Targeting human urinary metabolome by LC–MS/MS: a review
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Summary

Urine is a biological matrix that contains hundreds of metabolic end-products which constitute the urinary metabolome. The development and advances on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have revolutionized the analytical study of biomolecules by enabling their accurate identification and quantification in an unprecedented manner. Nowadays, LC-MS/MS is helping to unveil the complexity of urine metabolome, and the results obtained have multiple biomedical applications. This review focuses on the targeted LC-MS/MS analysis of the urine metabolome. In the first part, we describe general considerations (from sample collection to quantitation) required for a proper targeted metabolic analysis. In the second part we address the urinary analysis and recent applications of four relevant families: amino acids, catecholamines, lipids, and steroids.
Graphical Abstract

Sample collection and time → Stability and storage → Sample treatment → Chromatographic separation

URINARY TARGETED METABOLOMICS

Data interpretation → Data normalization → Method validation → Mass spectrometry detection
Executive summary

Introduction

- Metabolomics is a discipline with increasing interest and biomedical applications, which range from diagnosis of diseases to personalized medicine and doping control.
- In contrast with untargeted approaches, targeted metabolomics by LC-MS/MS allows for the verification or rejection of a predefined hypothesis by the accurate quantification of a specific part of the global metabolome.
- Urine analysis has multiple advantages (e.g. non-invasive collection, collection of large volumes, containing a broad metabolic profile) but several challenges have to be faced (e.g. difficulty to standardize concentrations, wide dynamic range of concentrations).

General considerations

- The targeted study of the metabolome should be performed only by using fully validated methods and taking into consideration multiple variables (e.g. sample collection, sampling time, storage, sample treatment and instrumental analysis) as we thoroughly review.

Families

- The analytical requirements and strategies required for a proper targeted metabolomic analysis (from pre-sample analysis to data generation) are described with a focus on four relevant families of compounds: amino acids, catecholamines, lipids, and steroids.
- Recent biomedical applications of urinary targeted metabolomics for these families of compounds are also discussed.

Future perspectives

- Two major challenges in the research on human metabolome are (i) the inter-laboratory reproducibility of results and (ii) the determination of healthy physiological
concentration ranges of biomarkers to distinguish pathological concentrations from normal variations (e.g., induced by physical activity or diet).

- The accurate information provided by targeted metabolomics (either alone or in combination with other omics techniques) constitutes a powerful tool that will offer a better understanding of the complexity of the human metabolome and will broaden the current spectrum of biomedical applications.
Key terms

**Urine analysis**: analysis of the aqueous biofluid that contains the kidney-mediated filtration product of blood. It contains hundreds of metabolic end-products which constitute the urine metabolome.

**Targeted metabolomics**: quantitative approach for the accurate and precise measurement of known and predefined group of metabolites.

**Untargeted metabolomics**: qualitative or semi-quantitative approach for the comprehensive measurement of known and unknown metabolites.

**LC-MS/MS**: gold-standard analytical tool in targeted metabolomics. It combines liquid chromatography with tandem mass spectrometry, allowing for the simultaneous measurement of a large number of analytes with high sensitivity and specificity.

**Amino acids**: organic compounds containing and amino and carboxyl functional groups which constitute the protein building blocks.

**Monoamine neurotransmitters**: endogenous neurochemicals involved in cognitive processes and responsible for transmitting signals across neurons.

**Lipids**: water insoluble molecules that constitute cell membranes, are a source of energy storage, and play key role as signaling molecules.

**Steroids**: organic compounds with a cyclopentanoperhydrophenanthrene nucleus which are produced mainly in the adrenal cortex, gonads or liver. They regulate multiple biological functions acting as hormones and interacting with nuclear receptors.

Key words: targeted metabolomics; untargeted metabolomics; urine; LC-MS/MS; amino acids; neurotransmitters; lipids; steroids; quantitation
1. **Introduction**

Metabolomics refers to the systematic identification and quantification of the metabolic products (the metabolome) in a biological system (tissue, biological fluid, or organism) at a specific time. By focusing on the comprehensive and simultaneous analysis of hundreds of small molecules in a system, metabolomics provides a direct signature of biochemical activity.

Metabolome profiling is typically addressed by powerful analytical techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy [1].

Since its start as a field of research, the diversity of challenges faced by metabolomics has increased. As a consequence, new sub-divisions with enough entity have been established within the metabolomic field. Sub-divisions might be related to the aim of the study (i.e. to study diverse types of cancer), to the type of instrument employed (NMR, MS), to the type of biological specimen analyzed (blood, urine, cells), to the scope of the analysis (untargeted or targeted), or a combination of them.

The term metabolome is commonly associated to the exploration of the biological material in an open way, meaning that no restrictions in terms of the nature of the monitored molecules are established. On the other hand, the aim of a targeted metabolomic analysis is to accurately quantify all the components of a particular family of the global metabolome. Thus, targeting approaches are less comprehensive, and require of an *ex-ante* knowledge about the relevant metabolites in the studied samples.

By analogy with other ‘omics’ sciences (genomics, transcriptomics, proteomics) we tend to believe that a metabolomic analysis will cover the complete or near-complete metabolic inventory. However, that is far from being the case. Even in the best scenario, only a part of the vast complexity of urinary products will be revealed. Thus, in any type of metabolome study (untargeted or targeted), the results will only show the tip of the iceberg. The size of
the tip, and the part of the iceberg showed will always depend upon to the instrumental approach employed [2].

A milestone in metabolomics was the development of databases which provides reference compound spectral data to identify metabolites. Some of metabolomics databases include Metlin, MetaboLights, the Metabolomics Workbench, Lipid Maps and The Human Metabolome Database (HMDB). First described in 2007, the HMDB (www.hmdb.ca) is a freely available web-enabled resource with comprehensive information about human metabolites along with their biological roles, physiological concentrations, disease associations, chemical reactions, metabolic pathways, and reference spectra. Over the past decade the HMDB has continued to grow and evolve, currently including information about more than 114000 known, expected and predicted metabolites [3].

Untargeted metabolic approaches (also referred to as “open”) lead to long lists of analytes that can be identified by these databases, and quantified with different degrees of accuracy. On the other hand, in targeted metabolomics, the amount of data is less, but the information obtained might be more valuable. This is due to the fact that researchers can optimize the sensibility and the accuracy of the analytical methods based on the chemical features of that particular family of compounds. By focusing on a subset of analytes, some metabolites that might have been ignored by the vast scrutiny of an untargeted approach are now revealed.

As the title indicates, the present review will focus on the use of LC-MS/MS instruments for the characterization of the metabolome in urine samples in a targeted manner. As an analytical platform, LC-MS/MS has a long history of achievements in the description of complex biological systems. Together with NMR, LC-MS/MS is the preferred technique for conducting metabolome investigations. LC-MS/MS is superior in versatility and sensitivity, whereas NMR offers higher reproducibility, straightforward sample preparation, and the advantage of being nondestructive [1, 4, 5].

In human studies urine is a convenient biofluid for metabolomics studies for several reasons: it can be collected non-invasively in large quantities, and its continuous collection over a
period of time offers a more complete picture than blood which provides a snapshot. However, metabolomics in urine are not free of difficulties, one of them being the standardization of concentrations which vary significantly due to personal hydration status [6].

In addition to that, urine analysis has some particularities which have to be considered when digging for information in a comprehensive way. Among them the vast chemical diversity and dynamic concentration range of the metabolome, the integrity of the metabolites under some collection and storage conditions, and the presence of isobaric compounds which makes their separation a key aspect in order to develop a suitable method.

In the present revision we will first describe some general considerations that are important in urinary targeted metabolomics. Afterwards, we will depict four families of molecules exemplifying the vast polarity array of the metabolome: amino acids and carnitines, catecholamines, lipids and steroids. For each family, a description of the analytical requirements, from sample collection to quantitation will be discussed, and illustrated with recent applications.

2. General considerations

One of the main goals of metabolomics is the differentiation between conditions based on the distribution of metabolites. Thus, results from metabolomics studies are commonly considered as quantitative. Nevertheless, many aspects of the analytical procedure can critically affect the precision and accuracy of LC-MS/MS methods. Their effect in the quantitative results is analyte-dependent and can vary from individual to individual and even from sample to sample. Therefore, the influence of these factors on metabolomics studies should not be underestimated.

This impact substantially differs depending on the metabolomic approach selected. The driven force of untargeted metabolomics is that all detectable metabolites are included as
potential biomarkers. Therefore, this strategy aims at the detection of virtually all biomarkers. The huge number of urinary products makes the control of the quantitative results an impossible task. On the other hand, targeted metabolomics implies the quantification of a limited number of metabolites. Targeted metabolomics studies can be conducted by using fully validated methods and, therefore the influence of each aspect of the whole procedure could be theoretically determined (Table 1).

In summary, the influence of factors altering the quantitative data is sometimes under-considered in metabolomic workflows, mainly when untargeted metabolomics is performed. In the next sections, the main factors affecting the quantitative measurements of urinary metabolites by LC-MS/MS are presented and their impact on metabolomic studies is discussed.

2.1. Pre-analysis

2.1.1. Sample collection

The first step in the whole analytical procedure is the collection of the sample. Contrarily to the standard procedures in blood/plasma/serum collection, the use of standardized collectors and/or stabilizers is less common in urine sampling. Thus, although sterile collectors made of non-adsorbing material are normally used in metabolomic studies, some light-sensitive analytes can be altered by the use of common transparent tubes [7]. The use of preservatives may be helpful for the conservation of particular analytes. For example, the addition of HCl to the sample has been found to be suitable for the preservation of some urinary metabolites such as citric acid [8]. Unfortunately, a universal preservative for the complete preservation of urine metabolome does not exist [9]. Due to these considerations, some of the results obtained by untargeted metabolomics might, to some extent, be questionable. On the other hand, the effect of sample collection on quantitative methods should be compulsory checked before its application to targeted metabolomics studies.
2.1.2. Sampling time

Although urinary metabolome of a specific individual can be seen as static, it is affected by several factors both external such as diet and internal such as circadian rhythms. Thus, sampling time might have a critical impact on metabolomics results. The detection of adrenal hormones is a typical example for circadian variation. For these compounds, urinary concentrations varying up to ten fold can be obtained depending on the sampling time [10]. The collection of all samples from a study at the same time can minimize this circadian effect, but differences on circadian rhythms depending on life-pattern e.g. night-shift vs. day-shift workers, cannot be discarded [11]. The collection of 24-hours urine samples is then preferred to neutralize circadian fluctuations [12]. Besides, the use of 24-hours urine samples also facilitates the normalization of the results (see section 2.4.3). However, this selection made the sample collection more difficult.

Metabolomic studies aiming to determine specific biomarkers for a characteristic diet have revealed how important it is on the metabolomic outcome [13, 14]. Various families such as lipids, amino acids or acylcarnitines have been reported to be altered by dietary habits [15]. Therefore, these considerations should be taken into account both when interpreting untargeted metabolomic results and during method validation in targeted approaches.

2.1.3. Stability and storage

Besides the sampling, the time passed between collection and analysis might also be critical for the evaluation of the urinary metabolome. Ideally, urine samples should be analyzed within two hours after collection [9]. From a metabolomics point of view, this ideal situation is difficult to be put into practice since collection of an adequate number of cases and controls usually requires a large period of time. Immediate freezing of the sample is recommended in order to avoid undesired microbial degradation (see below). However, urine metabolome
shows an adequate short-stability at 4 °C and it remains mostly unaltered during the first 24-hours [16]. Regarding sample storage, Laparre et al. suggested to store samples below -20 °C for short periods of time (< 30 days) while -80 °C storage has to be considered for longer periods [17]. As a summary, a short-term storage at 4 °C previous to the long-term storage at -80 °C seems to be an adequate protocol for metabolomic studies. In targeted metabolomics, the short-term and long-term stability of the selected metabolites should be tested during method validation.

One of the main problems regarding stability of urine is the occurrence of microbial contamination. Microorganisms present in the sample can alter the concentration of a several metabolites. Thus, undesired secondary reactions such as deconjugation, oxidation, demethylation or hormone deterioration have been reported to occur in urine [18]. Microbial degradation can be minimized by the addition of some preservatives although, as stated above, a universal preservative capable of preserving the complete metabolome does not exist.

2.2. Sample treatment

2.2.1. Filtration

In metabolomic studies, the preservation of the entire metabolome is one of the key steps. For this reason, unselective sample treatments are often preferred [19]. Although the direct injection of the urine sample into the LC-MS/MS system is possible [20], a previous filtration to remove materials in suspension can be employed. This filtration is commonly performed on filters of cellulose with 0.2 µm-0.45 µm pore size [21]. The use of special membranes with cutoff of 3-30 kDa has also been reported in order to cover hydrophilic metabolites [22]. Additionally, filtration also prevents bacterial growth during storage [23].

2.2.2. Hydrolysis of conjugates
Intrinsic complexity of urine is a double-edged sword in metabolomics. On the one hand the large number of metabolites and their relatively high concentrations make urine ideal for metabolomics. On the other hand, the large variability of metabolites and their frequent indirect implications on the tested status hamper the direct usefulness of the results obtained. Phase II metabolism is a key example of the latest.

A large number of endogenous compounds undergoes phase II metabolism consisting on the conjugation with polar groups. Due to this conjugation, many endogenous components are not exclusively excreted unconjugated but also as glucuronides or sulfates. Conjugation with other compounds such as N-acetylcysteine or the amino acids taurine and glycine are common for species like volatile organic compounds [24] or bile acids [25], respectively. The relative abundance of the different conjugates may depend on several factors like polymorphisms on specific enzymes or the diet. Therefore, the implication of a metabolite in the studied status might remain hidden by the between-group variability of the conjugation reactions.

One possibility to minimize this effect is to perform a hydrolysis step previous to the LC-MS analysis. In untargeted metabolomic studies one of the goals during sample preparation is to keep the sample as intact as possible. Thus, in order to preserve all unknown metabolites in their original form [26], hydrolysis is not common. Contrarily, this practice is frequent for targeted approaches designed to investigate metabolic pathways in which phase II plays an important role.

Both enzymatic and chemical hydrolysis can be used. In order to select the most appropriate one, the analyst should take into consideration factors like the hydrolysis efficiency, the potential generation of artifacts and the production of additional interferences altering the LC-MS/MS response. As an example, enzymatic hydrolysis is preferred for steroid analysis [27] whereas chemical hydrolysis is considered more effective for the urinary detection of catecholamines [28].
2.2.3. Preconcentration/dilution

An additional parameter which intrinsically affects the evaluation of the urinary metabolome is the broad range of concentrations in which the potential biomarkers are present (Figure 1). Some urinary metabolites are present in the μg/mL range. These major metabolites can be detected by both targeted and untargeted strategies. The main problem associated with their quantification deals with the poor linear behavior exhibited at high concentrations by LC-MS/MS methods. This fact is easily overcome by dilution of the sample with dilution factors up to 100 fold being common [20]. Quantitative results obtained for major components in low-diluted samples should be carefully interpreted.

On the other side, urine contains uncountable metabolites at the low ng/mL or even lower range. Despite their low concentrations, monitoring them might be critical in order to evaluate metabolic pathways. Most of these metabolites are under-considered in untargeted approaches due to instrumental sensitivity limitations. The development of adequate targeted LC-MS/MS approaches can improve their detectability. A preconcentration step is commonly used for this purpose.

Several strategies can be used to preconcentrate the analytes. Among them solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are the most widely used [29]. Thanks to the large volume available, a 10-50 fold preconcentration is normally employed. The application of these sample preparation steps increases the overall sensitivity thanks to the concentration of the analyte but also to the clean-up of the sample which eliminates interfering compounds and favors analyte ionization [30].

2.2.4. Derivatization

One of the well-known advantages of LC-MS/MS procedures is the absence of a derivatization step as an analytical pre-requisite. However, derivatization can provide substantial benefits to metabolomic strategies. The most evident is the possibility to improve
the quantification limits of targeted metabolites. Besides, specific derivatization reactions can improve key steps of the procedure such as the clean-up, the extraction and the chromatography. Derivatization is also a convenient resource in order to circumvent the lack of ionization in electrospray ionization (ESI) of some chemical features such as carbonyl or hydroxyl groups [31]. Thus, although the direct quantification of some metabolites of the tricarboxylic acid cycle has been reported [32], several aspects such as the chromatography, the sensitivity of the method and the potential separation of isomers clearly improve after derivatization [33].

Remarkably, derivatization also provides new insights to untargeted metabolomics. The most important one is the development of stable-isotope labeling studies [34]. As an example, the use of dansylation allowed for labeling untargeted metabolites containing primary amine, secondary amine, or phenolic hydroxyl group(s). This strategy improves both sensitivity and LC behavior of selected sub-metabolome. Additionally, the derivatization with a labeled dansyl chloride improved also the quantitative parameters obtained in untargeted metabolomics approaches [35]. Several derivatization reagents have been developed to apply this strategy to different branches of the metabolome. Thus, 5-diethylamino-naphthalene-1-sulfonyl chloride has been described as alternative to dansyl derivatization [36]. Additionally, p-dimethylaminophenacyl bromide and omega-bromoacetonylquinolinium bromide have been described for the determination of carboxylic acids and thiols respectively [37, 38].

### 2.3. Instrumental analysis

#### 2.3.1. LC considerations

Contrarily to other chromatographic systems, LC has a broad portfolio of stationary phases, column dimensions and mobile phase additives which can be used to found optimal chromatographic conditions. Among them, the use of C18 stationary phases is the default
choice for untargeted metabolomics approaches. However, these columns are unable to properly resolve polar compounds. For this reason, the use of hydrophilic interaction liquid chromatography (HILIC) is becoming a popular complement in untargeted approaches. These columns are capable of retaining polar/ionic metabolites which, combined with the results on C18 columns, allow for the determination of the largest number of metabolites in one sample [39].

Despite the comprehensive coverage obtained by the combination of both chromatographic methods, specific chromatographic conditions are still required for the proper determination of some metabolites. As an example, proper reversed-phase chromatography of urinary bis-sulfates require a large amount of salts in the mobile phase [40].

In summary, targeted approaches allow for the selection of optimal chromatographic conditions for the selected metabolites whereas the use of general conditions in untargeted studies might hamper the proper determination of some parts of the urinary metabolome.

2.3.2. Ionization

Although LC-MS/MS is generally considered as a universal technique, it has its main bottleneck in the ionization process. Indeed, only those molecules which are ionized in the interface can be detectable by LC-MS/MS. Several atmospheric pressure interfaces (API) have been developed to cover the ionization of the widest range of chemical structures. Among them, ESI is by far the most popular one for metabolomic investigations. Nevertheless, some studies used other ionization sources such as atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) [41]. In general, these interfaces are considered complementary to ESI as some analytes are efficiently ionized only by one of them. Therefore, the selection of the interface in untargeted methods will limit the part of the metabolome detected. A combination of the three interfaces has been
reported as the most comprehensive strategy for the detection of biomarkers in untargeted metabolomics [42].

Similar to other analytical characteristics, the pre-selection of the metabolites in targeted methods will also guide the selection of the most adequate interface. Thus, ESI is preferred in the detection of phase II metabolites [43] whereas the use of field free-APPI has been reported to increase the sensitivity for some steroids [44].

2.3.3. Analyzers

From a metabolomics point of view, LC-MS analyzers can be divided between low resolution and high resolution analyzers. High resolution analyzers are preferred for untargeted approaches due to the structural information of the potential biomarkers provided by the molecular formula calculated based on the accurate mass measurements. Hence, most of the untargeted approaches use either time-of-flight (TOF) or Orbitrap analyzers [45]. On the other hand, structural information is less critical for targeted approaches since comparison of retention times and ions abundances with reference allow unequivocal identification. Thus, sensitivity became the most important parameter for targeted studies. For this reason, selected reaction monitoring (SRM) approaches using triple quadrupole analyzers are preferred for targeted methods. The use of this approach also enhances specificity since the selection of low abundant but specific transitions can help in discerning between analytes and endogenous isobaric interferences [46].

2.4 Data generation and quantification

2.4.1. Internal standard

Quantitative analysis in LC-MS (mainly when using ESI as interface) is prone to ion suppression/enhancement due to effects of the matrix components on the ionization process [47]. This matrix effect can substantially affect the quantitative performance of LC-ESI-MS
based methods. Whereas sample treatment is the most appropriate way to minimize this effect [30], the most suitable way for compensating it is the addition of appropriate internal standards [48]. For obvious reasons, the selection of internal standards in untargeted approaches is difficult since the actual nature of the potential marker is unknown at the moment of the analysis. In contrast, the previous knowledge of the potential markers in targeted metabolomic approaches facilitates the selection of the internal standards. In fact, the use of isotopically labeled internal standards is a common strategy for targeted quantitative methods as significantly enhances the quantitation parameters.

2.4.2. Validation

Method validation is the analytical tool required to confirm the suitability of an approach to quantitatively determine some analytes in a specific matrix. There are several guidelines dealing with the validation of quantitative methods in biological fluids [49, 50]. All of them are based on spiking blank samples with known amounts of the analytes. This fact makes the validation of untargeted approaches virtually impossible. Regarding targeted methods, a full analytical validation is required before application to metabolomics. The main limitation is the common absence of blank samples (i.e. samples free of all analytes) to perform validation. Analysts might face this limitation by employing different approaches such as (i) the use of artificial matrices [51], (ii) the use isotopic labeled analogues as analytes [52], or (iii) the use of standard addition strategies [53]. Every approach has their own limitations but in general all of them are suitable for method validation. In summary, targeted metabolomics benefit from the fact that accuracy and precision of the results are known.

2.4.3. Normalization

In urinary analysis normalization of the results is one of the biggest challenges regardless of the analytical approach. Besides many factors such as gender, age, ethnicity, diet, and
exercise level, variance in the volume excreted is the most significant issue. This variation, as high as 15-fold, makes difficult to compare concentrations expressed as mass per unit of volume (e.g. g/L) [54]. As a consequence, it is of paramount importance to normalize the results of urinary metabolomic studies.

In untargeted approaches, normalization based on the sum of the areas of all detected peaks is common [55]. In targeted approaches, flow rate correction, electrical conductivity and osmolarity have been proposed, but the use of specific gravity or the creatinine normalization are the most commonly employed [56].

Specific gravity correction, measured by refractometry, has been recognized as an adequate way of normalizing. Most commonly, the correction is applied post-acquisition by applying the following formula to the urinary concentrations:

\[
C_{1.020} = (1.020 - 1) \cdot C_{\text{sample}} / (SG_{\text{sample}} - 1)
\]

Where \( C \) stands for concentration and \( SG_{\text{sample}} \) is the specific gravity of the sample. Moreover, some authors have proved that a pre-acquisition normalization (through dilution of urine samples to the lowest specific gravity measured) leads to even better results [57]. Nonetheless, the simple creatinine correction remains as the most used correction methodology. It is based on the relatively constant amounts of creatinine excreted [58]. However, this approach is not ideal since creatinine excretion varies according to renal problems, muscle mass, physical activity, sleep deprivation, diet, and pregnancy [56].

Obviously, none of these methods will overcome the circadian variation that affects some of the metabolites. Thus, the normalization by the volume excreted in a 24-hours period is the best option (see section 2.1.2).
2.4.4. Concentration and ratios

Usually, the final product of a metabolomic study is a big matrix of data containing normalized peak-areas or normalized concentration values for each metabolite in all samples. In untargeted approaches the data is mainly based on peak-areas (since no absolute quantitation is available). In targeted metabolomics reliable concentrations are obtained. But, this might not be the last step before jumping to conclusions on differences between groups of individuals. Another way of looking at metabolomics data is by realizing that the ratios between some metabolites concentrations are the information-carrying features.

Instead of looking at that matrix of data holistically, it is possible to have a more oriented look by using the knowledge on the human metabolic pathways. By doing so, is possible to specifically calculate the precursor/product ratios of urinary metabolites concentrations. These ratios allow for estimating the activity of particular enzymatic processes. Precursor/product ratios have proved to be the best markers for the diagnosis of many conditions [59-61].

Since targeted metabolic studies are devised based on a driven-hypothesis about specific biological pathways, it is reasonable to think that figures based on concentration ratios will be the best way to detect imbalances in known metabolic routes.
3. Families

3.1. Amino acids and carnitines

3.1.1 Definitions

Chemically, amino acids are molecules that have a carboxyl group or acidic character, along with an amino group. This definition encompasses a large number of molecules, not all of which are naturally occurring. Amino acids play a critical role in human biochemistry, being present in virtually all metabolic and cellular functions. In addition of being the primary building blocks of proteins and acting as neurotransmitters and as a source of energy, they are involved in the regulation of gene expression, cell metabolism and signaling. Amino acids are essential in lipid transports, and are implicated in several metabolic defects [62].

L-Carnitine (3-hydroxy-4-N,N,N-trimethylaminobutyrate) plays an important role transporting long chain fatty acids from the cytosol into mitochondria for β-oxidation. It has both an exogenous (dietary, mainly from meat and dairy) and endogenous (synthesized from L-lysine and L-methionine) origin. It can be found either in its free form or esterified with fatty acids forming acylcarnitines, which have multiple biological actions [63]. Depending on the length of the acyl chain, the structure of acylcarnitines can be similar to amino acids (when the chain is short) or similar to lipids (in the case of long chain acylcarnitines). In this review we have included representative examples of both of these families. However, this subheading is mainly focused on the 21 proteinogenic amino acids, which are the building blocks of proteins (Figure 2).

Amino acid analysis is necessary for the diagnosis of a variety of inborn errors of metabolism [64]. More broadly, amino acid analysis is part of metabolomic research studies of both in vivo and in vitro model systems [65]. Owed to their importance and thanks to their relatively high concentration (ranging from of 0.2–200.0 μM) they always appear in untargeted metabolomic studies.
Due to the fact that an early detection of some aminoacidurias may have serious implications in the patient survival, their determination in biological fluids was faced decades ago. Historically, methodologies have employed cation-exchange chromatography followed by post-column derivatization and UV detection [66].

Over the years their quantification has evolved, and nowadays LC-MS/MS is recognized as the technique of choice for both serum and urine analyses [67].

3.1.2. Sample collection and preservation

According to the National Metabolic Biochemistry Network guidelines, urine samples should be collected into preservative free bottles [68]. However, some authors employ a more effective bacteriostatic transport medium by adding boric acid to the urine samples upon collection [69]. The use of merthiolate or thymol as a preservative it is also recommended if the sample cannot be frozen intermediately after collection [68].

For clinical purposes, random urines are generally accepted, since the urinary concentrations are corrected by creatinine. However, 24-hours urine collection is more adequate since as many other metabolites, amino acids levels can fluctuate in response to daily activity, particularly feeding [15] (see section 2.4.3).

Regarding sample conservation, clinical laboratories require that urine samples must be shipped frozen. Depending on the laboratory the stability of the specimen is set between 7 to 30 days. According to a recent study, the amino acids more affected by temperature storage are; arginine, valine, leucine and isoleucine. They concentrations decreased by 40% when stored at 9ºC for 24 hours, whereas serine and methionine concentrations were reduced up to 60% when stored at 20 ºC [70].

It is essential that specimen quality is checked by testing for nitrite and pH. If a specimen shows signs of deterioration, some amino acids may be falsely low. Specimen deterioration may cause decreased serine, increased or decreased alanine, increased glycine,
decarboxylation of glutamic acid to form γ-aminobutyric acid, and hydrolysis of peptides causing increased proline [68]. Finally, some drugs (e.g. valproate, vigabatrin) and dietary products (e.g. heat treated milk products) may cause apparent amino acid abnormalities [68].

3.1.3 *Sample treatment*

Typically sample preparation prior to analysis does not include any extraction. Amino acids can be quantified by LC-MS/MS with or without derivatization. Obviously, if amino acids are just a part of the metabolic pool investigated, the underivatized protocol must be selected. The derivatization of samples prior to LC-MS/MS improves the sensitivity which may be important for some amino acids excreted in low amounts. However, for the majority of amino acids the derivatization can be avoided [67]. In these cases, the sample preparation is basically the addition of appropriate internal standards and the dilution of the sample.

For those protocols including a derivatization the reaction takes some extra time. For instance, the protocol employing the AccQ•Tag derivatization agent takes 10 minutes [71]. Other types of derivatization reactions, such as butylation are associated with decreased reproducibility.

3.1.4 *Instrumental analysis, data generation and validation*

Leaving aside the separation of stereoisomeric D- an L forms of amino acids (only L-forms are used by cells), analysts must carefully select the chromatographic conditions in order to guarantee adequate retention, and proper separation of structural pairs of isomers such as leucine and isoleucine.

Several types of stationary phases can be employed for achieving appropriate separation, including structural isomers. A method without derivatization employing a C18 column and
using perfluoroheptanoic acid and trifluoroacetic acid as mobile phase modifiers was validated by Qu et al. [72]. However, some ion-pairing agents can generate problems. For instance, tridecfluoroheptanoic acid effectively removes interfering compounds and reduces ion suppression, but the associated solvent conditions result in reduced sensitivity for amino acids such as glycine, due to poor ionization in the detector [73].

The separation of isomeric amino acids in their underivatized forms can be accomplished by selecting proper chromatographic conditions. In that sense, some commercially solutions for the chromatography allowing the separation of all isomers are already in the market [74]. Some examples of these isomers including both proteinogenic and non-proteinogenic amino acids are: 1-methylhistidine and 3-methylhistidine; α-aminobutyric acid, β-aminobutyric acid, β-aminoisobutyric acid, and γ-aminobutyric acid (GABA); alanine, sarcosine and β-alanine; o-methylserine, allo-threonine, and homoserine; norvaline, and valine; leucine, alloisoleucine, and isoleucine [74]. Figure 3 illustrates the separation of pairs leucine/isoleucine and glutamic acid/glutamine after derivatization with AQC reagent [69]. In the case of the pair leucine/isoleucine, the baseline separation can be achieved either by the selection of specific transitions or by derivatization. Regarding the interference of glutamine into the glutamic acid transition, it can be avoided by the selection of amide column or by derivatization.

In a similar way, a good chromatographic separation is also needed to separate isobaric acylcarnitines with same nominal mass but different molecular structure [75] and the accurate measurement is essential to avoid false positives in the diagnostic of inborn errors of metabolism [76]. As an example, Minkler et al. developed a 14-minutes chromatographic separation of 4 different C5 acylcarnitines and included it to a method that measures 66 acylcarnitines after derivatization with pentafluorophenacyltrifluoromethanesulfonate [76, 77].

In cases where some constitutional isomers and diasteriomeric acylcarnitines exist, the
diagnostic of some inborn errors of metabolism lies on the appropriate chromatographic separation of the compounds [78].

The correction by mg of creatinine is generally accepted as the method for normalizing the results. The quantification is conducted by using the area ratios of the analytes to its corresponding labeled internal standard.

Regarding validation, most of the LC-MS/MS methods employed in investigations and in routine clinical laboratories have been validated by evaluating the robustness, accuracy and precision of all amino acids [69].

3.1.5. Applications

Amino acid analysis is necessary for the diagnosis of a variety of inborn errors of metabolism. These include: phenylketonuria, tyrosinemia, citrullinemia, maple syrup urine disease, and homocystinuria. The assay is also key for the continued monitoring of treatment plans for these disorders and useful for assessing nutritional status of patients [64].

General elevations in urine amino acid levels, i.e. aminoaciduria, can be seen in disorders with amino acid transport defects such as lysinuric protein intolerance and Hartnup disease, as well as in conditions with renal tubular dysfunction [64]. Although analysis of amino acids is commonly based on serum analysis, the analysis of urinary levels is also relevant. For instance, diagnosis of disorders of amino acid transport and other renal tubulopathies requires analysis of amino acids in urine [68].

Moreover, quantification of amino acids in urine may aid in evaluation of endocrine disorders, liver, muscle, and neoplastic diseases, neurological disorders, nutritional disturbances, renal failure, and burns [79-81].

In a similar way to proteinogenic amino acids, the measurement of acylcarnitines by LC-MS/MS is applied in newborn screening to detect and to confirm inborn errors of metabolism
and metabolic diseases (mainly disorders of fatty acid oxidation and organic acid metabolism) [63, 78] although recent studies are also evaluating their impact on drug-induced hypocarnitinemia [82], insulin resistance [75] or neurological diseases [63].

3.2. Biogenic amines/Monoamine neurotransmitters

3.2.1. Definition

Monoamine neurotransmitters are a group of molecules containing an aromatic ring connected to one amino group by a two-carbon chain (Figure 4 A). They include catecholamines (dopamine, noradrenaline and adrenaline), tryptamines (serotonin) and histamine. Monoamine neurotransmitters are synthesized in our body by decarboxylation of aromatic aminoacids (tyrosine, tryptophan and histidine) and they have a critical biological role.

The accurate measurement of catecholamines in urine has been a major challenge due to their instability, low excretion, high polarity and poor ionizability [83]. Historically, the analytical techniques employed to measure catecholamines have been HPLC coupled to electrochemical or fluorescence detection. However, more recently the new advances have placed LC-MS/MS in a central position due to its higher sensitivity and selectivity.

3.2.2. Sample collection and preservation

Some of these compounds (like noradrenaline or adrenaline) are known to follow circadian rhythms. Therefore, the conventional strategy for sample collection follows 24-hours urinary excretion. Interestingly, it has been recently shown that the second morning urine sampling could be employed as an alternative to the 24-hours urine collection due to the good correlation existing between both measurements [83].
Catecholamines are labile compounds that can be easily oxidized into the corresponding quinones. Some studies have shown that oxidation of these compounds in biological samples takes place if they are kept at 37ºC for 24-48 hours [84]. In order to overcome the oxidation in urine it is recommended to preserve the samples by acidification (as the degradation is higher in alkaline pH than in acidic media [85]) or by adding preservatives (i.e. EDTA and sodium metabisulfite), as this has shown to increase their stability and the reproducibility of results [84]. In the case of acidification, the addition of concentrated inorganic acids (e.g. HCl or H$_2$SO$_4$) can lead to the deconjugation of sulfated metabolites generating misleading results. Recent findings have shown that the use of monobasic citric acid allows the acidification of the urine sample without leading to deconjugation of sulfated metabolites [85].

**3.2.3. Sample treatment**

Most reports of urinary catecholamines analyze the levels of free forms [86-88] although hydrolytic treatments have also been described [89]. The strategies employed for purifying the sample in order to avoid matrix effects include mainly solid-phase extractions [83, 86-88, 90, 91] but liquid–liquid extractions and have also been developed [89, 92]. Recently, Konieczna et al. employed dispersive liquid-liquid microextraction (using ethanol as disperser solvent and dichloromethane as extraction solvent) to analyze catecholamines, serotonin, and their metabolites in human urine [92]. Some authors use diphenylboronic acid derivatives to generate boronate complexes prior to the extraction step [83, 89-91].

**3.2.4. Instrumental analysis, data generation and quantification**

The polarity and hydrophilicity of catecholamines confers them a poor retention on conventional reverse-phase columns. For this reason, other stationary phases including HILIC, pentafluorophenyl propyl, polar C18 and porous graphitic carbon have been applied
to separate and retain appropriately both catecholamines and metanephrines (O-methylated metabolites of adrenaline and noradrenaline) [88, 92]. The chromatographic separation of these compounds plays an important role, as some of them (e.g. adrenaline and normetanephrine) are isobaric and the accurate quantification could be affected if they are not properly separated.

Catecholamines can be monitored in both positive and negative mode due to the presence of both hydroxyl and amino groups, but they are generally monitored in positive ESI mode [83, 86-90], as it offers more sensitivity [88].

Nowadays, the availability of labeled compounds for the analysis of catecholamines enables to have appropriate internal standards for their accurate quantification.

3.2.5. Applications

The measurement of the urinary levels of catecholamines and methanephrines has been commonly used for the diagnosis of neuroendocrine tumors (especially for pheochromocytome but also for others like parangagliome or neuroblastome). In this regard, some methods for the detection of urinary catecholamines are being used in routine clinical analyses [88, 89]. Besides this classical practical application, the measurement of catecholamines could be applied to other conditions such as depression, anxiety, and ADHD, as well as the regulation of blood pressure or the levels of glucose.

3.3. Lipids

3.3.1. Definition

Lipids are hydrophobic or amphiphilic molecules that are insoluble in water and soluble in organic solvents. They comprise a great variety of molecular structures that differ between them, resulting in a challenge for their simultaneous analysis (Figure 4 B). The study of their
changes is a branch of metabolomics, although the diversity and large number of lipids has driven some authors to consider lipidomics as an independent *omics* itself. In this subheading, we will focus on lipids with a special emphasis on targeted lipid analysis (as opposed to untargeted lipidomics) considering it as subclass of targeted metabolomics. The analysis of steroids, which in a strict sense are lipids, is reviewed separately in subheading 3.4.

Traditional lipid analyses were performed by NMR or thin layer chromatography (TLC), which are limited by their lack of sensitivity and accuracy [93]. Gas chromatography-mass spectrometry (GC-MS) has also been used in lipid analyses but its main drawback is that only volatile compounds (with or without derivatization) can be detected. Recent advances of soft ionization techniques, mainly ESI and to a minor extent matrix-assisted laser desorption/ionization (MALDI), have represented a revolution in the lipid analysis. These techniques allow the identification and quantitation of a larger number of lipids with a higher sensitivity.

Although lipids are more abundant in biological samples other than urine (e.g. plasma, brain, liver) untargeted lipidomic analyses have estimated that the total urinary 24-hours lipid excretion is around 1 µM and have confirmed the presence of hundreds of lipids in urine. Furthermore, according to these studies, total lipid urinary excretion presents a sexual dimorphism (the excretion being higher in women) but it does not follow a circadian rhythm [94].

3.3.2. Sample collection and preservation

The sample collection of urine for lipid analyses does not have any special requirements, other than using sterile cups and, in some cases, centrifuging the samples and transferring the supernatants to appropriate tubes before storing them at -20°C or -80°C.
3.3.3. Sample treatment

The isolation of lipids from biological matrices has traditionally been done by using liquid-liquid extraction procedures based on chloroform/methanol/water mixtures that were developed in the 1950's [95, 96]. Nowadays, these extractions are still used although other organic solvents like methyl-tert-butyl ether are also employed [97]. A recent report evaluating six different liquid-liquid extraction procedures of lipids from urine evidenced that the best recovery depends on the particular class of lipids analyzed [98]. For this reason, the extraction efficiency is generally evaluated using different organic solvents to find the most appropriate for the specific analytes [99, 100].

Alternatively, SPE has also been used in urinary lipidomic analyses [101, 102]. In some cases, samples have been analyzed by simple dilution of the sample [103], although when targeted lipids are present at low concentrations it is common to optimize the extraction procedure by means of SPE. Anyhow, the efficiency and reproducibility of the extraction procedure should be carefully evaluated during the validation of the method. This is the case of most targeted approaches, but in many untargeted approaches is not examined, compromising the appropriate quantification.

Hydrolytic treatments are not commonly used in lipid analysis, with the exception of total fatty acid estimations as well as in the analysis of oxidized lipids which have been described to be excreted conjugated with glucuronic acid [104].

3.3.4. Instrumental analysis, data generation and quantification

One of the strategies employed in lipid analysis does not use a chromatographic separation but a direct infusion (generally of the reconstituted chloroform extracts) into the mass spectrometer. In this approach, known as shotgun lipidomics, the identification and quantification is based on precursor-ion scans and/or neutral loss scans of specific fragments [105]. Shotgun lipidomics has the main advantage of providing a high-throughput
vision of the lipid composition in a biological sample. Despite this, it presents some
drawbacks like (i) ion suppression (that affects sensitivity and quantification), (ii) the proper
quantitation of isobaric compounds (e.g. stereoisomers, structural isomers) [106], and (iii)
the measurement of low abundant analytes. For this reason, the gold standard for the
quantification of lipids that are present at low concentrations and/or often occur in
isobaric/isomeric forms (e.g. eicosanoids) is based on LC-MS/MS approaches using
selected ion monitoring or multiple reaction monitoring, as they provide the highest
sensitivity.

Depending on the nature of the lipids and the required separation, several chromatographic
modes can be used, including reverse phase, normal phase (generally used for non-polar lipids), or HILIC [107]. The type of targeted lipids also guides the mode of ionization (positive or negative). For instance, leukotrienes [108] and prostaglandins [109] can measured using negative ionization mode due to the presence of a carboxylic group, while underivatized acylcarnitines can be measured using positive ionization mode due to the presence of the charged amino group [110].

The quantification of lipids in biological samples represents a challenge as relative intensities of different lipids do not reflect their abundance. Indeed, the instrument response varies between lipid families (e.g. it increases with unsaturation), within families (e.g. it decreases as the acyl chain length is higher) and even within the same lipid species (e.g. it decreases when the lipid concentration is higher) [111]. This fact evidences the importance of using appropriate (ideally isotopically labeled) internal standards for all the analytes, a fact that is even more challenging and often disregarded in untargeted analyses. In the case fatty acids, some authors have used as an internal standard compounds with odd number of carbons instead of their corresponding isotopically analogues. This is because compounds with even number of carbons are common in nature whereas those with odd number of carbons are rare.
Over the last decade, some commercially available kits allow the measurement of multiple lipids (in some cases hundreds) of several categories (e.g. fatty acyls, glycerophospholipids, sphingolipids) by LC-MS. It should be noted that these kits, although appropriate as a first screening or for a high-throughput analysis, provide only semi-quantitative information as they generally have only a few internal standards (generally one corresponding to each family). As an example, in the case of lipid biomarkers measured by direct injection-MS/MS using commercially available kits, the unique structure of each lipid is not known as each lipid identified by these kits could correspond to 5-10 different chemical structures [2]. Typically, this is the case of isobaric/isomeric lipids like phosphatidylcholines, sphingolipids and lysophosphatidylcholines [112]. This fact is of importance since sometimes the results obtained by these kits are inappropriately considered quantitative.

3.3.5. Applications

The interest in lipid analysis has increased exponentially during the last decade due to the convergence of three factors: (1) the already mentioned advances in soft ionization techniques and chromatography, (2) the discovery that lipids can act as signaling molecules, and (3) the fact that the metabolism of lipids is dysregulated in multiple diseases.

Recent applications of lipidomics include the identification of biomarkers in some pathologies (e.g. metabolic syndrome, neurological disorders, cancer, eye diseases), nutrition (to evaluate dietary-induced changes), drug discovery and personalized medicine [113]. Some recent examples of targeted urinary lipid applications are summarized in Table 2. In the work of Gorden et al. the authors analyzed a large number of lipids belonging to different families (including eicosanoids, neutral lipids and sphingolipids) by MRM, with the aim of identifying new biomarkers of non-alcoholic fatty liver disease. To do so, each family of compounds was pretreated accordingly (using SPE, o liquid-liquid extractions) and with the use of internal standards [101].
Despite recent advances, urinary lipidomic analysis is still in its infancy, and one of the major current limitations is the limited knowledge of normal ranges so that normal variations (e.g. those due to nutrition or physical activity) can be differentiated from those due to a pathological condition. In this regard, some studies evaluating and estimating normal values are of relevance [94, 103, 109].

3.4. Steroids

3.4.1. Definition

Steroid compounds are a group of molecules with a cyclopentano-perhydrophenanthrene skeleton. They include a large variety of compounds such as steroid hormones (corticosteroids, progestogens, androgens and estrogens), sterols or bile acids (Figure 5 A). Although, strictly they are considered as lipids, some of their special characteristics (low concentration, abundant conjugation) hampered their detection by common lipidomic approaches. For this reason, steroids are commonly determined by targeted specific methods and, therefore, they are treated in an individual section in this review. In fact, some authors have described the word “steroidome” referring to the detection of the steroid part of the metabolome [114, 115].

Since the discovery of the steroid hormone structures at the beginning of the XX century, the accurate measurement of steroids in human fluids has been linked to several application fields like disease diagnosis, prognosis, or doping control. From the 1960’s analysis of steroids is routinely performed by GC-MS after performing laborious sample preparations including hydrolysis and derivatization steps. Although this strategy is still used in most of the steroid analysis laboratories [116], the introduction of LC-MS/MS in the late 1980’s provided new insights in the steroid determination. Among them, the detection of steroids with unaffordable derivatization [117] or the direct detection of steroid conjugates [51, 118, 119] are the most remarkable ones.
3.4.2. Sample collection and preservation

Some of the steroid hormones suffer from a substantial circadian rhythm being cortisol a clear example for this behaviour. Thus, urinary concentrations of unconjugated cortisol might vary up to ten times depending on the collection time (Figure 5 B). This fact makes the interpretation of steroid concentrations in spot urine samples difficult. The collection of 24-hours urine samples is preferred in order to compensate for circadian variations. If that is not possible, spot urine samples must be collected at the same time in order to provide comparable results. Interestingly, the excretion of the main hepatic steroid metabolites is less affected by circadian variations [120] (Figure 5 B). For this reason, the detection of a large number of urinary metabolites might help to establish an impairment in the steroid synthesis since a simultaneous decrease of all steroid metabolites cannot be explained only by circadian variations. Thus, a decrease in cortisol synthesis was confirmed in acute intermittent porphyria patients by the decrease on the urinary concentrations of 20 cortisol metabolites [121].

Steroids urinary concentrations use to be stable over the time under common storage conditions (-20ºC, -80ºC). However, under other conditions (e.g. at 4ºC or room temperature transport) urinary concentrations for some steroids can vary by microbial degradation. As stated above, urine is the ideal medium for microorganisms’ growth. It has been reported that some of these microorganisms can alter the urinary steroidome. That is critical in some application fields in which the concentration of minor metabolites needs to be quantified e.g. doping control. The occurrence of abundant androstanelone in urine samples is indicative of microbial degradation and the presence of this marker at high concentrations would question the results of the complete steroidome [122, 123]. A chemical stabilization mixture has been developed in order to minimize the effect of this degradation [124].
Regarding phase II metabolites, most of them are stable under common storage conditions. However, some sulfates have been reported to be unstable in urine [125], and the spontaneous release of cysteine conjugates has also been reported [126].

### 3.4.3 Sample treatment

Due to the low polarity of steroids, they are commonly excreted as phase II metabolites. Thus, steroid hormones are mainly excreted as glucuronides and sulfates [127] although other conjugates such as cysteine [126] or N-acetylglucosamines [128] have been also reported. In the case of bile acids they are commonly excreted as glycine or taurine conjugates [129, 130]. Due to this conjugation, two main analytical options are available: (i) detection of the unconjugated steroid after the release of the conjugate or (ii) direct determination of the steroid conjugates.

The classical approach for steroid determination implies the release of the conjugate. For this purpose, enzymatic preparations containing β-glucuronidase and/or sulfatase are used [118]. Although results use to be reproducible and reliable, these enzymatic preparations are associated to well-defined problems such as the transformation of some urinary steroids by specific enzymes [131, 132] or the inability of the enzymes to hydrolyze some of the conjugates [133, 134]. After releasing the conjugate(s), unconjugated steroids might be extracted from urine by common extraction procedures like LLE or SPE. This step is also useful to preconcentrate the sample facilitating the detection of minor metabolites. Additionally, the clean extract obtained after LLE facilitates the derivatization step. In this sense, several derivatizing agents have been described to improve the sensitivity on LC-MS/MS determination of steroids [135-137].

The second approach implies the direct detection of phase II metabolites. The high polarity of these compounds represents one of the most characteristics limitations in their extraction. However, the apolar characteristics of the steroid part of the phase II metabolite helps in the
extraction of the conjugate from urine. Thus, SPE is preferred for this purpose [51, 138] although LLE extraction with ethyl acetate has been reported for the extraction of steroid sulfates [118]. Some extraction methods based on ionic exchange columns have also been described for the specific and fractionated extraction of some phase II metabolites including glucuronides, sulfates and/or bissulfates [139, 140].

3.4.4. Instrumental analysis, data generation and quantification

Due to their low polarity, unconjugated steroids and metabolites show a proper chromatographic behavior when using common C18 columns [141]. The main limitation is the need to separate several isobaric steroids. Although this separation is feasible with common chromatographic set-ups, it usually involves longer chromatographic runs. Thus, gradients up to 30 min are usual for the LC-MS/MS determination of the urinary steroidome [142]. More problematic is the chromatography for phase II metabolites. Thus, chromatographic peak shapes for sulfates use to suffer peak tailing in the absence of organic modifiers. This situation is even more critical when resolving bis-sulfates [40]. The addition of relatively high amount (10-50 mM) of ammonium into the mobile phase is the most straightforward approach to minimize this effect although the presence of this amount of salts usually implies a reduction on the method sensitivity.

Regarding ionization, several steroids lack an easily protonable moiety. This structure is therefore guiding the suitability of ESI to determine unconjugated steroids [143]. Although the use of adducts can help in the determination of some of these compounds [143, 144], ESI still has substantial limitations in the ionization of fully reduced metabolites. For this reason, despite the forward leap in steroid analysis provided by the introduction of LC-MS/MS, the detection of the complete phase I steroidome remains a challenge and the complementary use of GC-MS(/MS) is still required. Regarding phase II metabolism, it usually implies the conjugation of the phase I metabolites with polar or ionic groups. These groups are easily ionized by ESI either in negative mode (glucuronides and sulfates) or in
positive mode (some glucuronides, cysteiny1, glycine and taurine conjugates). Therefore, in contrast with the stated for unconjugated steroids, LC-ESI-MS/MS should be considered the platform of choice for the direct detection of steroid conjugates irrespective of their structure. Several methods have been published using either triple quadrupole [51, 138, 145] or high resolution [146-148] analyzers.

Similar to other groups of analytes, the availability of labeled compounds for steroid hormones enables to have appropriate internal standards for their accurate quantification. However, there is a lack of labeled compounds for steroid urinary metabolites. Thus, analytical methods dealing with the LC-MS/MS determination of urinary steroid metabolites commonly use few available internal standards to quantify the whole steroidome. That can make that some compounds might be slightly over- or under-quantified [142]. This situation is even more pronounced when dealing with the direct determination of phase II metabolites since only few phase II metabolites are commercially available as standards. Thus, most of the methods dealing with their detection only quantify a limited number of analytes. Several research groups are working on finding optimized strategies to synthesize these standards [149, 150]. It is expected that once they are available, the comprehensive quantification of the phase II steroidome will be feasible.

3.4.5. Applications

Steroids are involved in the control of a large variety of biological processes from the homeostatic regulation of blood pressure to the control of sexual characteristics. Thus, it is not surprising that they have been reported as potential markers for a large variety of status/diseases. Recent examples of clinical applications of the determination of urinary steroids are summarized in Table 3.

On the one hand, some steroid metabolites have arisen as potential biomarkers after the application of untargeted metabolomics approaches. Thus, some bile acids have been
reported as potential biomarkers for prostate cancer, chronic kidney disease or dioxin contamination. Progestogens and metabolites have been associated with status as different as kidney disease or idiopathic Parkinson’s disease.

On the other hand, variations on the urinary steroidome has been observed by targeted quantitative mass spectrometric methods in several diseases related with the steroidogenesis such as 21-hydroxylase deficiency, polycystic ovary syndrome or adrenocortical carcinoma. Additionally, alterations in the urinary steroidome has been also observed in non-related diseases such as acute intermittent porphyria or ovarian granulosa cell tumors. Finally, variations on the urinary steroidome have been reported in different status such as pubertal or night shift workers.

4. Conclusions and Future perspective

In the present review, we have described the most relevant issues to be taken into account when performing targeted metabolomic analyses of human urine by LC-MS/MS. The importance of each one of these aspects, from sample collection to data quantitation, has been explained in detail for five families of metabolites. The study of this five families leads to the conclusion that each step of the process may be critical depending on the type of targeted metabolites, and therefore only fully validated methodologies are appropriate for quantifying the urinary metabolome.

Regarding the near future, LC-MS/MS will remain the favored analytical technique to evaluate the metabolome in any biological specimen, and in particular in urine. Alone, or in combination with other types of instrumentation it will play a pivotal role in this field [2, 4, 151-153].

The inherent limitations of untargeted metabolomics will persist. Thus, it is likely that the need of reliable data will lead the scientific community towards targeted approaches. Untargeted strategies will be employed in the quest for unknown metabolites, and for investigations looking for a general view of the major metabolites in a biological system.
Researchers aiming at the finest metabolomic results, a two-steps protocol appears as the most promising option. In the first step samples will be analyzed by an untargeted approach, which will point out at the part of the metabolome of interest in the investigated system. In the second step, samples will be re-analyzed by validated targeted methods developed to accurately quantitate those metabolic families.

It is expected that the portfolio of commercial labeled internal standards, and commercial kits for metabolomic profiling will grow, meaning that targeted methods will become more comprehensive by adding more metabolites to already existing methods. Moreover, increased throughput in terms of automated MS assays will be common place. This fact will surely make LC–MS/MS more accessible for laboratories that do not have the expertise to develop methods.

This will surely make possible that by running the samples with a few LC–MS/MS methods, a wide coverage of the human urinary metabolome will be obtained. However, the goal of computing the complete urinary metabolome will be far from being accomplished due to its extreme complexity.

The current lack of reproducibility when comparing data from different laboratories [154] will be corrected in the near future, by the use of fully validated methodologies. Full advantage of metabolomics can only be accomplished by comparing the data obtained with normal ranges in healthy populations. Thus, it is expected, that the same validated methodologies will be employed to conduct studies aiming at the generation of reliable normal ranges data, and to investigate the effect of physiological or environmental factors (e.g. age, sex, ethnicity, diet, and physical activity).

Regarding instrumentation, it is likely that a new generation of more affordable new high resolution mass spectrometers will gain presence in the laboratories. As a consequence, in addition to the three precious qualities that define LC-MS/MS (sensitivity, selectivity and dynamic range) the advent of HRMS analyzers will allow to measure accurate masses while keeping the current triple quadrupole sensitivity. Furthermore, other technologies such as
ion-mobility mass spectrometry might contribute to the targeted metabolomic field [155, 156], but it would be interesting to see the extent of this new analytical platforms.

As a consequence, it is anticipated that targeted methods by LC-MS/MS will be increasingly used for the study of the urinary metabolome. The derived results will have incidence in many fields from personalized medicine, to clinical diagnosis, to doping control analyses. More importantly, they might help researchers by providing new hints on the biological interactions, which in turn might lead to the discovery of new biological mechanisms.

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