total protein concentration refers to quantification of the total protein in the urine sample. Digested protein refers to the protein concentration calculated after the enzymatic digestion was done over the protein separated in the vivacon.

Urine Specimen	Urine Protein Concentration (µg/mL)	In-membrane Vivacon Digested Protein (µg)
1	25 ± 1	29 ± 2
2	49 ± 1	58 ± 1
3	347 ± 13	380 ± 16
4	108 ± 3	129 ± 3

IV.2. Urine protein digestion and peptide quantification

After protein concentration and stabilization, the urine proteome was digested using a Filter Aided Sample Preparation (FASP) method, which was developed by Wiśniewski *et al*. Trypsin was used as the proteolytic enzyme with a 1:30 enzyme-to-protein ratio. It specifically hydrolyses peptide bonds at the lysine and arginine residues, generating peptides that contains an Arg or Lys at the C terminal which

are suitable for mass spectrometry applications. FASP have already been applied to urine proteomics studies, such as 96-well filter-plate for urine sample preparation [52] and in-urine proteome annotation [39]. For this experimental study, the initial method described by Wiśniewski *et al*.,[49] was modified accordingly to standard protocols used in our lab. Briefly, reduction and alkylation were performed in 45 min and 35 min respectively. We also decide to use the vivacon 500 membrane with a molecular weight cut-off of 10,000 Daltons to ensure that most of the proteins stayed in the membrane for preservation.

After digestion, the resulting peptides were quantified by the Quantitative Colorimetric Peptide Assay® from Thermo Scientific. For each urine a different standard calibration curve was done (**Figure VII.SM. 2**). **Figure IV.1** shows the concentrations obtained.

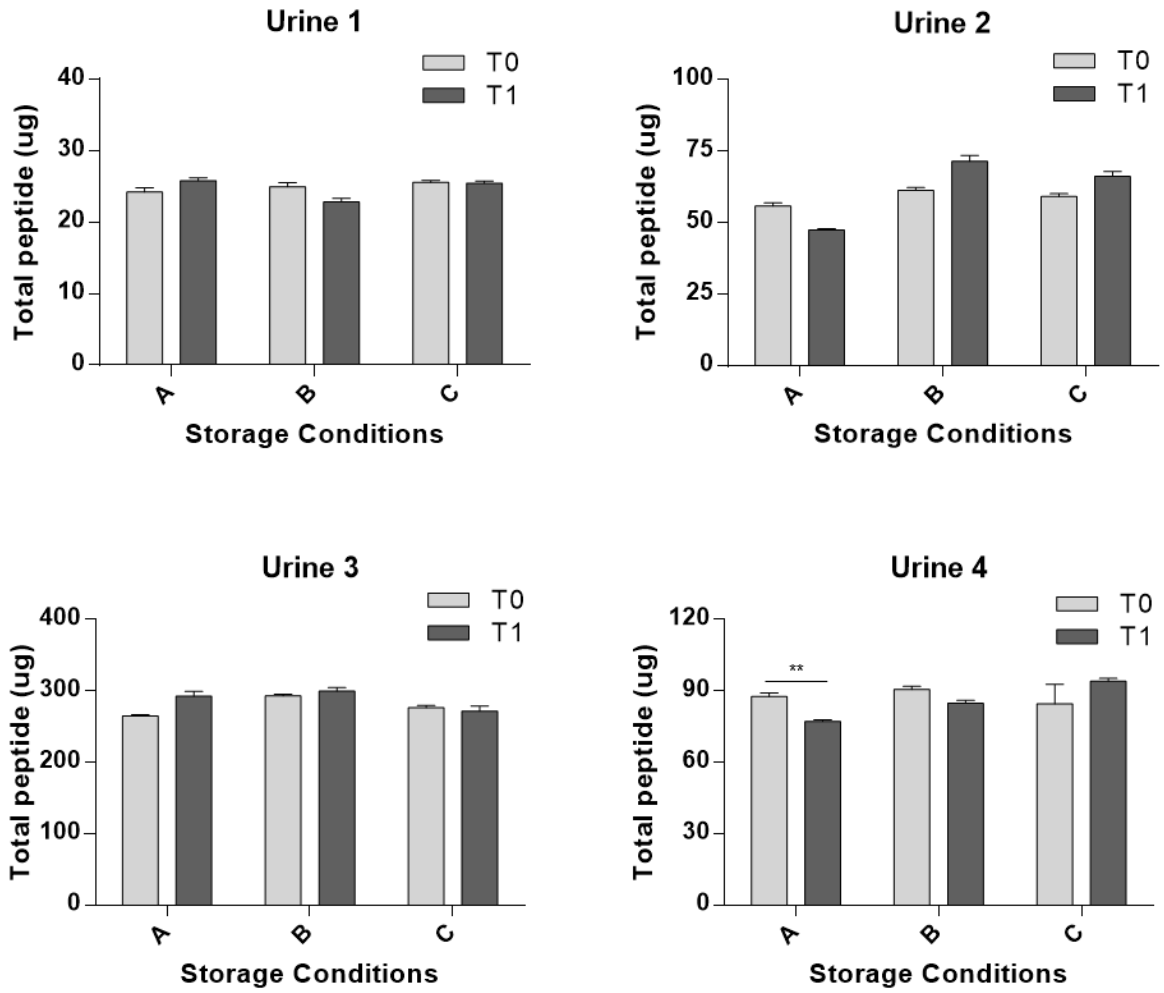

Figure IV.1 – Peptide quantity (µg) found for each storage method after in-membrane protein digestion at the T0 and T1 times of storage. It is represented the peptide quantification for all urines **1**, **2**, **3** ,**4**. In each graphic both storaged methods were quantified. **A:** Stabilizor T1 and room temperature storage.,**B:** Stabilizor and -60ºc storage and **C:** -20ºC storage. (** $p < 0.01$).

For each storage method, a paired T-Test was conducted to compare the total protein found for time T0 and time T1. For all the times and methods of storage studied, the levels found of total protein were similar ($p < 0.01$) regardless the urine and method assessed, with the exception of condition A, stabilizor T1 and room temperature storage, in urine 4. Yet, we hypostatise that this exception can be attributed to a random error of the operator during sample processing.

Although different quantities of protein were digested for each urine sample, the same quantity of peptides (540 ng) was, however, injected into the reverse phase LC Column.

IV.3. Standard urine storage

The most common method for urine short and long-term storage is freezing the urine at -20˚C or -80 ˚C. The problem of freezing samples is that many proteins are degraded if they are exposed to many repetitive freeze/thaw cycles [61]. In our short-term urine storage (condition C, -20˚C), we could observe the formation of a protein precipitate (turbidity) once the sample was thawed (**Figure IV.2 A**). To overcome this problem, sample pH was changed to 8.8 with Tris-HCl. As shown in Figure IV.3, almost all the precipitate was resuspended again. (**Figure IV.2 B**). However, for the more concentrated urines (U3 and U4), complete resuspension of the precipitate was not possible. It was possible to observe the presence of a small precipitate even after the addition of Tris-HCl. (**Figure IV.2 C**).

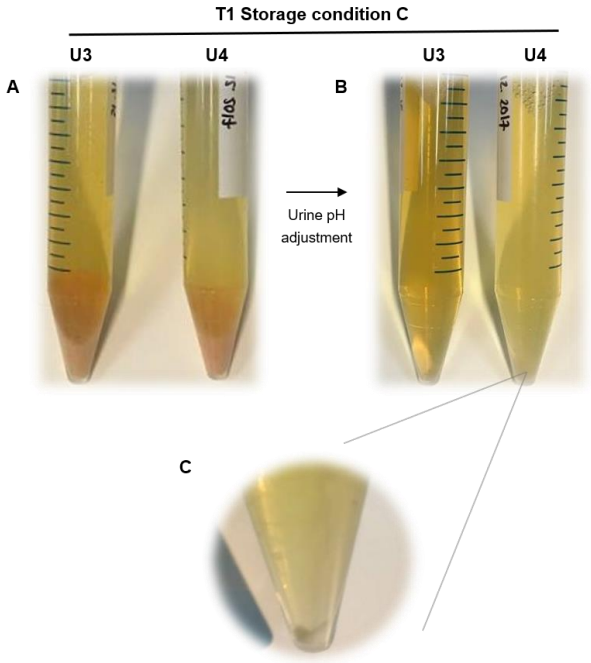

Figure IV.2 – Samples stored at -20ºC after one freeze/thaw cycle. Sample 3 shows that the solid was not entirely resuspended.

To avoid changes in the proteome due to freeze/thaw cycles, it has been recommended to limit the number of such cycles to 5. It has been consistently proved that freeze/thaw cycles cause protein degradation. In specific, was observed that albumin and total protein present in urine samples stored at -20 ˚C and - 80 ˚C, significant decrease after 6 freeze/thaw cycles [46].

IV.5. Effect of each storage method in the number of sequenced peptides and identified proteins

To evaluate the effect of storage over the urine proteome, proteins were quantified using label-free mass spectrometry. Briefly, an aliquot of each digested sample containing 540 ng of peptides were analyzed using high-resolution mass spectrometry. The numbers of identified peptide sequences (Peptide Spectrum Matches, PSMs) for the identified proteins were determined for each storage condition (A, B, C) and time (T0, T1). **Figure IV.3** shows higher data dispersion and a decrease of PSMs in condition C in both time points of analysis. Despite this observation, the PSMs in condition C is not statistically significant (Student's T-test, p-value > 0.05), and therefore cannot offer information about the performance of each preservation method. Moreover, the number of identified proteins is comparable among preservation conditions (A, B, C) and time of preservation (T0, T1).

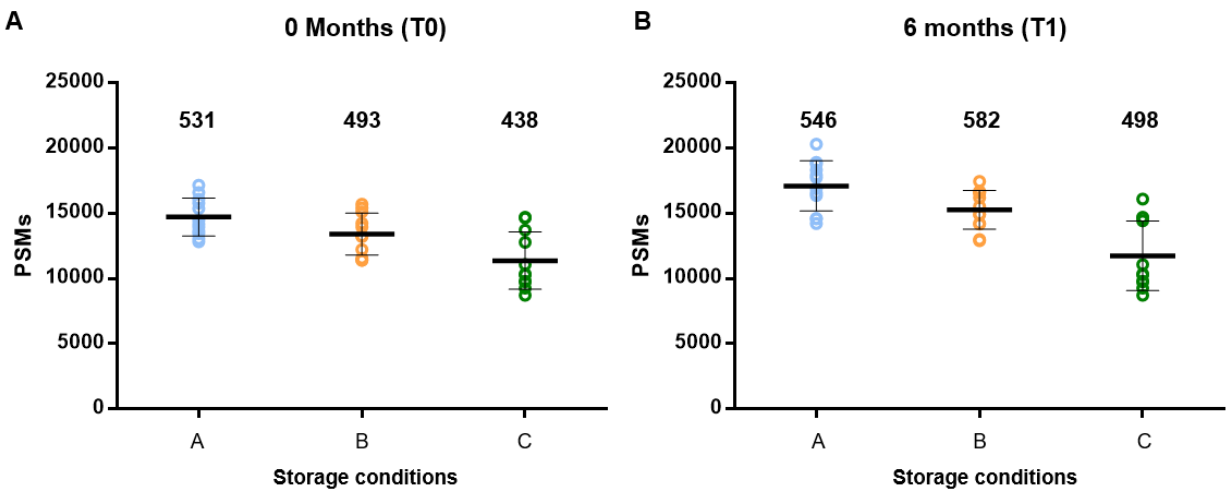

Figure IV.3 – Number of proteins and peptides identified for storage condition and time. Representation of the number of peptides spectrum matches (PSMs) with the storage time and conditions used in all urine specimens. The numbers in the graph represent the number of identified proteins (Four urine samples + 3 technical replicates). Storage condition **A:** Stabilizor T1 and storage at RT; Storage condition **B:** Stabilizor T1 and storage at -60 ºC; Storage condition **C:** Storage at -20 ºC.

IV.6. Label-free quantification of the urine proteome

IV.6.1. Correlation between the storage methods at T0

Different quantitative comparisons between urine storage methods and the analysis time points were taken into consideration (**Figure IV.4)**.

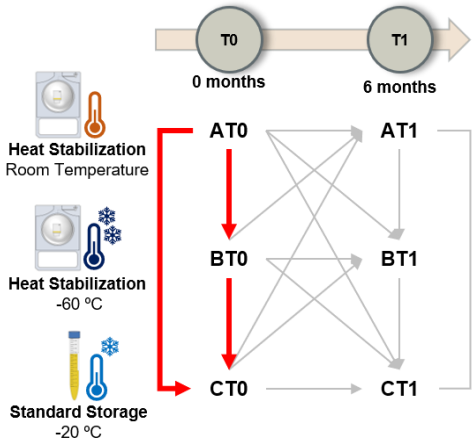

Figure IV.4 – Quantitative comparison in time 0 between storage methods. Comparison underlying the storage time T0 (48 hours) and the 3 different storage methods.

Figure IV.5 and **Figure IV.6** depict the volcano plots regarding the comparison of the proteins identified and quantified for each T0 storage method. No significative differences were found for the majority of the proteins (Paired Student's T-test, FDR 0.05, S0>1.5), meaning the statistical assays address that almost all the proteins compared do not differ in terms or relative abundance. As a matter of fact, the few proteins found down- or over- preserved was below 1%. Only for the case of the comparison of AT0 versus CT0 (urine 1) this value was slightly higher below 3%. For all other cases the differences were below 1%. These results were expected as at T0, the proteins were only stored for 2 days before analysis. This period of time is not large enough to expect significant changes between the urine storage methods. The aforementioned results showed that the Stabilizor T1 at room temperature works as a storage method for at least 48 h.

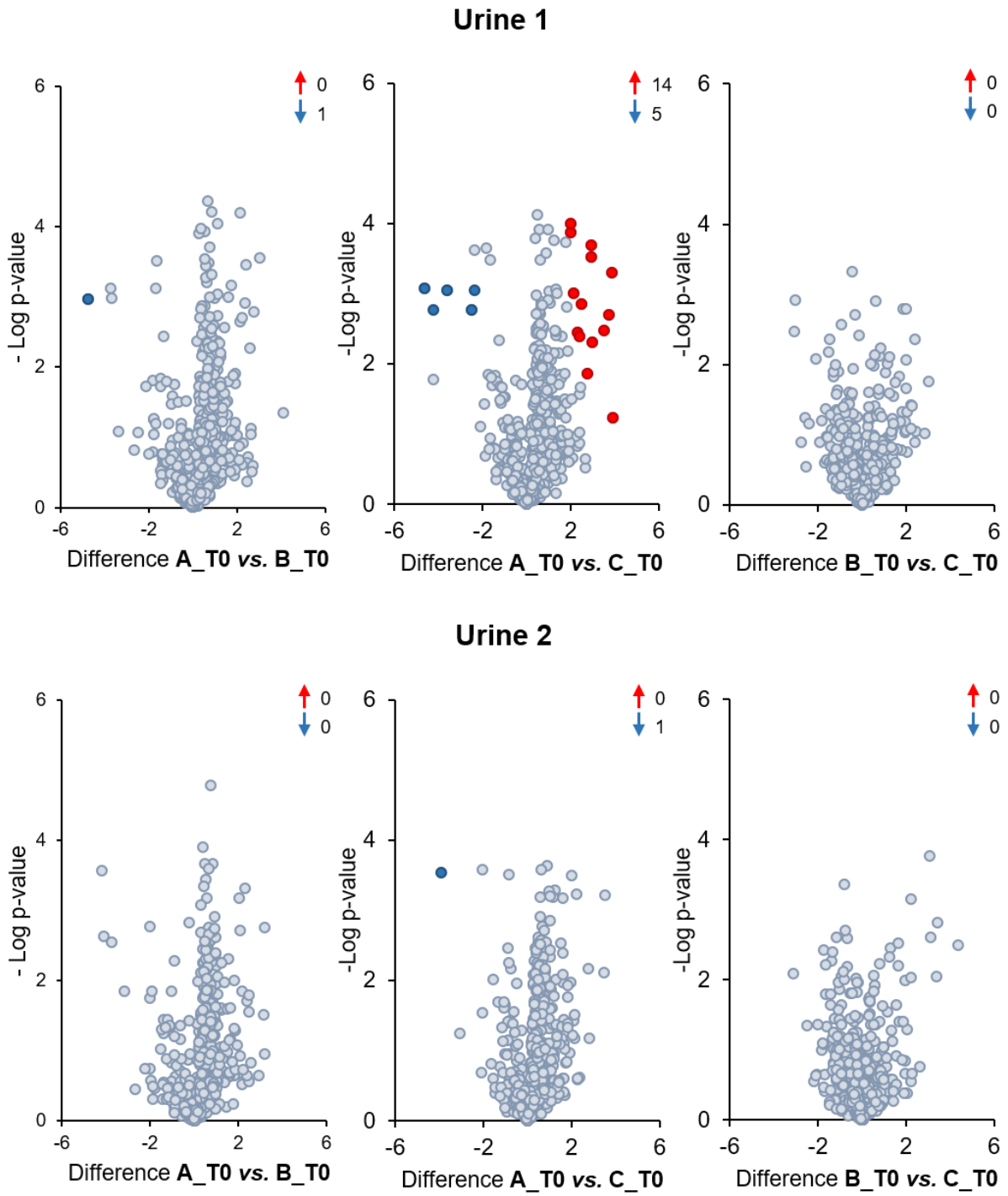

Figure IV.5 – Volcano plots from the normalized MaxQuant LFQ intensities for T0 and for urines 1 and 2. A: Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºc storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

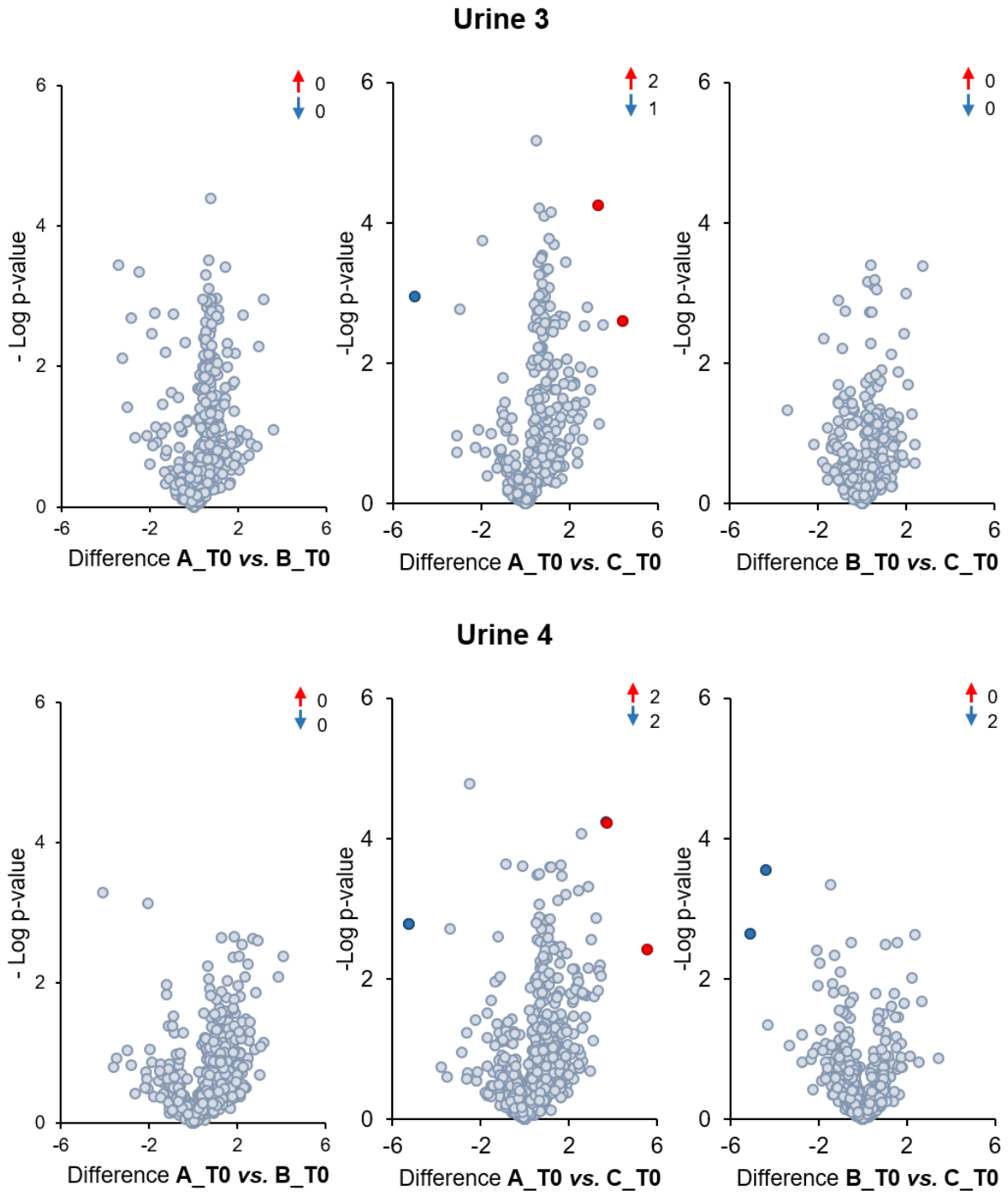

Figure IV.6 – Volcano plots from the normalized MaxQuant LFQ intensities for T0 and for urines 3 and 4. **A:** Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºc storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

IV.6.2. Correlation between the storage methods at T1(6months)

Figure IV.7 shows the comprehensive scheme for the comparison of the storage during 6 months.

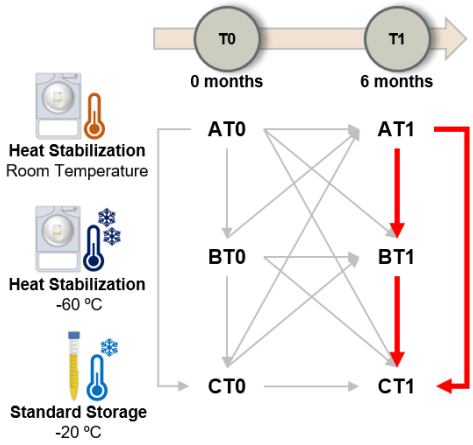

Figure IV.7 – Quantitative compararison in time 1 (6 months) between storage methods. Comparison underlying the storage time T1 (6months) and the 3 different storage methods.

Figure IV.7 and **Figure IV.8** depict the volcano plots regarding the comparison of the proteins identified and quantified for each T1 storage method. No significative differences were found for the majority of the proteins (Paired Student's T-test, FDR 0.05, S0>1.5), meaning the statistical assays address that almost all the proteins compared do not differ in terms or relative abundance. As a matter of fact, the few proteins found down- or over- preserved was below the 3% for three out of four urines. Only in the case of urine four he comparison of AT1 versus CT1 showed a difference of 8%. The increase from 1 to 3% in differences when comparing T0 versus T1 is an indicator that with time the proteome is degrading, even at -60 ºC. Interestingly, this degradation was the of the same level for all the storage system tested. The difference in urine four AT1 versus CT1 is attributed to a random error in the handling of the sample.

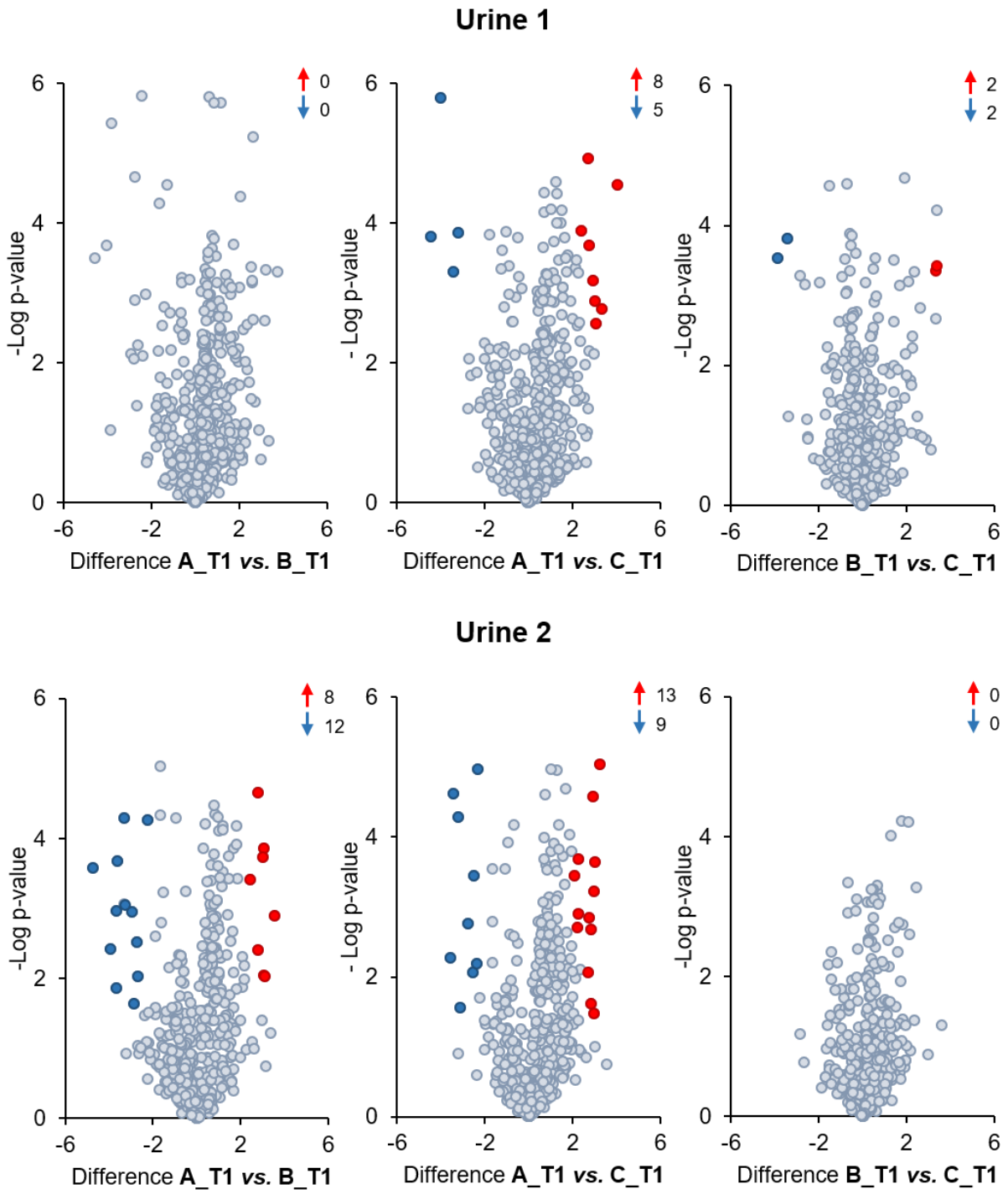

Figure IV.8 – Volcano plots from the normalized MaxQuant LFQ intensities for T1 and for urines 1 and 2. A: Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºc storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

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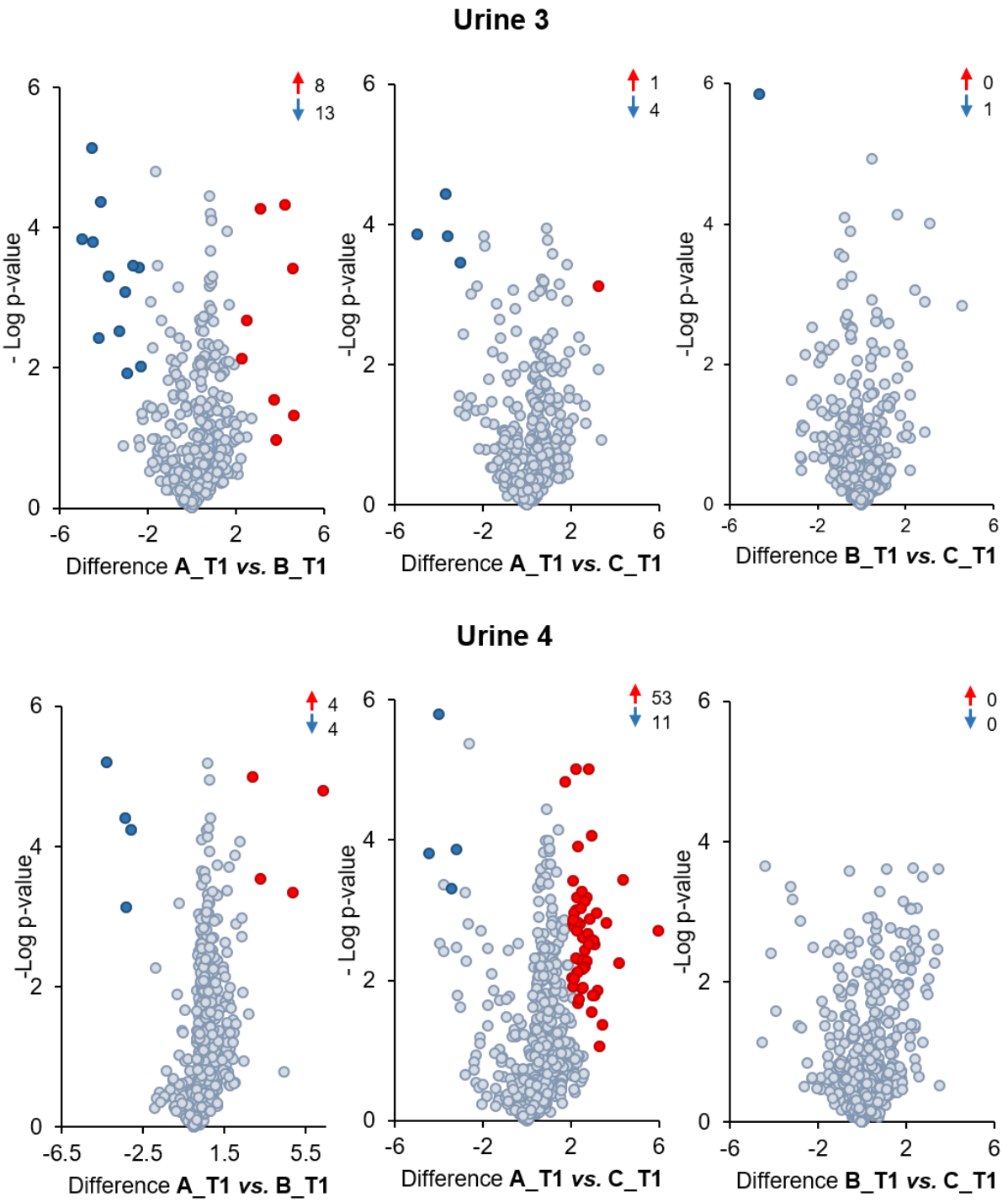

Figure IV.9 – Volcano plots from the normalized MaxQuant LFQ intensities for T1 and for urines 3 and 4. **A:** Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºc storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

IV.6.3. Performance of protein preservation method over time

Figure IV.10 shows the comprehensive scheme for the comparison of each storage method during 6 months.

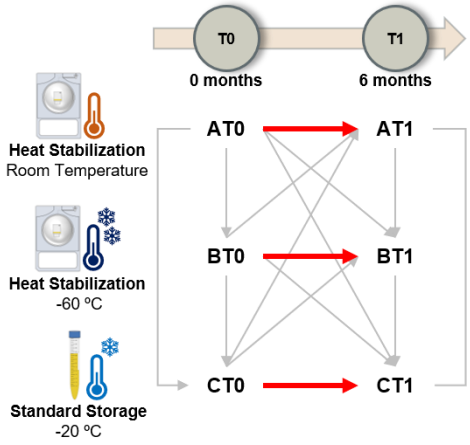

Figure IV.10 – Longitudinal quantitative comparison between storage methods. Urine samples were compared at T0 (48 hours) with T1 (6 months) for the evaluation of urine proteome preservation with the methods A, B and C.

As can be seen in **Figure IV.11** and **Figure IV.12,** depict the volcano plots regarding the comparison of the proteins identified and quantified for each storage method between T0 and T1. No significative differences were found for the majority of the proteins (Paired Student's T-test, FDR 0.05, S0>1.5), meaning the statistical assays address that all the proteins compared do not differ in terms or relative abundance. As a matter of fact, the few proteins found down- or over- preserved was ≤1% for three out of four urines. Only for the case of urine one the comparison of AT0 versus AT1 showed a difference of 4%. This case is attributed to a random error of handling.

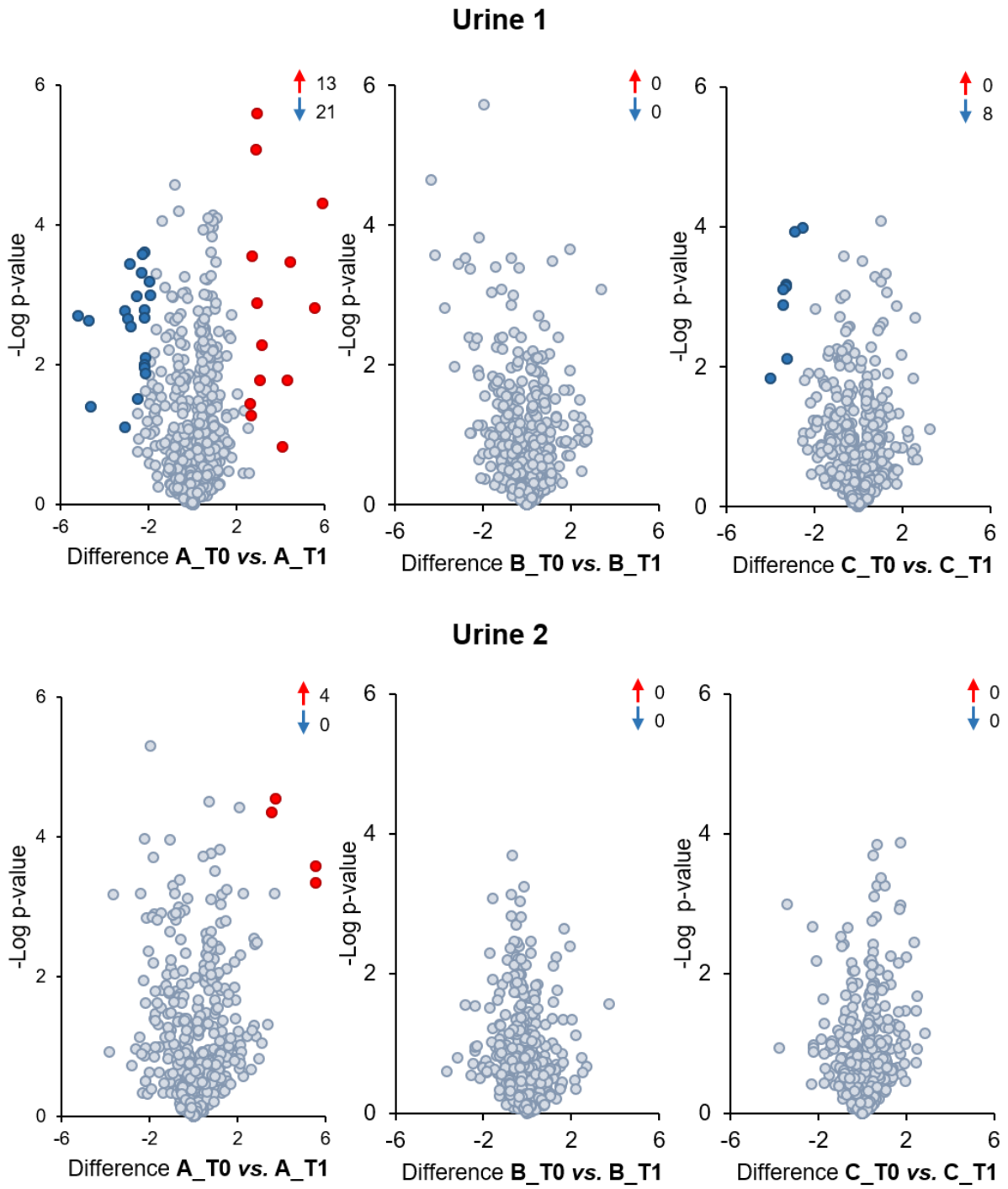

Figure IV.11 – **Volcano plots from the normalized MaxQuant LFQ intensities for T0 *vs.* T1 comparison in for urines 1 and 2. A:** Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºc storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

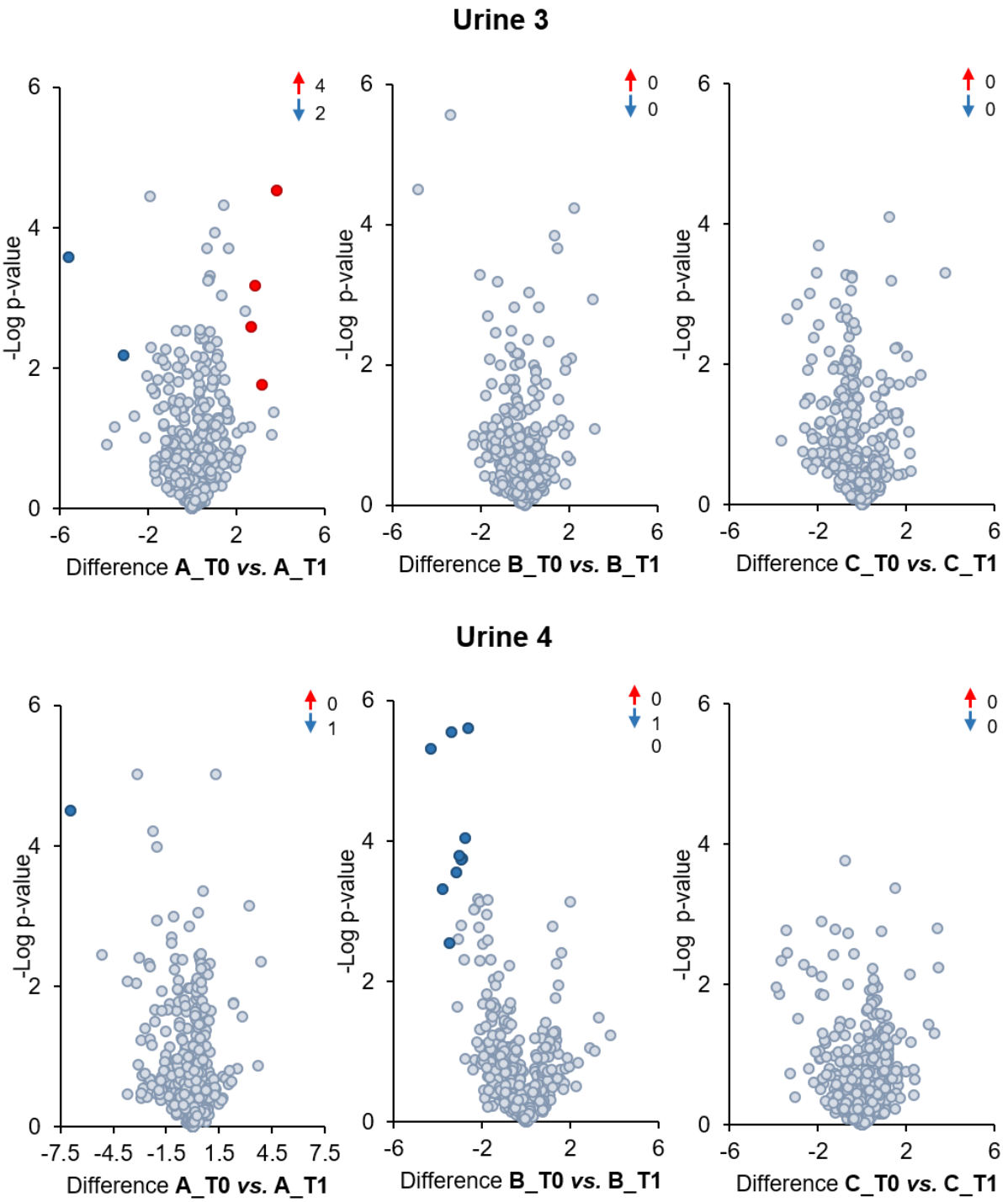

Figure IV.12 – Volcano plots from the normalized MaxQuant LFQ intensities for T0 *vs.* T1 comparison in for urines 1 and 2. A: Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºc storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

IV.7. Hierarchical cluster analysis of each storage methods and time

Figure IV.13 and Figure IV.14 depict the hierarchical clusters obtained when all the data series for each urine at different times and storage methods are compared. The LFQ data of each preservation method for T0 and T1 was compared using an ANOVA test and the significant proteins were normalized by Z-scores. The Z-score offers information about how many standard deviations differ from the mean. Once this data for each technical replicate is selected as explained above, they are grouped by storage method and time. Then, all are compared by unsupervised clustering. The hierarchical clusters suggest that data obtained with method of storage A (Stabilizor T1 and room temperature) tends to group together whilst data obtained with methods B (Stabilizor T1 and -60 ºC) and method C (-20ºC) tends to group together. This second group cannot be grouped correctly for urines 1, 2 and 3. In urine 4, method A and B are mixed together. The aforementioned results obtained with the hierarchical cluster analysis are in agreement with the information retrieved from the volcano plots, that address that the three methods perform equally in terms of preservation.

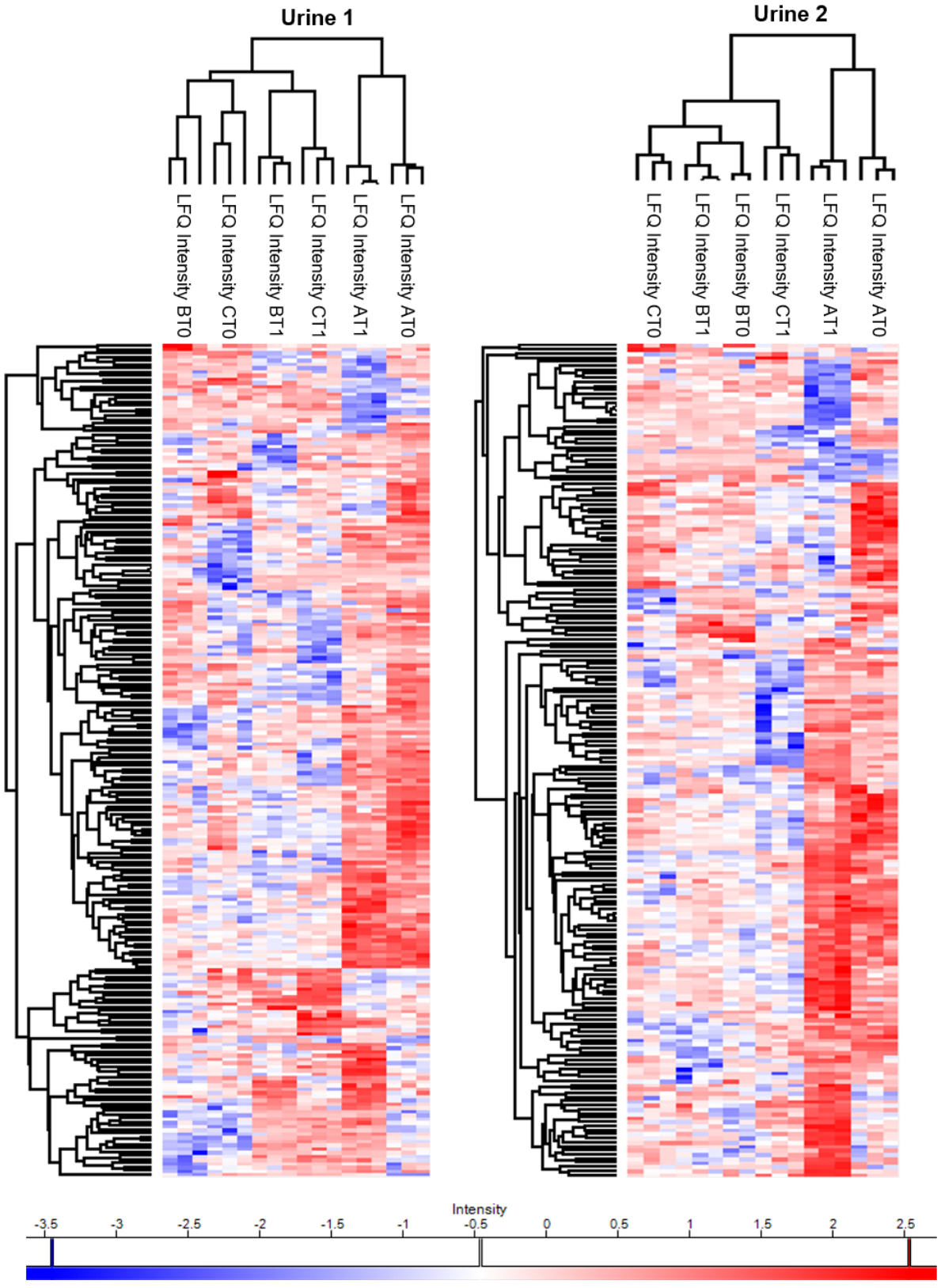

Figure IV.13 – **Hierarchical-cluster analysis of all the signicant proteins in urines 1 and 2**. Storage condition **A:** Stabilizor T1 and storage at RT; Storage condition **B:** Stabilizor T1 and storage at -60 ºC; Storage condition **C:** Storage at -20 ºC.

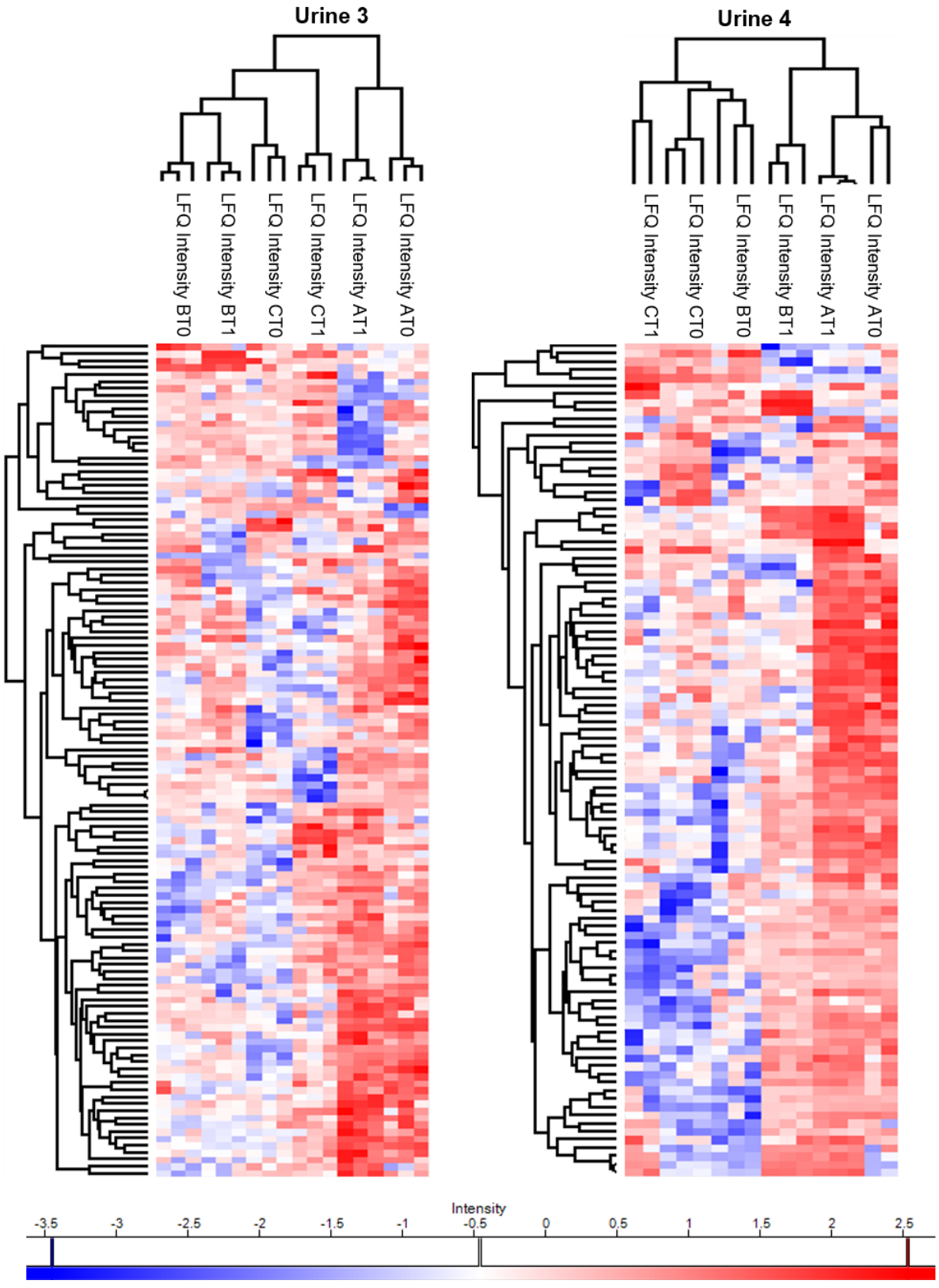

Figure IV.14 – **Hierarchical-cluster analysis of all the signicant proteins in urines 3 and 4**. Storage condition **A:** Stabilizor T1 and storage at RT; Storage condition **B:** Stabilizor T1 and storage at -60 ºC; Storage condition **C:** Storage at -20 ºC.

IV.8. Proof-of-concept: preservation of Acute Kidney Injury urinary biomarkers.

To further investigate the preservation methods studied in this work we decided to check the integrity with the time of different urinary biomarkers of Acute Kidney Injury, AKI.

Over the last decade, different biomarkers have been studied and identified to diagnose this AKI [62]. A study was done by Liu *et al*.,2016 [63] demonstrated, the effect of urine storage at - 70˚C on the preservation of different AKI biomarkers. Thus, it was observed that to storage urine at -70 ˚C for 18 months affect the biomarkers levels up to 1% per month, with a total variation of 18% after the time period of study [63]. Following this study, we decided to look specifically to the levels of the following AKI biomarkers: Neutrophil gelatinase-associated lipocalin (NGAL), liver fatty acid binding protein (L-FABP) and Cystatin-C (CyC) by determining their quantitative changes due to the storage method (A, B, and C) or due to time (T0 and T1).

IV.8.1. NGAL preservation during the time of storage in all the methods

Neutrophil gelatinase-associated lipocalin (NGAL) is a biomarker for AKI that already passed through pre-clinical and is facing clinical testing stages for biomarker validation [64]. In **Figure IV.15** shows the variation of LFQ intensities for NGAL in all urine storage conditions tested. And, as it may be observed, some variations were found for all storage conditions. Yet these variations were not statistically significative (Paired Student's T-test Log_2LFQ Intensity, FDR 0.05, S0>1.5) and we assigned them to the experimental error associated with the sample preparation.

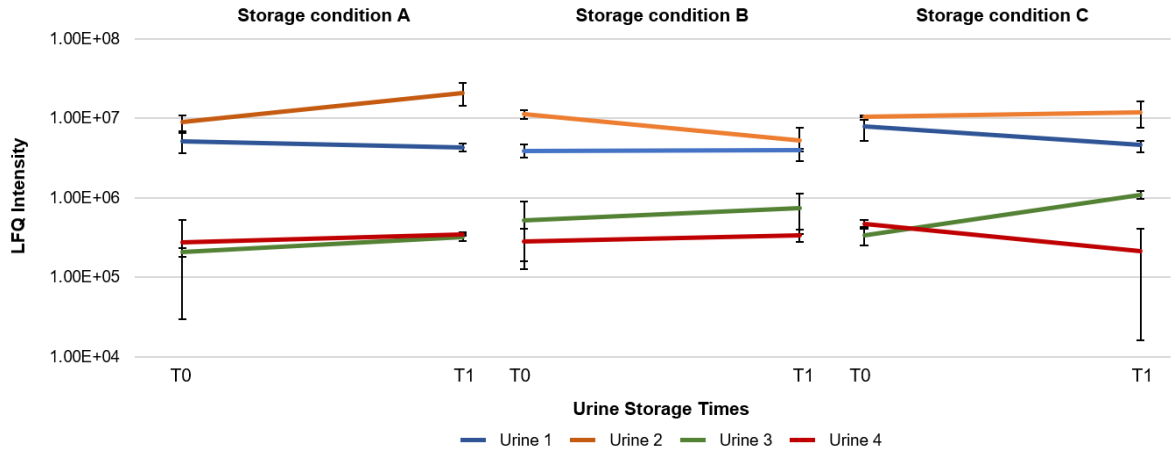

Figure IV.15 – **NGAL levels in all storage methods. NGAL was identified in all urine specimens.** The effect of 6 months of storage in NGAL was evaluated by 3 different methods of urine storage and preservation. Storage condition **A:** Stabilizor T1 and storage at RT; Storage condition **B:** Stabilizor T1 and storage at -60 ºC; Storage condition **C:** Storage at -20 ºC.

IV.8.2. L-FABP preservation during the time of storage in all the methods

Liver fatty acid binding protein (L-FABP) has been pointed by several groups as a candidate biomarker for AKI [65]. The variation with time and storage condition presented in **Figure IV.16** reveals some differences in all storage conditions and urines. However, as in the case of NGAL biomarker, the differences were non-significant (Paired Student's T-test Log_2LFQ Intensity, FDR 0.05, S0>1.5) and, we assigned them to the experimental error associated with the sample preparation.

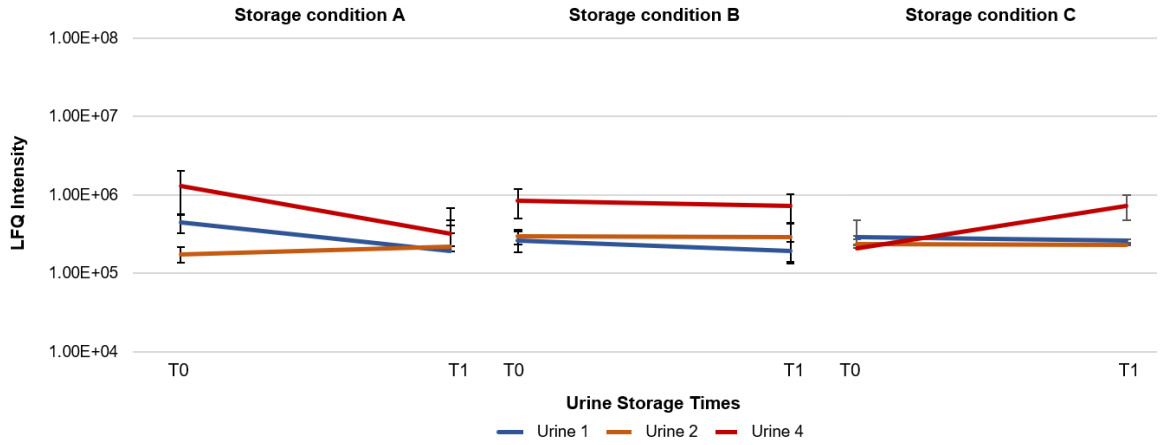

Figure IV.16 – L-FABP levels in all storage methods. L-FABP was identified in 3 out of 4 urines. The presence of FABP2 was not detected in urine specimen 3. The effect of 6 months of storage in L-FABP was evaluted by 3 different methods of urine storage and preservation. Storage condition **A:** Stabilizor T1 and storage at RT; Storage condition **B:** Stabilizor T1 stabilization and storage at -60 ºC; Storage condition C: Storage at -20 ºC.

IV.8.3. CyC preservation during the time of storage in all the methods

Cystatin-C (CyC) is a biomarker for early detection of AKI [66]. We also evaluated the variation of abundance for this specific biomarker in our storage methodologies over 6 months of storage. Such variation is presented in **Figure IV.17**. Some variations were found with time for all the urines, as for the NGAL and L-FABP. The differences found were not statistically significant (Paired Student's T-test Log_2LFQ Intensity, FDR 0.05, S0>1.5)

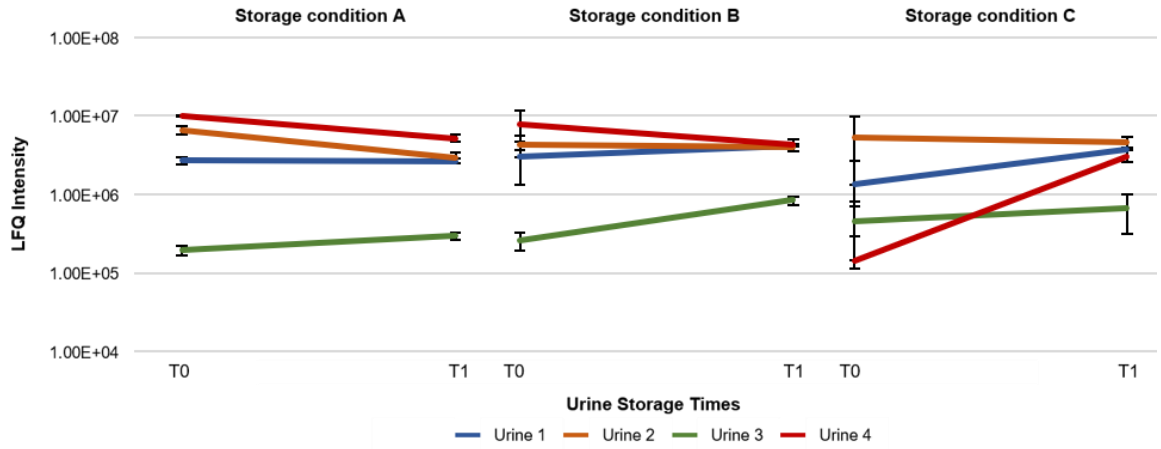

Figure IV.17 – CyC LFQ intensities of CyC for the three storage methods for T0 and T1. CyC was identified in all the 4 urines specimens. The effect of 6 months of storage in CyC was evaluted by 3 different methods of urine storage and preservation. Storage condition **A:** Stabilizor T1 stabilization and storage at RT; Storage condition **B:** Stabilizor T1 and storage at -60 ºC; Storage condition **C:** Storage at -20 ºC.

V. Conclusions and Future Perspectives

The work here presented shows clearly that the three methods studied preserve the urine proteome conveniently for at least 6 months. This means that the use of the Stabilizor T1 system in combination with the FASP approach allows the preservation of the entire proteins within 6 months without the need of storing the sample in a freezer. Such a novel approach is going to change the way the urine proteome is preserved as the space and conditions needed for storage are dramatically simplified. This is especially important for biobanks, due to the large number of samples stored. The risks of sample degradation caused by misfunction of the freezer are also overcome, decreasing the cost of storage and minimizing the care that samples need. Furthermore, the use of the FASP approach to separate the proteins it allows to efficiently process further the sample in time and space, at least within a period of 6 months.

This approach opens new perspectives in sample preservation. It would be interesting to test for how long samples can be preserved with the Stabilizor T1 system. Ideally, this study should be extended for at least periods of 1, 1.5, 2, 2.5, 3, 3.5 and 4 years.

This method has been applied to urine however, it can be extended to any liquid biopsy such as serum and plasma. Therefore, it is expected that the study reported in this work will be applied to such samples within the next years.

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VII. Supplementary Material

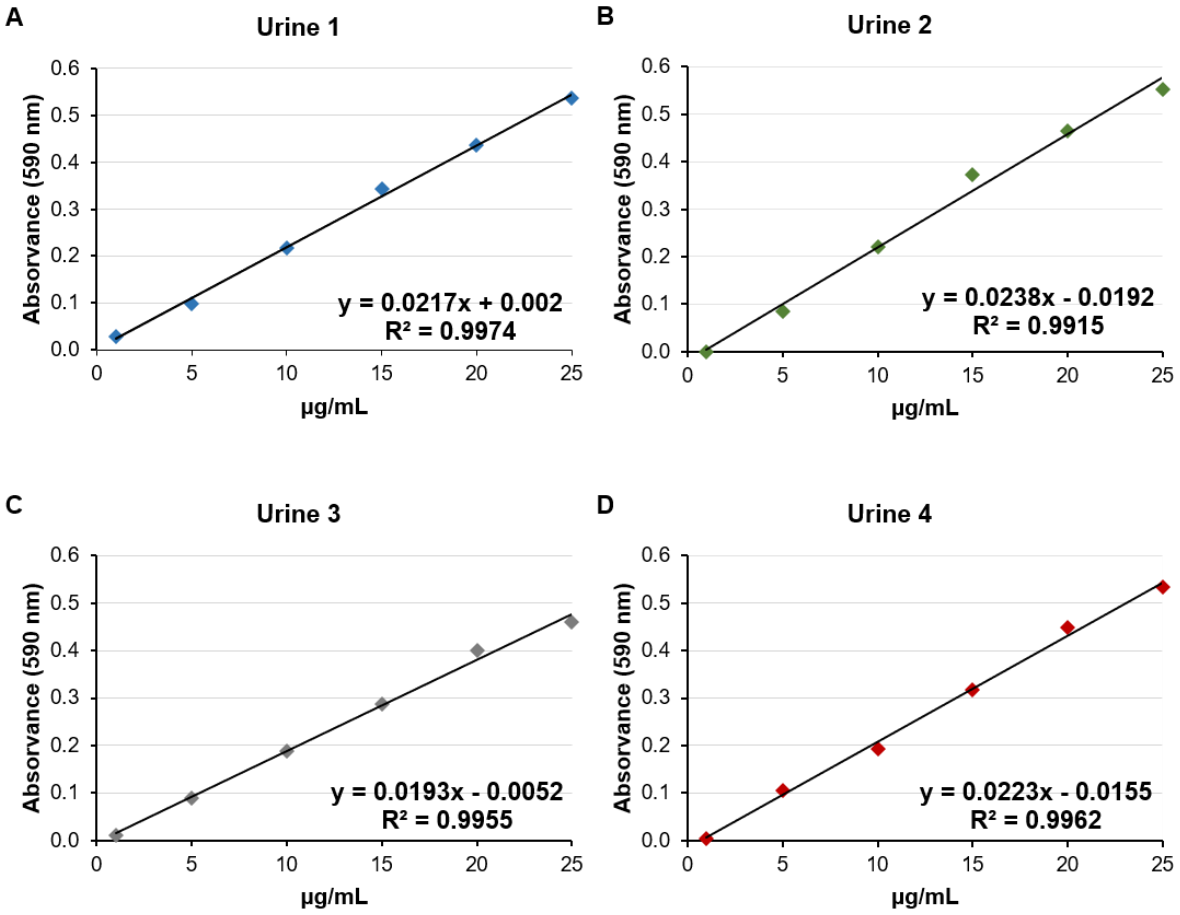

Figure VII.SM. 1 – Bradford assay standard curves. Total protein of each urine 1, 2, 3 and 4 were obtained, respectably, with the standard curves A, B, C, D.

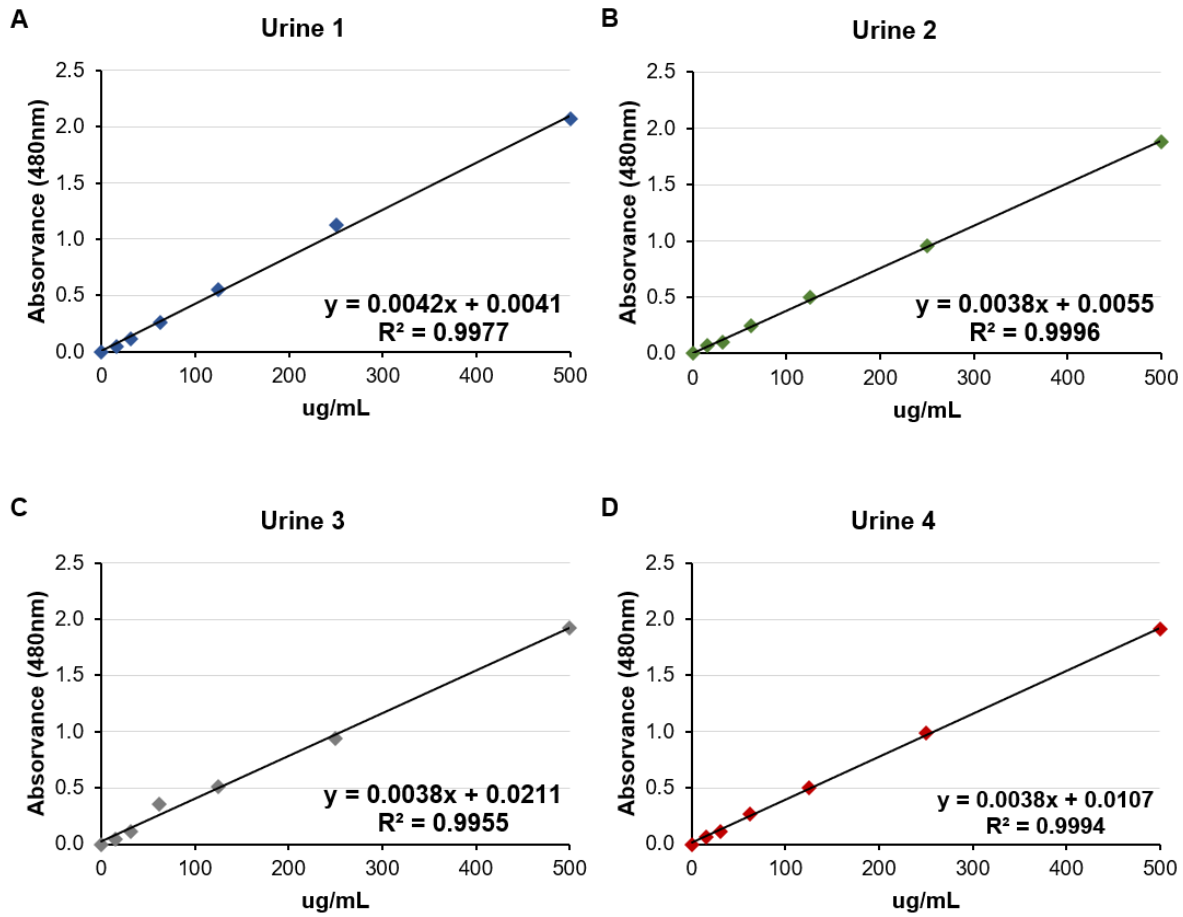

Figure VII.SM. 2 – Quantitative Colorimetric Peptide Assay® standard curves. The total peptide of each urine 1, 2,3 and 4 was obtained, respectably, with the standard curves A, B, C, D.

Table VII. SM. 1 – Peptide Quantification Table. Peptide concentration (mg/mL) and total peptide (µg) obtained in the storage conditions A, B and C and time T0 and T1. It is represented in both replicates (n=3) for each condition in all urine specimens (U1, U2, U3 and U4).

Specimen	Analysis Time	Condition	Peptide concentration (mg/mL)	SD	Total peptide (µg)	SD
U1	T0 0 months	A	0.273	0.010	27.281	1.047
			0.219	0.003	21.852	0.256
			0.234	0.001	23.424	0.096
		B	0.244	0.009	24.376	0.948
			0.239	0.001	23.948	0.078
			0.265	0.003	26.495	0.325
		C	0.216	0.001	21.614	0.145
			0.244	0.000	24.424	0.040
			0.307	0.004	30.662	0.416
	T2 6 months	A	0.243	0.003	24.342	0.262
			0.265	0.002	26.474	0.248
			0.265	0.006	26.526	0.641
		B	0.307	0.002	30.684	0.206
			0.111	0.001	11.132	0.129
			0.266	0.008	26.605	0.819
		C	0.258	0.002	25.763	0.152
			0.243	0.003	24.263	0.271
			0.263	0.004	26.342	0.401
U2	T0 0 months	A	0.539	0.006	53.947	0.556
			0.568	0.016	56.816	1.632
			0.567	0.009	56.658	0.857
		B	0.593	0.002	59.316	0.241
			0.563	0.013	56.263	1.338
			0.683	0.009	68.316	0.889
		C	0.557	0.010	55.711	0.982
			0.618	0.009	61.763	0.897
			0.600	0.010	60.000	0.970
	T2 6 months	A	0.399	0.002	39.947	0.246
			0.416	0.007	41.579	0.672
			0.609	0.003	60.921	0.297
		B	0.739	0.010	73.895	0.963
			0.694	0.017	69.447	1.700
			0.708	0.031	70.763	3.111
		C	0.701	0.026	70.132	2.572
			0.659	0.011	65.921	1.110
			0.624	0.006	62.395	0.584

U3	T0 0 months	A	1.412	0.013	282.400	2.611
			1.335	0.007	266.926	1.450
			1.228	0.002	245.558	0.347
		B	1.456	0.017	291.137	3.484
			1.464	0.005	292.821	0.911
			1.481	0.001	296.295	0.229
		C	1.373	0.026	274.505	5.296
			1.413	0.005	282.611	0.970
			1.363	0.005	272.611	1.026
	T2 6 months	A	1.540	0.021	307.979	4.203
			1.483	0.035	296.505	6.954
			1.369	0.042	273.768	8.447
		B	1.481	0.010	296.189	1.986
			1.464	0.024	292.821	4.709
			1.556	0.032	311.242	6.336
		C	1.406	0.038	281.137	7.667
			1.343	0.040	268.505	8.073
			1.332	0.026	266.400	5.143
U4	T0 0 months	A	0.426	0.003	85.116	0.550
			0.406	0.003	81.221	0.697
			0.485	0.011	96.905	2.201
		B	0.429	0.002	85.747	0.484
			0.480	0.001	95.958	0.113
			0.451	0.012	90.274	2.338
		C	0.407	0.069	81.326	13.807
			0.442	0.007	88.379	1.380
			0.421	0.010	84.168	2.080
	T1 6 months	A	0.361	0.005	72.274	1.029
			0.386	0.004	77.116	0.764
			0.411	0.002	82.274	0.302
		B	0.455	0.006	90.905	1.193
			0.376	0.006	75.221	1.124
			0.443	0.005	88.589	1.099
		C	0.486	0.003	97.221	0.571
			0.465	0.007	93.011	1.361
			0.461	0.007	92.168	1.425

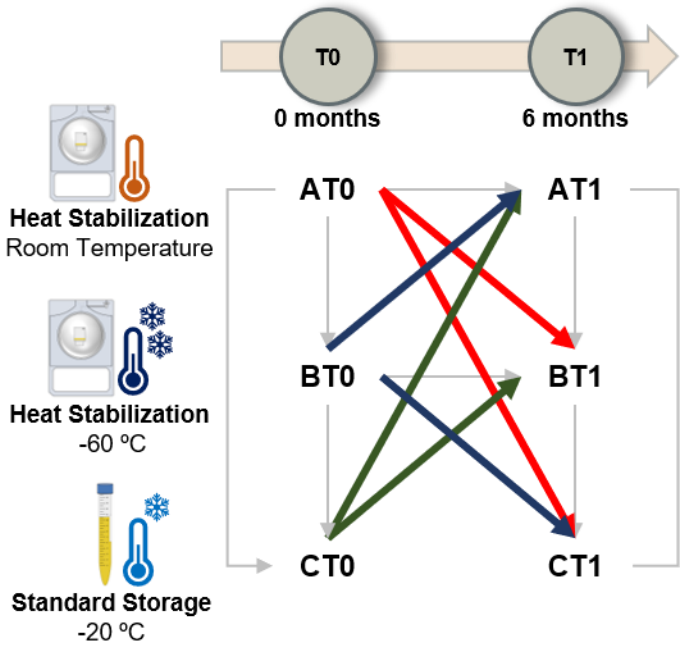

Figure VII. SM. 3 – Comparison between all the methods and time points of analysis. Comparison between all storage methods and time points in all urines specimens.

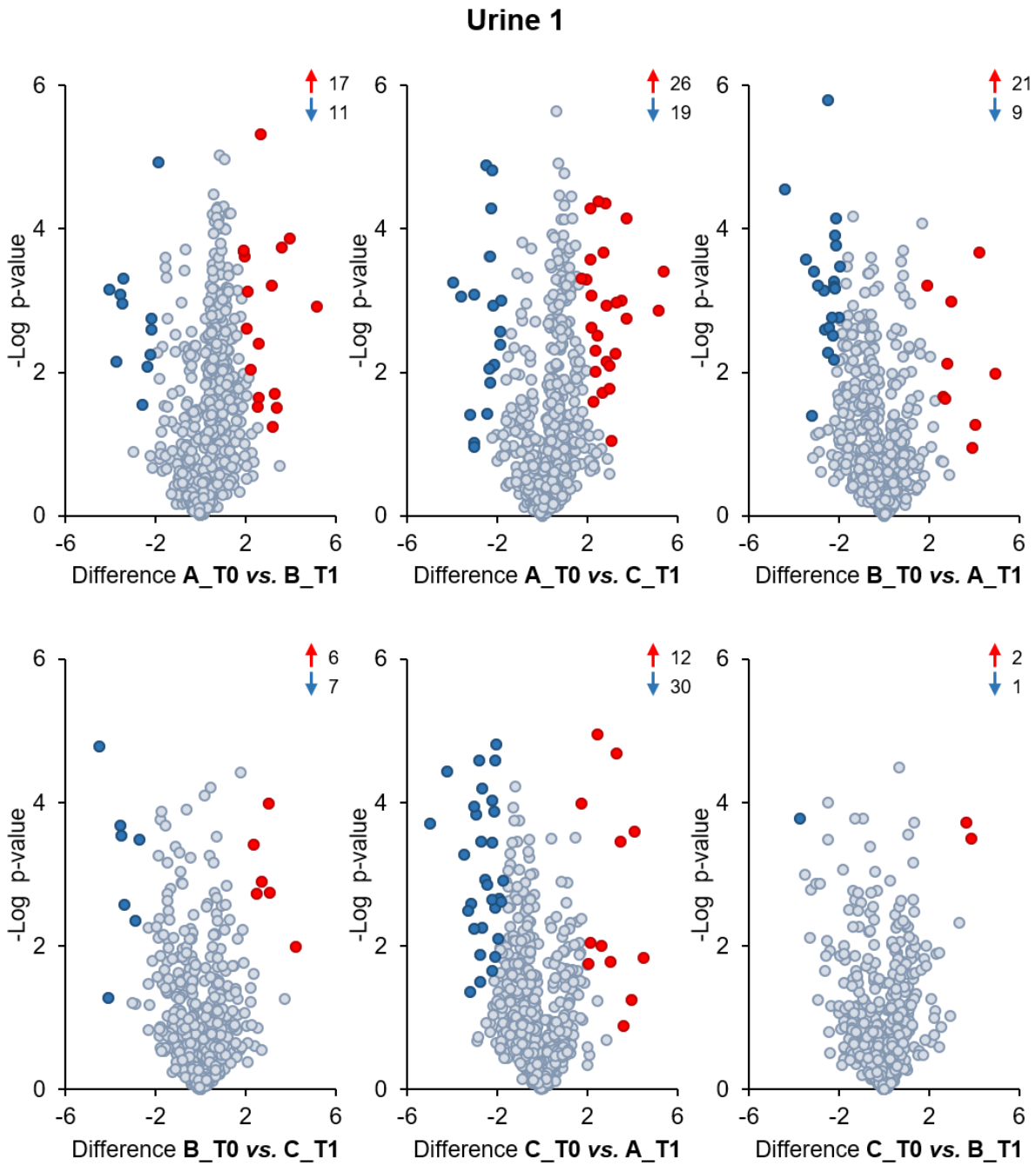

Figure VII. SM 4 – Volcano plots from the normalized MaxQuant LFQ intensities for all storage methods and time points in urines 1. A: Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºc storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

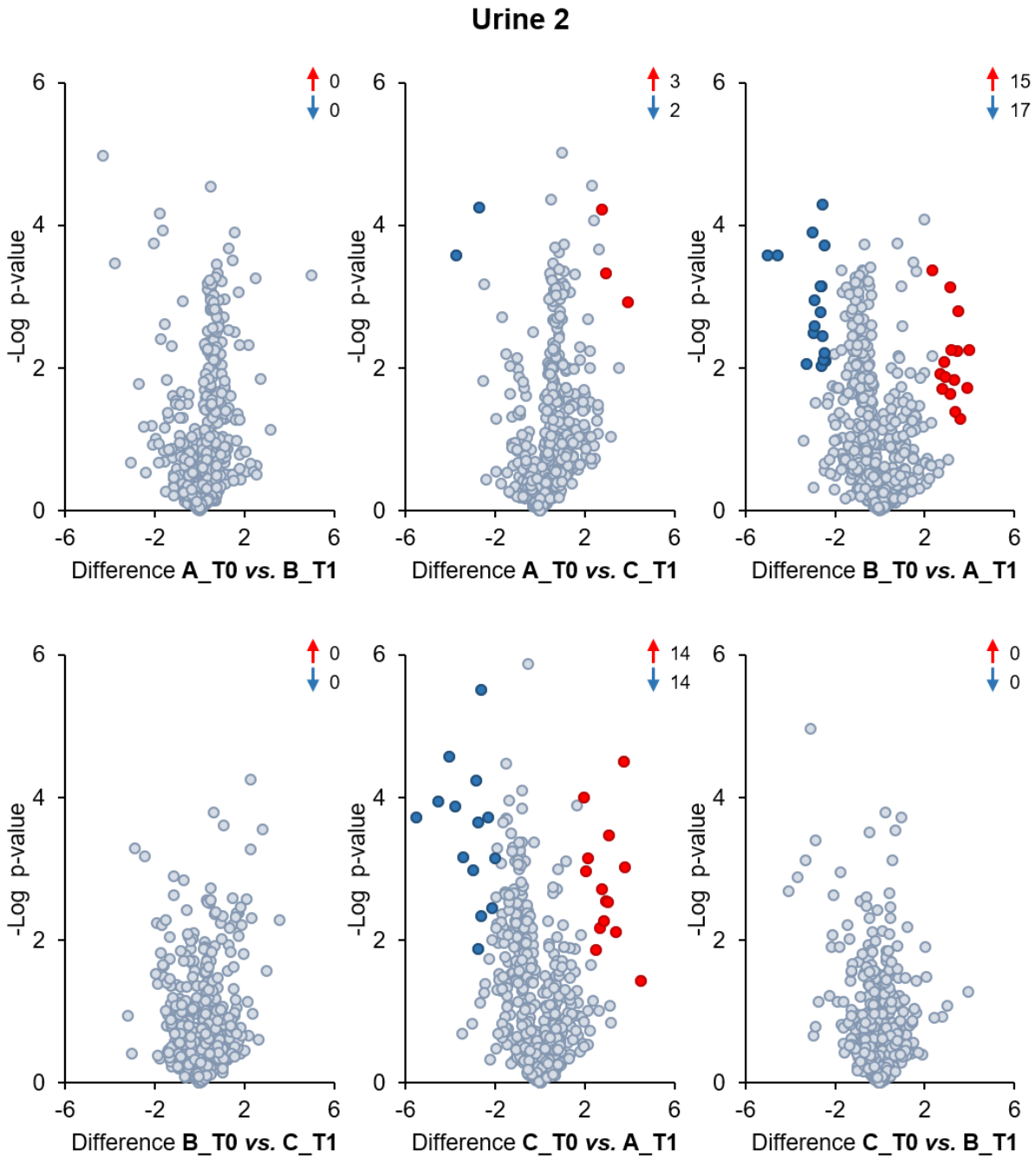

Figure VII.SM. 5 – Volcano plots from the normalized MaxQuant LFQ intensities for all storage methods and time points in urines 2. A: Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºc storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

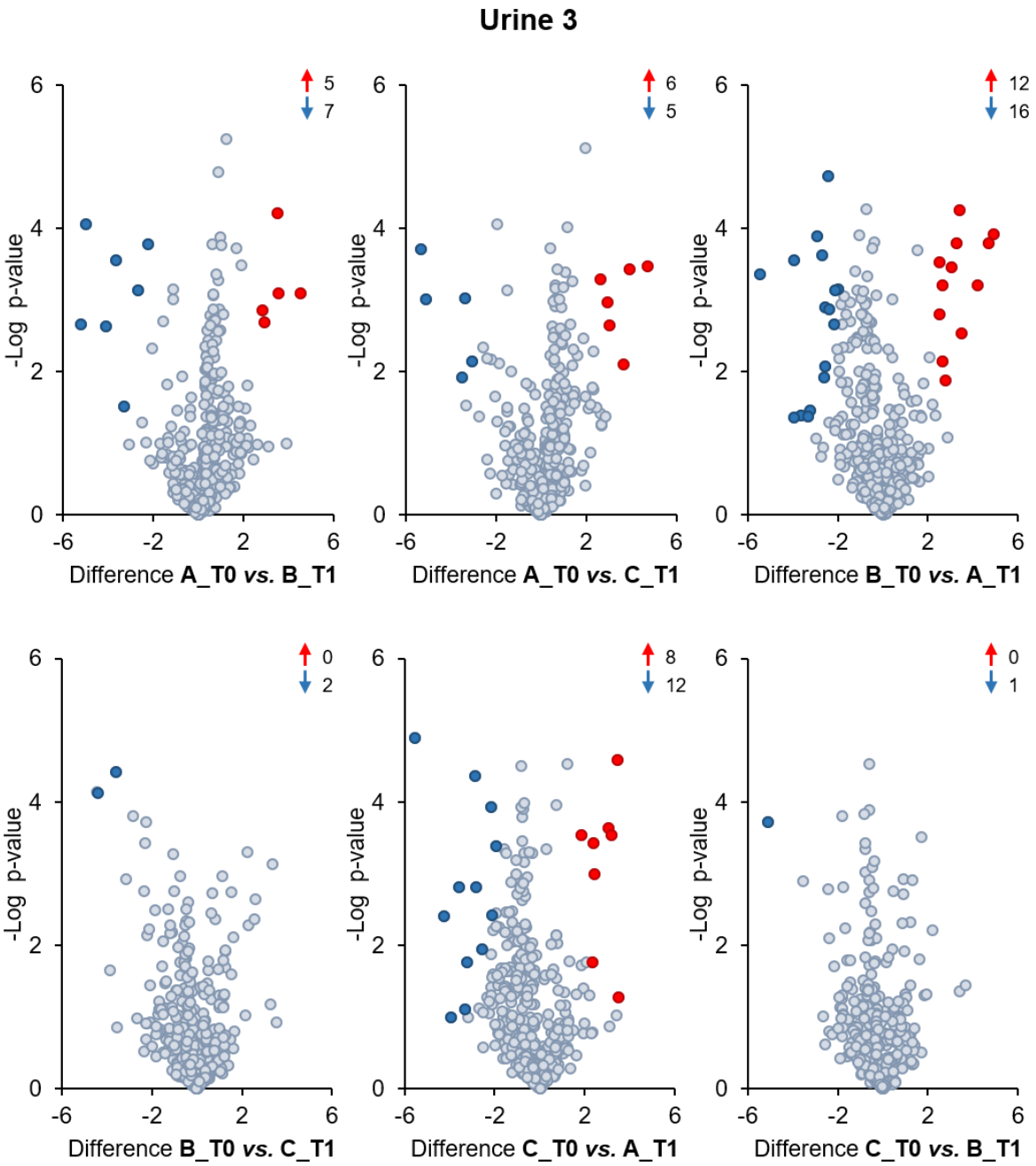

Figure VII. SM. 6 – Volcano plots from the normalized MaxQuant LFQ intensities for all storage methods and time points in urines 3. A: Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºC storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).

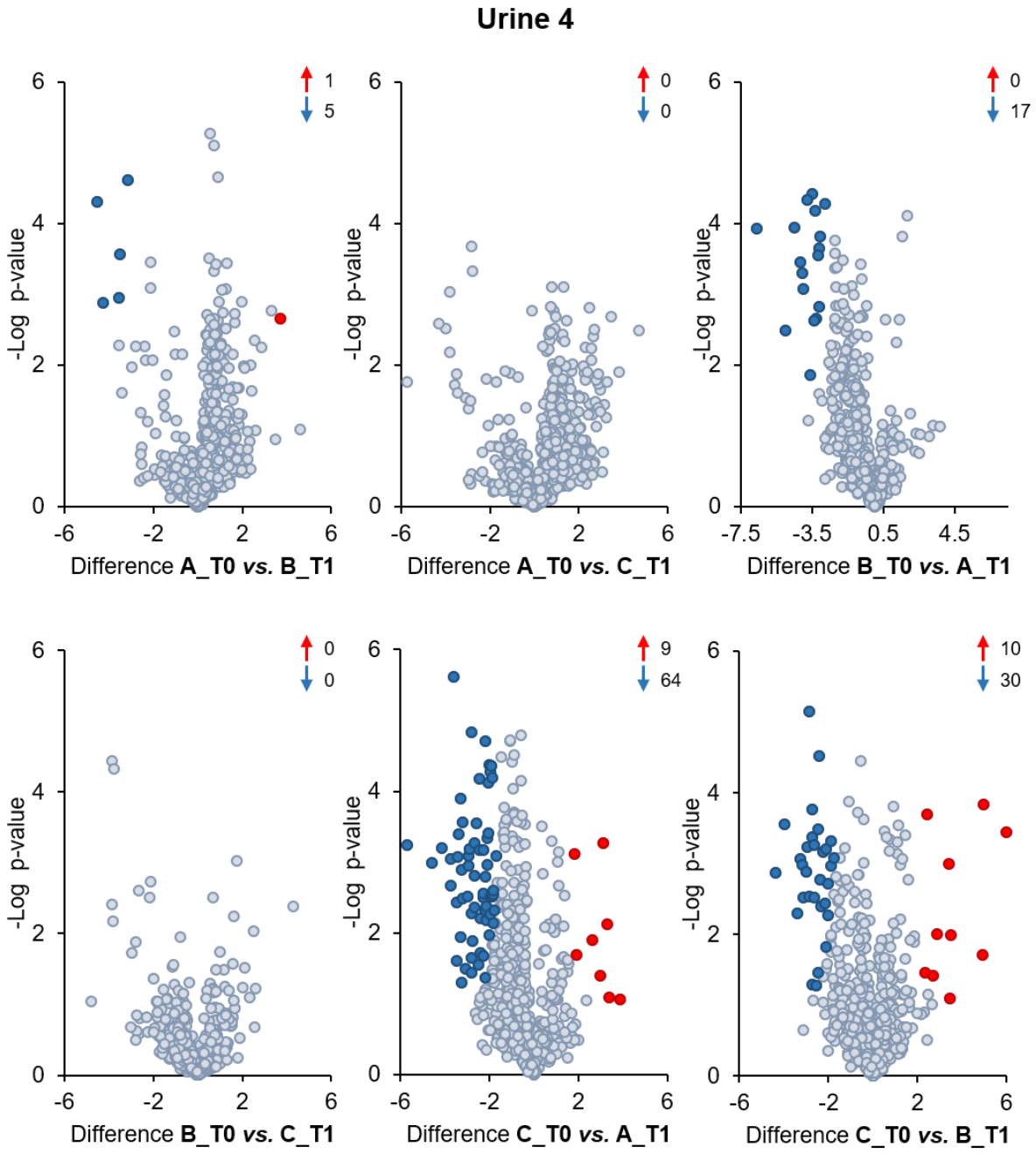

Figure VII. SM. 7 – Volcano plots from the normalized MaxQuant LFQ intensities for all storage methods and time points in urines 4. A: Stabilizor T1 and room temperature storage, **B:** Stabilizor T1 and -60ºC storage and **C:** -20ºC storage. Protein abundance was considered statistically different when fold change was higher than 1.5 for an FDR=0.05. (Paired Student's T-test, FDR 0.05, S0>1.5).