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Review

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Recommendations and Standardization of Biomarker Quantification Using NMR-based Metabolomics with Particular Focus on Urinary Analysis

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Abstract

NMR-based metabolomics has shown considerable promise in disease diagnosis and biomarker discovery because it allows one to non-destructively identify and quantify large numbers of novel metabolite biomarkers in both biofluids and tissues. Indeed, precise metabolite quantification is a necessary prerequisite to move any chemical biomarker or biomarker panel from the lab into the clinic. Among the many biofluids (urine, serum, plasma, cerebrospinal fluid and saliva) commonly used for disease diagnosis and prognosis, urine has several advantages. It is abundant, sterile, easily obtained, needs little sample preparation and does not require any invasive medical procedures for collection. Furthermore, urine captures and concentrates many "unwanted" or "undesirable" compounds throughout the body, thereby providing a rich source of potentially useful disease biomarkers. However, the incredible variation in urine chemical concentrations due to effects such as gender, age, diet, life style, health conditions, and physical activity make the analysis of urine and the identification of useful urinary biomarkers by NMR quite challenging. In this review, we discuss a number of the most significant issues regarding NMR-based urinary metabolomics with a specific emphasis on metabolite quantification for disease biomarker applications. We also propose a number of data collection and instrumental recommendations regarding NMR pulse sequences, acceptable acquisition parameter ranges, relaxation effects on quantitation, proper handling of instrumental differences, as well as recommendations regarding sample preparation and biomarker assessment.

Keywords: NMR, urine, metabolomics, biomarker, disease, metabolites, quantitative analysis, recommendations, standardization, quantification

1. Introduction

Metabolomics (also known as metabonomics) is the study of global metabolite profiles in biological samples such as bio-fluids, cell extracts and tissues. Metabolomics can be integrated with other omics sciences such as genomics, transcriptomics, and proteomics to facilitate a more complete understanding of global biological systems. Metabolite concentrations and perturbations represent a snapshot of the metabolic dynamic that reflect the response of living systems to environmental factors, pathophysiological stimuli and/or genetic modification. In order to characterize the vast array of metabolites found in any given biosample, metabolomics researchers must utilize a wide range analytical platforms including high performance liquid chromatography (HPLC) [1], liquid chromatography with mass spectrometry (LC-MS) [2], gas chromatography with mass spectrometry (GC-MS) [2a, 2b, 3], tandem mass spectrometry [4], and NMR spectroscopy [5]. Each technique has its own advantages and disadvantages, and the choice of a given analytical platform often depends on the focus of the study and the samples [6].

The use of NMR in metabolomics is particularly appealing to many researchers because of its non-destructive, quantitative nature and its ability to identify novel compounds via their unique spectral patterns [7]. In NMR-based metabolomics biological fluids such as serum, plasma, urine, saliva [8], cerebrospinal fluid [9], amniotic fluid [10], synovial fluid [11], exhaled breath condensate [12], cell extracts [13] and tissue extracts [7g, 14] have all proven to be particularly amenable to NMR analysis. Urine, in particular, is a very appealing biofluid for analysis because it is abundant, can be collected non-invasively, and is particularly rich in terms of its chemical diversity. Consequently, urine offers significant opportunities for data mining, data modelling and biomarker discovery, particularly with respect to human health and disease [15]. Furthermore, urine exhibits a strong phenotypic or metabotypic stability [16], which strengthens its potential for biomedical research and clinical utility.

Urine is composed primarily of small hydrophilic molecules such as sugars, organic acids, amino acids, soluble lipids, organic amines, etc., along with inorganic salts that are small enough to

have successfully passed through the body's reticuloendothelial filtration system. Whilst the non-invasive collection of urine is advantageous for many metabolomics applications, the major spectroscopic challenge associated with analyzing urine by NMR is the tremendous variation in its chemical concentrations. There are a number of factors, including sample collection and processing, as well as data acquisition and processing parameters, that need to be considered to enable accurate and precise quantitation of urinary metabolites by NMR-based metabolomics. Metabolic profiling of urine gives a time-averaged representation of an individual's recent (typically within 24 hrs) homeostatic condition. Some metabolites may associate with individual's physiological or pathological state, whereas others may associate with an individual's genotype, environmental exposures, dietary habits, or drug intake, [17] as well as the time (season, hour of the day) of collection. Indeed one of the most significant unresolved issues in urinary metabolomics lies in the remarkable variance in urinary excretion volumes and subsequent variations in metabolite concentrations [18]. As such, it is critical that in quantitative metabolomics the inter- and intra-individual metabolite variance within the normal/control group be properly identified, defined [18] and as much as possible minimized. This may be facilitated by requiring a 12 hour fast prior to urine collection, or restricting the consumption of supplemental protein performance enhancing food, such as protein shakes, prior to any sample collection [19]. Even with these controls in place, the variance in urine metabolite concentrations is still quite significant.

In light of these challenges, a coherent standard protocol is particularly important for the analysis of urinary biomarkers. To ensure robust and accurate quantification of potential urinary biomarkers by NMR, each step of the analytical protocol must be carefully performed and evaluated. This includes appropriate consideration for the context of the chosen application. For example, in nutritional intervention studies, it is important that a standardized diet is introduced to participants prior to the intervention and subsequent sample collection in order to minimize dietary effects confounding the results. Specifically, sample collection, preservation, preparation as well as instrumental optimization, NMR pulse sequence selection, choice of acquisition parameters, data

processing parameters, peak/metabolite identification confidence, and final reporting results must be undertaken with a high level of consistency and an appropriate degree of scientific rigor to ensure the validity of the results. This process is not entirely straightforward and much research has been devoted to refining, testing and optimizing each step of the NMR analytical and data processing protocols [7d, 20]. Indeed, more than 70 papers have been published on the subject since 1990 with one of the most widely cited of these is the publication by Beckonert *et al.* [20b]. While advocating the need for standardized protocols in metabolomic applications (*i.e.* the Metabolomics Standards Initiative) [21], these authors also noted that it may be "detrimental to the exploratory nature of the subject to allow only 'validated' or 'approved' procedures to be used in experimental metabolism studies." This acknowledges the importance of further research and improved methodologies for sample handling, data acquisition and data analysis.

In a previous review [22] we proposed several recommendations regarding the standardization of the experimental conditions for using urine in NMR-based metabolomic studies. We highlighted the effects of diet, sample collection time (of day), age, gender, gut microflora, individual metabotypes, physical activity, subject selection, sample storage, salt and pH effects as well as acquisition temperature with regard to urine metabolite composition and concentrations. We also provided recommendations regarding ethical guidelines for sample acquisition, the establishment of written SOPs, the selection of containers/consumables, patient/sample selection protocols, sample collection handling methods (centrifugation, additives, storage protocols), sample transfer methods, sample pH, chemical shift referencing, minimum sample numbers, sample randomization, magnetic field strength, optimal NMR pulse sequences, acquisition temperature and results or reporting standards.

In this review, we will discuss the pertinent issues regarding NMR-based urinary metabolomics with a specific emphasis on quantification for disease biomarker applications. We first review and discuss some of the key issues relating to: 1) biomarker assessment; 2) urinary biomarkers and the need for metabolite quantification, 3) metabolite quantification methods for

NMR; 4) examples of NMR-derived biomarkers and 5) concentration normalization methods. Consensus recommendations will then be made regarding: concentration normalization, suitable NMR pulse sequences, acceptable parameter ranges, the effects of relaxation on quantitation, and the utility of data acquired on different instruments

2. Biomarkers and Biomarker Assessment

A biomarker is a measurable substance in a biofluid or biological tissue that can be used as an indicator of some biological perturbation caused by a disease, a change in biological state, or an environmental exposure. Biomarkers may be used for disease diagnosis, prognosis, prediction or monitoring as well as for measuring biological responses from various drug, toxin or environmental exposures. Biomarkers have a wide range and may include chemicals, metabolites, genes/mutations, RNA transcripts, proteins, cell counts or cell types, karyotypes or just about any other detectable substance or measurable biological feature. As a general rule, a single biomarker often corresponds to a single medical test, with a threshold value (concentration or number) being used to distinguish between healthy and diseased states.

2.1. Biomarker Sensitivity and Specificity

The performance of a biomarker is typically evaluated by its sensitivity and specificity. Sensitivity relates to the biomarker's ability to identify positive results and specificity relates to the biomarker's ability to identify negative results. More specifically, sensitivity can be defined as "*the proportion of patients who are known to have the disease who test positive for it*" while specificity can be defined as "*the proportion of patients that are known not to have the disease who will test negative.*" The mathematical definition of sensitivity and specificity is given below:

$$\text{Sensitivity} = \frac{number\ of\ true\ positives}{number\ of\ true\ positives + number\ of\ false\ negatives} = \frac{number\ of\ true\ positives}{number\ of\ positives} \quad (1)$$

$$\text{Specificity} = \frac{number\ of\ true\ negatives}{number\ of\ true\ negatives + number\ of\ false\ positives} = \frac{number\ of\ true\ negatives}{number\ of\ negatives} \quad (2)$$

In common medical practice, sensitivity is generally not sufficient to assess the diagnostic performance of a test where the test has no negative predictions (from a theoretical point of view, 100% sensitivity). Therefore, both sensitivity and specificity should be examined together and reported in disease diagnostic studies. Figure 1 demonstrates the assessment of biomarker performance, in the context of a biomarker's ability to differentiate between diseased and healthy subjects [23]. Table 1 summarizes the relationship between positive and negative test outcomes and of true/false positives and negatives.

Simultaneous measurement of both sensitivity and specificity with respect to different separation threshold values is often best illustrated using a receiver operating characteristic (ROC) curve. A ROC curve shows how the sensitivity and specificity change as the classification decision boundary is varied across the range of available biomarker scores. A ROC curve is not dependent on the prevalence of a given outcome, and because it shows the performance of a biomarker test over the complete range of possible decision boundaries, it allows the optimal specificity and/or sensitivity to be determined *post-hoc*. ROC curves are often summarized into a single metric known as the "area under the curve" (AUC). For a perfect biomarker test, the AUC is 1.0. An AUC of 0.5 is equivalent to randomly classifying subjects (*i.e.* the classifier is of no practical utility). A rough guide for assessing the utility of a biomarker based on its AUC is as follows: 0.9-1.0 = excellent;

0.8-0.9 = good to very good; 0.7-0.8 = fair; 0.6-0.7 = poor; 0.5-0.6 = fail. ROC curve analysis and using ROC-AUC is widely considered to be the most objective and statistically valid method for biomarker performance evaluation. A much more detailed review of ROC analysis along with general recommendations for biomarker quantification and statistical strategies for multi-biomarker models is provided elsewhere [23].

3. Biomarkers and Metabolite Quantification

Metabolites and metabolite concentrations can be particularly sensitive to modest physiological or subtle genetic perturbations. Observed changes to urine have historically been accredited to attempts to diagnose ailments as far back as the ancient Greek physician Hippocrates (400 BC) and the Arabian/Persian alchemist Avicenna (11th century). Indeed, as Sir Archibald Garrod (the founder of modern clinical chemistry) noted in 1908, changes in metabolite concentrations often start before the onset of clinical symptoms [24]. This simple fact has served as the basis to the development of more than 180 different chemical or metabolite biomarker tests that are commonly used today (https://labtestsonline.org). Indeed, there are more approved metabolite or chemical-based clinical tests than approved genetic, protein or karyotype tests (https://labtestsonline.org). While most clinical tests are blood-based, an increasing number of clinical assays that use urine currently exist. The very first clinical assay (and the first genetic disease test) was designed for detecting homogentisic acid among individuals with a rare inborn error of metabolism called alkaptonuria [24]. Among the most commonly used metabolite tests today are urinary glucose tests for monitoring diabetes [25], 24 hour urinary creatinine tests to measure kidney function [26], urinary cortisol tests to diagnose Cushing's or Addison's disease, urinary nitrite tests to detect bacterial infections and urinary bilirubin tests to assess liver function (Table 2).

Almost all approved clinical chemistry tests, whether they measure chemical or protein biomarkers, are required to be quantitative or at least semi-quantitative (via some ratio measurement). This requirement has allowed universal reference values for different ages and

genders to be developed and applied for routine medical diagnoses. In many cases physicians and clinical chemists are required to know and/or memorize threshold values for a large number of medically important metabolites and proteins. Absolute quantification not only gives physicians useful reference or decision-making thresholds, but also ensures consistency and reproducibility from instrument to instrument, lab to lab, city to city and country to country. As stated above, the issue of quantification has turned out to be an Achilles heel for many biomarker tests developed using "omics" platforms [15b, 27]. This is because proteomic, transcriptomic and even most metabolomic assays were not originally designed with comparative quantitation in mind, nor were they designed to be compared accurately and precisely between different instrumental platforms.

However, there is now a growing shift in the "omics" community towards developing quantitative proteomic and quantitative metabolomic assays [5g, 23, 28] with a long-term goal of using these quantitative assays for biomarker discovery, development and clinical translation [23]. The need for quantification, either relative or absolute, is particularly acute for metabolite-based urine analysis where individual metabolite concentration values can vary by a factor of 10 or more due to dilution, gender, diet or diurnal effects [29]. As a result there have been a number of papers, NMR-specific software tools (Chenomx, AMIX, Batman [30], Bayesil [31], *etc.*) as well as a number of commercial kits that have been developed (such as the BioCrates kit for mass spectrometers) to perform absolute metabolite quantification. Methods to facilitate urinary metabolite quantification, with a specific focus on NMR, are briefly reviewed in the next section.

4. Quantitation of Metabolites by NMR

NMR spectroscopy is a powerful analytical approach for both identification and quantification of analytes with superior advantages, such as being non-destructive, highly reproducible and, most importantly, requiring minimal sample preparation [32]. Indeed, NMR is inherently a quantitative technique as the intensity of an NMR signal is proportional to the concentration of detectable (usually ^{1}H) nuclei in the receiver coil [33]. The 1D ^{1}H NMR spectra of urine samples are highly

complex, with thousands of distinct signals visible in a single spectrum. Consequently, signal overlap and signal distortions from nearby (strong) peaks are often evident even in 1D ^{1}H NMR spectra collected at 800 MHz and above. Over the past two decades, several methods had been proposed or developed in order to address these issues. In particular, an exhaustive technical review on this subject has recently been published by P. Giraudeau [34]. This paper nicely summarizes the strengths and weaknesses of various two-dimensional NMR methods eg. 2D-Jresolved, COSY, TOCSY, 2D INEPT, HSQC, HMBC etc. with regard to resolution in the second dimension and with respect to compound identification and quantification in complex mixtures. Common shortcomings identified for all (conventional) 2D NMR methods were the lack of sensitivity, the lack of speed and the difficulties in reproducible quantitation compared to 1D NMR. However, this paper also highlighted several novel 2D NMR methods or data processing techniques that appear to solve or at least address some of these issues. For instance, 2D ^{1}H INADEQUATE with sparse sampling/non-linear sampling (NLS) as proposed by Hybert *et. al.* [35] seems to be very promising for characterizing and quantifying low abundance metabolites. More recently, another approach has been proposed [36] wherein three different collection and processing techniques were combined, including J-compensated 2D HSQC, NLS and forward entropy (FM) reconstruction. This combination resulted in a 22-fold reduction in NMR recording time (relative to a conventional HSQC spectrum), while at the same time yielding precise metabolite quantitation in both native and lyophilized urine samples. The authors report a lower limit of detection of “tens of micromolar”. However, these types of experiments are not straight-forward and significant prior knowledge about the sample and expected spectral windows is needed to properly implement the method. Furthermore, while these approaches are promising, the, have yet to be implemented in a real, non-targeted metabolomic study nor have they been comprehensively evaluated and validated. Ultrafast 2D NMR spectroscopy [37] is yet another promising method. However, this approach requires sophistication in pulse field gradient performance and specific processing software, presently not available on most commercially available NMR spectrometers. Nevertheless, given its speed,

resolution and sensitivity advantages over fast heteronuclear NMR and even 1D homonuclear NMR methods, this approach may soon become the method of choice for identifying and quantifying low abundance metabolites in urine samples.

Unlike MS-based quantitative methods, that usually require expensive isotopically labelled standards and time-consuming chromatographic separations, NMR spectroscopy offers the possibility of quantifying many metabolites simultaneously without the need for any prior chromatographic separation. However, accurately and reproducibly quantifying NMR signals from different instruments at different field strengths and using different pulse sequences or pulse widths and delay times is often challenging. Indeed, despite a steady increase in the number of publications arising from NMR-based metabolomics focused on diagnostic biomarkers, only a few of these report quantified metabolite levels [23, 29a, 38]. This paucity of quantitative data could be due to the fact that metabolite quantification is both instrumentally challenging and manually intensive work.

In terms of instrumental issues, the greatest challenge in NMR-based quantification lies in consistency and reproducibility. To obtain consistent and reliable results, identical NMR tubes must be used as well as identical instrument parameters including temperature, chemical shift referencing, tuning, shimming, magnetic field drift compensation/lock, pulse sequences, water suppression methods, acquisition times, and data processing parameters (Table 3). These conditions must be strictly adhered to from sample to sample. This not only allows for rigorous quality assurance, it also allows robust intra-laboratory and inter-laboratory comparison. Variations in the ionic strength of different samples can have dramatic effects on the ability to tune and match the spectrometer (assuming the probe range is sufficient) leading to profoundly different pulse widths and/or spectrometer responses. Specifically, what are assumed to be infinitely short "on-resonance" pulses become ineffective (much less than 90°) and contain larger and larger off-resonance pulse effects (evolution). This can have dramatic consequences regarding relaxation of the NMR signal in-between data acquisitions. In addition, some instrumental effects (*e.g.* field instability, temperature variations, incorrect referencing) can cause chemical shift changes.

Recently, Sokolenko *et al.,* evaluated the sources of quantification variability in NMR and determined that such seemingly mundane issues as sample insertion methods and/or shimming protocols as well as the choice of NMR pulse sequence could lead to significant differences in the resulting metabolite identification and quantification [39]. It has also been shown that the data acquisition parameters, spectral processing parameters and the choice of water suppression method can also substantially affect metabolite quantification results [40].

As a general rule, a single excitation pulse sequence with a relatively low-power water pre-saturation is the default method for performing quantitative metabolite analysis of biofluids and biological extracts. Historically, quantitation in NMR required the use of long pulse delays to allow signals to fully recover, but more recently the use of a low flip angles (*i.e.* << 90°) to accommodate faster signal recovery along with suitable signal correction methods has allowed much more rapid data acquisition [41]. For most biological samples, there is a substantial amount of water present, so strong solvent suppression must be employed to obtain good metabolite signals. Therefore a one-dimensional NOESY pulse sequence with either water pre-saturation (see [42] and references therein), excitation sculpting [43] or WATERGATE solvent suppression [44] is preferred. Due to solute/solvent hydrogen exchange, the pre-saturation sequence itself can dramatically alter quantitation results. Specifically, the exchangeable proton signals from urea and other water exchangeable solutes are often suppressed by pre-saturation making their quantification difficult or inconsistent. While more complex solvent suppression schemes like PURGE [45], excitation sculpting, or variations of WATERGATE [46] are potentially better choices for metabolite quantification, these pulse sequences all have auxiliary regions of signal suppression which makes them more difficult to optimize and harder to use. This added complexity can also affect inter-lab and intra-lab consistency or reproducibility. Pulsed field gradient (PFG) versions of NMR pulse sequences (*e.g.* one-dimensional NOESY pulse sequence) are available, but these may lead to problems with quantification due to both lock instability and gradient ring down periods [47]. These problems manifest as peak distortions (i.e. semi-dispersive line shape) to the internal chemical shift and

quantitation reference (such as 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS)). For these reasons gradient pulse sequences, despite their advantages [48], **are not recommended**.

In terms of spectral analysis, metabolite quantification requires meticulous consistency and considerable attention to detail. Prior to spectral collection, each biofluid sample must be "spiked" with an exact amount of a known quantification standard (usually a chemical shift reference standard such as tetramethylsilane (TMS) for organic solvents or trimethylsilyl propanoic acid (TSP) and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) for water based experiments) [49]. Once the NMR spectra have been collected, the data must be properly phased, baseline corrected and any residual interfering solvent signals digitally filtered. Then individual "sentinel" peaks corresponding to the specific compounds must be identified and the peak area(s) carefully integrated [50]. This identification and quantification step (also called spectral deconvolution) can be done manually, semi-automatically or automatically (vide infra). The concentration of a given metabolite can be directly calculated from the spiked standard using the following formula [51]:

$$\frac{[\mathrm{M}]}{[\mathrm{Std}]} = \frac{\mathrm{Im}}{Is} \cdot \frac{Nm}{Ns} \qquad (3)$$

Where [M]; is the metabolite molar concentration, [Std] the spiked standard's known molar concentration, "I_m" the metabolite intensity, "I_s" the intensity of the spiked standard's peak, "N_m" the number of nuclei contributing to the metabolite peak and "N_s" the number of nuclei contributing to the spiked standard's peak. In some cases it is not necessary to use an internal standard to quantify metabolites but rather one may use an external reference or an electronic reference signal (59, 60), or even the solvent resonances [52].

A key challenge with quantifying metabolites in biological samples, and especially in urine, is the considerable degree of spectral overlap seen in 1D ^{1}H NMR spectra. For example, urine NMR spectra typically consist of >2000 detectable peaks corresponding to >200 detectable

compounds [53]. The positions and shapes of these peaks can vary with pH, temperature, salt concentrations, magnetic field strength, sample stability and homogeneity. In addition, common ^{1}H-^{1}H dipolar or quadrupolar (*e.g.* ^{14}N-^{1}H) couplings further complicate the observations. Consequently, it is very difficult for even a trained individual to identify a single set of "sentinel" peaks that can be unambiguously identified and properly integrated for any given urine sample. To assist with this peak identification and metabolite identification process it is possible to use several approaches. One approach is spectral simplification through statistical analysis or chemometric analysis using spectral binning/alignment and statistical total correlation spectroscopy- STOCSY [54]. These methods statistically align and compare the NMR spectra between two groups (diseased and healthy) and the most significantly different peaks are then identified. This leads to a reduction in the number of peaks that need to be analyzed or identified. A disadvantage of this approach is that it can lead to problems in compound identification and quantification, as the spectra have been extensively averaged or "warped" as part of the statistical processing [54].

Another approach involves identifying and quantifying as many compounds as possible prior to determining any statistical differences between groups. This leaves the NMR spectra in a relatively pristine state so that compound identification and quantification is easier and more accurate. However, the process is time consuming as dozens of compounds and hundreds of peaks must be identified and quantified through a process known as spectral deconvolution [54a]. Several companies have developed spectral deconvolution tools and software (*e.g.* Chenomx NMRSuite – from Chenomx Inc.; AMIX – from Bruker Inc.; MnovaScreen from Mestralab Research; CRAFT or Complete Reduction to Amplitude Frequency Table from Agilent) that makes this process easier. These packages include carefully collected reference NMR spectra of hundreds of common metabolites at different field strengths and pH values. These programs and their corresponding libraries can allow 50-75 compounds to be manually identified and/or quantified from urine spectra in about 1-4 hours. However, this process must be performed by well-trained individuals. The requirements of manual phasing, manual baseline correction, manual solvent removal and manual

spectral deconvolution often mean that quantification inconsistencies and even compound identification errors can be introduced. For example, one published study looked into these issues of consistency and reproducibility [39] and found compound quantification by some of these methods is only accurate to within 15%. More recently several groups have attempted to develop fully automated methods that remove any human variability in spectral processing and compound identification [30b, 55]. These methods appear to be very promising, especially for simpler biofluids such as serum, saliva or cerebrospinal fluid, but they have yet to be shown to be effective for biofluids as complex as urine.

4.1. Sample-to-Sample Normalization

The large differences in metabolite concentrations observed in different urine samples can complicate comparisons between samples and may lead to false conclusions. Thus, normalizing NMR spectra is a critical step for eliminating systematic errors. In addition to normalizing concentrations relative to creatinine (which is commonly done in clinical practice) several other normalization methods have been developed, including total integral intensity (usually the total spectral area). This is one of the most commonly used methods in NMR [56]. Other methods have been proposed as an alternative to the total intensity method, including probabilistic quotient normalization (PQN) [57] and histogram matching (HM) [58], that effectively make the data more suitable to identifying potential metabolic biomarkers for disease.

Normalization can be problematic due to the potential influence that the method might have on compound identification and/or quantification. While one would hope that compound identification is unaffected (see below), the relative proportions certainly might. This assumes that normalization would occur only on the quantitation of unambiguous peaks (*i.e.* no overlap) and would apply to all such positively identified metabolites. This might seem obvious, but it needs to be explicitly stated as normalization can also refer to frequency dependent compensation for NMR pulse sequence effects, *e.g.* T_1 dependent changes. Any changes to the NMR pulse sequence or

parameters will perturb peak heights. Any compensation for pulse sequence differences would also affect the peak ratios for atoms in the same molecule, as well as the overall metabolite quantitation. For example, a compensation for different pre-saturation power levels would correct amplitudes differently close to the carrier position versus the edges of the spectra. A compound containing resonances both close to and far from the water resonance would then have an intramolecular integration ratio that would not make sense and could have its identification changed or moved to an ambiguous/unassigned grouping.

This is not a problem unique to NMR. Mass spectrometry (MS) has been dealing with this issue for years. In MS, control samples are introduced into the sample queue to compensate for changes in the chromatographic steps and equipment necessary to separate metabolites. Specifically, quality control samples are submitted at regular intervals, and/or samples are combined and run as reference samples for comparison. Interestingly, in NMR the use of control or calibration samples is not regularly done. While chromatographic columns are not normally used in NMR, batched samples run on robotic sample handling systems must often sit for lengthy periods of time (hours) prior to loading and spectral acquisition. Control samples placed at the beginning, middle, and end of a long sample-loading run would therefore reveal any potential degradation/storage/handling problems or (less likely) changes occurring to the spectrometer/console/probe [59]. Compared to the extensive efforts used in quality control for MS, the use of quality control for normalization in NMR is, in our opinion, still under-developed.

5. Examples of Potential NMR-Derived Urinary Biomarkers

There is an abundance of literature linking urinary biomarkers with human disease states [7e, 15b, 60]. The analytical approaches used to collect these data are certainly diverse and this diversity serves to demonstrate the complexity associated with developing standardized approaches for urinary biomarker quantification. Recently Bouatra *et al.* [15a] collated and critically evaluated the existing information on human urine to establish a comprehensive and electronically accessible human urine

metabolome database (http://www.urinemetabolome.ca). This database includes quantitative concentrations of metabolites in normal and abnormal (*i.e.* disease-associated) urine samples and represents a significant development and resource for biomarker identification and quantification. This database also serves as a benchmarking and cross-referencing tool for future metabolomics approaches and will no doubt aid in efforts aimed at standardizing metabolomic approaches. While it is impractical to cover all the biomarker examples described in the human urine metabolome database, it is perhaps useful to highlight some of the studies where NMR-based metabolomics was used to identify novel and/or potentially important biomarkers in urine. These are summarized in Table 2 and explained in more details below.

As can be seen in this table, urinary biomarkers have been identified for a wide range of conditions, ranging from cancer to neonatal conditions to pancreatic disorders. For example, Lusczek and colleagues identified a useful set of potentially discriminating metabolites in the urine of pancreatitis patients compared to healthy controls [61]. Although the metabolites identified in this study cannot be conclusively defined as biomarkers of the disease, they do have the potential to become biomarkers once additional studies are carried out to validate their findings, ideally using a larger patient cohort. In similar study aimed at exploring other kinds of pancreatic disorders, Davis *et al.,* used NMR based metabolomics investigation on urine samples from age and gender matched patients with pancreatic ductal adenocarcinoma, compared to a healthy control group (82). These authors were able to easily differentiate between those with cancer and those in the control group (using both ROC curves and area under the curve [AUROC] calculations) using the set of strongly diagnostic metabolites listed in Table 2. As with the previous study, further validation using a larger cohort is needed to confirm the result. In another cancer-based study, urinary ^{1}H NMR spectra of bladder cancer patients versus non-cancer controls (healthy and those with urinary tract infections or bladder stones) was used to discriminate between the two groups, with taurine showing significant elevation in the urine of bladder cancer patients [62]. However, the authors of this study were not able to discriminate between different disease stages possibly due to cancer-specific

metabolic alterations or the low sensitivity of their particular instrument [62]. Urinary biomarkers were also used to successfully and accurately diagnose a cohort West African patients with hepatocellular carcinoma [63]. In this particular study the authors not only calculated ROC and AUROC values, they actually validated their findings using second larger cohort, a very good practice indeed. The metabolite biomarker panel identified in this study was also shown to perform much better than serum alpha-fetoprotein, a protein biomarker that is traditionally used for hepatocellular carcinoma diagnosis [63].

Another interesting set of studies highlighted in Table 2 concerns the application of NMR-based urinary metabolomics to neonatal diagnoses. One very interesting NMR-based study used urine samples from neonates to compare those with intrauterine growth retardation versus full-term normal-weight controls to better define the metabolic patterns associated with this pathology. The authors identified myo-inositol, sarcosine, creatine and creatinine as key metabolites that clearly differentiated between the two groups [64]. While the initial findings are promising, the results will need to be further validated on a larger cohort. A more recent study on influence of early nutritional metabolic programming and long-term health in infants was carried out by Moltu *et al.,* using NMR-based urinary metabolomics. In this intervention study, one group received significantly higher amounts of enhanced postnatal nutrition compared to the control group. The authors concluded that the enhanced nutrition did not appear to affect the urinary profiles to an extent exceeding the individual variation [65]. This particular study is a good example of a well-conducted nutritional intervention study.

While the list of examples highlighted here (and in Table 2) is not exhaustive, it does highlight some of the common issues and challenges with respect to designing, implementing and validating NMR-based urinary metabolomic biomarker studies. Based on these examples as well as an extensive review of the literature and a detailed assessment of the best practices conducted in our own laboratories and elsewhere, we have developed a set of consensus recommendations. These are summarized below.

6. Recommendations for Sample Collection and Processing:

The identification of new biomarkers, along with their validation and translation into practical clinical applications requires standardized pre-analytical procedures for sample handling, sample stabilization, sample transport and sample storage. Depending on the procedures employed, the detectable metabolites may be affected differentially by residual enzymatic activities and/or spontaneous chemical reactions that may alter the NMR profile [66]. These alterations could seriously bias the results of studies based on samples having different collection, treatment and storage histories. Other factors that may affect the concentration levels of urine metabolites include drug administration, health conditions, diet, physical activities and environmental stressors. Thus, it is crucial for any quantitative urinary analysis to standardize sample collection conditions. Our recommendations are as follows:

Overnight fasting: Overnight fasting prior to urine collection gives a more stable homeostatic picture of an individual's urinary metabolome. Consequently we recommend that all urine samples should be collected the morning after overnight fasting to reduce the effects of diurnal variations [67].

- ***Mid-stream urine:*** Collecting mid-stream urine is recommended to avoid contamination from epithelial cells and bacteria from the urinary tract. Sampling conditions should be similar in control groups including age and gender matched groups for comparative analysis.
- ***Medical procedures:*** Medical procedures (including drug intake) performed before sample collection should be recorded and properly taken into account as medical treatments could induce significant changes in metabolites levels. All medical procedures prior to sample collection should be reported.
- ***Aliquoting, centrifugation and filtering prior to storage:*** Urine samples should be processed and aliquoted within 2 hrs from the time of collection, but preferably faster. Samples must be kept refrigerated at 4 °C before processing and must not be frozen prior to

processing in order to avoid possible cell breakage [20d]. Before aliquoting and long term storage, urine samples should be centrifuged at 1,000–3,000 RCF (5 min at 4 °C) and (optionally) filtered using a 0.22 μm filter [20d] to remove cells and other particulates.

- ***Storage:*** For long-term storage, urine samples should be stored at -80 °C. If possible, for very long-term storage, it is better to use liquid nitrogen vapor [20d]. Appropriately labeled cryovials should be used to store urine samples.
- ***Preservatives:*** The addition of micromolar quantities of inorganic bacteriostatic agents such as sodium azide (to limit bacterial growth) is appropriate. The use of externally added organic preservatives (such as EDTA or glycerol) is strongly discouraged due to their possible interference with metabolite signals.
- ***Diet monitoring:*** Certain metabolites may be affected significantly by dietary intake. If possible, dietary information should be collected for 1-2 days before sampling. For example, taurine increases significantly after the consumption of taurine containing diet and energy drinks [68]. To reduce lifestyle-related variations, and to the extent experimental design allows, a standardized diet is recommended for donors at least one day before sample collection.
- ***Native samples:*** Performing urine metabolomics on native (unadulterated, unprocessed) samples provides more reproducible and precise data. Extraction procedures, along with multi-step sampling processes, increase the chance of metabolite loss. They also introduce analytical and operator errors [20d, 69].
- ***Buffering:*** An example basic protocol would be to mix 630 μL of human urine with 70 μL of phosphate buffer (prepared in ^2H_2O) to minimize the drift in chemical shift due to pH variations. A concentrated KH_2PO_4 solution (1.5 M, pH 7.0) is best. If the variation in pH between the samples is still significant after the addition of the phosphate buffer, sample pH may be adjusted by adding NaOH and/or HCl accordingly [15b].

- ***Final centrifugation:*** Centrifugation at 12,000×g for at least 10 min at 4 °C to remove any suspended particles is strongly encouraged [20d].
- ***Chemical shift referencing:*** A chemical shift referencing standard such as TSP or DSS should always be added prior to NMR collection. Because the peak is easily resolved and unique, chemical shift standards can also be used for metabolite quantification. Note that DSS and TSP do bind proteins and lipids, so if these macromolecules are present to any significant degree, they may give rise to errors in quantitation. A final TSP or DSS concentration ranging between 0.1-0.5 mM is sufficient for most urine samples.

7. Recommendations for Spectral Acquisition and Processing

In addition to the recommendations for spectral acquisition that have been proposed in our previous reference paper [22], there are a number of instrumental, acquisition and data processing parameters that can significantly affect quantitative accuracy and precision. These parameters need to be optimized prior to conducting quantitative analysis of urine samples. Here we provide recommendations and justifications related to these issues.

7.1. Acquisition of Urinary NMR Data.

- ***Selection of magnetic field:*** NMR-based metabolomics studies on urine benefit from the use of the highest accessible magnetic field strengths. Presently most NMR-based metabolomic studies are conducted using 600 MHz NMR spectrometers, as these instruments are relatively abundant and offer a good compromise between the cost, sensitivity and resolution needed for metabolomics experiments. If the purpose of the analysis is the quantification and/or the identification of low abundance metabolites, we recommend using more sophisticated two-dimensional experiments, higher fields (e.g. 800-1000 MHz) and/or increased sensitivity “cold-probes” or “cryoprobes”.

- ***Pulse sequence and general setup***: Automated pulse width calibration [70] is strongly recommended for any NMR-based metabolomic study of urine. This is because urine samples exhibit considerable variability in salt concentrations, which substantially affect pulse widths. Pulse width calibration also helps to compensate for differences in sample volume or drift/decay in NMR hardware performance. For example, if components of the spectrometer or probe begin to fail, then pulse width calibrations will immediately indicate a problem and prevent wasted time and resources. Most modern NMR spectrometers have the option of sample handling robots for sample insertion into the spectrometer. Most NMR instruments also support auto-pulse calibration, auto-shim and auto tune/match prior to data acquisition. If available, these automated approaches should be used for all urine-based metabolomics studies to reduce human error and ensure maximum consistency and reproducibility.
- ***Water suppression:*** As detailed previously, a one-dimensional NOESY pulse sequence with water pre-saturation, is the most commonly used NMR pulse sequence for metabolomics studies. While there are other excellent solvent suppression techniques such as excitation sculpting, or WATERGATE, the incorporation of pulsed field gradients can easily result in inconsistent spectrometer lock performance, the introduction of artifacts, and irreproducible solvent suppression. However, a pre-saturation pulse if done inconsistently can also dramatically alter quantitation results due to hydrogen exchange with the solvent during the long saturation period(s) [71]. We therefore strongly recommend the use of an absolutely consistent pre-saturation period (*e.g.* 1 second) with equally consistently delivered and calibrated power (*e.g.* 80 Hz gamma B_1 induced field). Of note, the exchangeable hydrogen signal from urea's NH2- groups and other water exchangeable signals can also be suppressed by time-shared multi-frequency saturation based sequences [72], but these also introduce additional regions of suppression that can alter quantitation. Therefore these types of sequences are not recommended for metabolomics.

- ***Sample temperature control:*** Variations in the sample temperature during spectral acquisition can significantly affect the precision and reproducibility of NMR data. A calibrated sample temperature of ~298 K (25°C) for urine is recommended during NMR spectral acquisition. If a robotic sample handling system is being used, where large numbers of samples are prepared in advance and stored at the instrument, the samples should be kept below room temperature (~5 °C) while waiting for sample insertion [29a, 73]. To ensure proper temperature equilibration, samples should be pre-warmed outside and/or in the probe for 5-10 min before spectral acquisition, especially if a cooling rack is used to maintain refrigerated samples. While samples will begin to show thermal equilibrium via lock monitoring in 60 to 120 sec, several minutes of equilibration are often necessary for convection currents to settle.
- ***NMR tubes:*** Higher quality NMR tubes yield higher quality spectra. Therefore, we recommend that the highest quality tubes with the lowest camber (straightest), thinnest, most consistent glass wall widths be used. Slightly curved tubes will wobble while spinning, which can lead to spinning side bands (extra peaks). Most NMR spectroscopists use 5 mm tubes, requiring volumes of 400-600 μL of material. If less material is available, the use of 3 mm or even 1.7 mm tubes is possible. Likewise, solvent-matched (Shigemi) tubes may also be used for low volume situations, however this option can increase the cost significantly. Prior to their use, NMR tubes should be cleaned to remove any film. Cleaned tubes tend to sheet refluxed material back down into the bottom of the tube thus reaching equilibrium faster. Dirty or stock tubes with the film remaining from manufacturing, will bead solvent further up the tube effectively shortening the sample length and changing the magnetic field homogeneity.

7.2. Recommendations for Optimized Instrumental Parameters:

- ***Time domain points (Bruker TD or Agilent np):*** Modern NMR spectrometers with high speed/high memory computers and digital oversampling can easily collect 64K or more data points. Considering the expected spectral windows and the desired resolution for NMR-based metabolomics work, time domain acquisitions ≥32K points are recommended for quantitative analysis of urine samples.
- ***Repetition time (T_R):*** This is also known as the relaxation delay or waiting period prior to the first hard excitation pulse. We define this period as the total time spent in acquiring a single scan spectrum, including the acquisition time, and acquisition delays prior to the next excitation/acquisition. This period strongly affects the absolute quantitation of metabolites. Ideally, T_R should be five times the longest T_1 in the sample. This typically provides enough time for complete relaxation of all resonances between every scan, resulting in good quantitation. In metabolomics studies, using a T_R that is five times the longest T_1 in a sample requiring 128 scans, would result in a sacrifice of enormous amounts of instrumental time that would dramatically increase *the* cost of the overall study. As a result a shorter repetition time (*e.g.* 2 - 4 sec) is often used for many NMR studies [69]. The key is that the total relaxation time used is consistent from experiment to experiment. For absolute quantitation of metabolites, a T_1 correction factor for spectra recorded over a shorter T_R time can be determined and applied to compensate for the effect of incomplete relaxation [74]. Application of this correction requires evaluation of the T_1 for all the resonances of interest on a representative sample prior to quantitation, assuming that the T_1 of the resonances would be the same in all samples for the study. Alternately, using reference spectra of compounds of known concentration that have been collected using the same TR and using these reference spectra to deconvolute the mixture (as done with Chenomx, AMIX, Batman, or Bayesil) would also allow one to accurately determine concentrations.
- ***Number of scans/Signal to noise ratio:*** The better the signal-to-noise (S/N) is for a NMR spectrum, the more reliable the absolute quantitation. The simplest way to increase (S/N) in

NMR is to increase the number of scans (also known as transients). However, adding more scans must be balanced by cost or time considerations. The S/N also depends on other factors like the magnetic field strength, stability of the spectrometer, probe type/quality, sample concentration, sample volume, excitation flip angle, *etc.* If time and resources permit, maximizing each of these factors (field strength, stability, probe quality, sample concentration, sample volume and flip angle) will yield significant gains in S/N without the need for additional acquisition time. Thirty-two to sixty-four scans are often considered enough for a normal urine sample on a 600 MHz (or greater field strength) instrument. Dilute urine will require more scans to achieve the desired S/N for quantitative NMR analysis. More scans (or higher fields) are also required to detect and quantify minor components (<10 uM) in urine and/or very dilute samples. Use of a cryogenically cooled probe can significantly (by a factor of 2-4X) increase the S/N for the sample.

- ***Receiver Gain (RG):*** NMR experiments can be run either on a constant receiver gain setting (optimized to a standard sample) or with an automatically optimized receiver gain for every sample. The former is preferred for pattern recognition analysis of urine NMR data; however it does not work on samples where concentration variation between samples is substantial. In these cases the auto receiver gain setting may be required for every sample prior to acquisition, or an efficiently low gain would be required.
- ***Shimming:*** Automatic shimming options in newer NMR spectrometers have made shimming much easier and faster for metabolomic analysis. NMR shims can be optimized by watching the line shape and width-at-half-height of the reference signal (such as DSS or TSP), or another common low molecular weight component. For DSS/TSP, observing the intensity and symmetry of the silicon (^{29}Si) satellite signal is also a good option to optimize the shimming process. The line width of chemical shift standards such as DSS or TSP should be shimmed to between 0.5-1.0 Hz. Adjustment of the field and lock position/phase during the shimming process can also improve the quality of the spectra.

- ***Tuning and matching:*** Probe tuning and matching (which is equivalent to tuning a radio) are essential to getting high quality NMR spectra. Many NMR instruments are tuned for salt-free solvents (chloroform or D_2O). However, buffered urine samples typically have high salt concentrations, this can lead to poor performance (low S/N, long pulses, poor solvent suppression, etc.) on high field NMR spectrometers that have not previously been tuned to accommodate high salt samples. These difficulties can become more evident when a user is acquiring data on a cryogenically cooled NMR probe. Ideally, manual probe tuning and matching for a given sample or solvent type (using a representative sample) should be performed prior to spectral acquisition for a large number of solvent-similar samples. Auto-tune capabilities and match accessories along with salt tolerant NMR flat sample tubes can help mitigate this problem.

7.3. Data Processing

Proper data processing can improve the S/N, resolution, visual appearance and the integration accuracy of 1D NMR spectra. Several simple data processing techniques may be employed, some of which are automatic while others require some manual effort and skill. Our recommendations are as follows:

- ***Windowing or apodization:*** A window function should be applied to the time domain data prior to Fourier transformation to improve the appearance of the spectra and emphasize either S/N or resolution (both are not possible). In a urine 1D spectrum, a free induction decay (FID) can be multiplied by an exponential window function. The artificially increased decay rate of an exponential windowing function (which minimizes later time points in the FID) will reduce the noise and broaden the resonance lines. On the other hand, a window function that enhances later time points in the FID will enhance resolution but at the expense of increasing spectral noise. Line broadening between 0.3-1.0 Hz is recommended for quantitative analysis of urine samples. The exact value will depend on the digital resolution

of the experiment. Line broadening equal to the digital resolution is generally recommended, as it provides no penalty in spectral separation but does improve general spectral appearance.

- ***Zero filling:*** FID data should be zero filled by a factor of 2 (*i.e.* twice the number of experimentally collected data points) before Fourier transformation to reduce noise and improve the visual appearance of the spectra. Zero filling does not increase the actual spectral resolution but functions to better interpolate between real data points in the original spectrum, thereby improving line-fitting and peak position determination. While often over emphasized, zero filling is still helpful for many NMR spectroscopists and does not require additional spectrometer time. Extending zero filling past a factor of two does little for the spectra, takes up more storage space, and slows software calculations.
- ***Phasing:*** Phasing is one of the most critical yet one of the more error prone steps in performing quantitative NMR. Errors in phasing can cause significant errors in absolute and relative peak area measurements [75]. Manual phasing is preferred over automatic phasing as there is a greater chance of distortion among lower intensity signals during automatic phase correction. Vertical expansion should be increased as much as possible during manual phasing to make the proper adjustments for smaller signals. Water peaks can be ignored during phasing and the water signal should be removed (through post-processing digital filtration) from the spectra to enable further qualitative and quantitative analysis. Experimental errors in the pulse sequence can often manifest as apparent "phase" distortions, but cannot be corrected with either frequency dependent or frequency independent corrections. Dispersive peak characteristics, especially on very intense peaks should be carefully examined and noted in case the NMR parameters/setup need to be altered.
- ***Base line correction:*** Proper base line correction are necessary for quantitative accuracy of peak integration and curve fitting methods applied to calculate peak areas. Most base line

corrections are performed semi-automatically through manual identification of base line regions followed by a computer-generated spline fit to the baseline regions. Several third-party software packages can also perform automated baseline correction [50].

- ***Chemical shift referencing:*** Every spectrum needs to be properly referenced to an internal chemical shift standard before qualitative/quantitative analysis. In many cases, since the precise concentration of the standard is known, the chemical shift standard can also be used to determine the concentration of other compounds by comparing the relative peak areas. Both trimethylsilyl propanoic acid (TSP) and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) can be used for chemical shift referencing in water (δ=0.0 ppm), although DSS is the IUPAC standard (80). While other peaks may be used in place of an internal standard if they are very insensitive to pH/ionic strength variations, this practice is definitely not recommended for most applications (water has been successfully used as a concentration reference) [52]. Chemical shift referencing to solvent peaks (i.e., water) should be avoided, since its peak position is sensitive to pH, salt and temperature effects.

Concluding Remarks

Despite the growing number of publications focused on diagnostic metabolite biomarkers appearing in the scientific literature, a surprisingly small number of these reports provide information on quantified metabolite levels, especially in urine. This situation indicates that metabolite identification and quantification in urine is still considered a challenging task. For metabolite biomarkers, accurate identification and quantification is essential for advancing biomarker discoveries to clinical practice. As noted throughout this review, NMR spectroscopy offers a robust route to the identification and quantification of metabolites. In this regard, we believe quantitative NMR-based metabolomics represents a superb (albeit under-used) platform for the discovery, development and translation of metabolite biomarkers to clinical practice. In this review, we focused on the use of urine as a biofluid for biomarker discovery and biomarker applications. In

particular, we explained how biomarkers should be evaluated or assessed. We also elaborated on several methods for metabolite identification, quantification and normalization by NMR and provided examples of a number of newly discovered urinary metabolite biomarkers. We further discussed a number of the most significant issues or challenges regarding the experimental aspects of quantitative, NMR-based urinary metabolomics. To address these experimental challenges, several consensus recommendations were provided. These included best-practice recommendations regarding: 1) sample collection; 2) sample processing and storage; 3) NMR data acquisition; 4) NMR instrument set-up; and NMR data processing. Detailed justifications were provided for each of these recommendations. We believe that if these recommendations are followed, they will help members of the NMR metabolomics community better validate and translate their biomarker discoveries from the lab into clinical practice.

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Table 1. Relationship between terms of positive and negative test outcomes.

	Condition positive	**Condition negative**
Positive test outcome	True positive	False positive
Negative test outcome	False negative	True negative

Table 2. Examples of urinary biomarkers of disease discovered using NMR based metabolomics in human studies

Condition	Comparison	Biomarkers	Reference
Pancreatitis	Pancreatitis patients vs controls	Citrate Adenosine	[61]
Bladder cancer	Bladder cancer patients vs controls	Hippurate Citrate Taurine	[62]
Hepatoceullar carcinoma	Hepatocellular carcinoma v cirrhosis vs non-cirrhotic liver disease patients vs controls	Inosine Indole-3-acetate Galactose NAA	[63]
Pancreatic ductal adenocarcinoma	Pancreatic ductal adenocarcinoma vs controls	Acetone Hypoxanthine o-acetylcarnitine dimethylamine	[76]
Esophageal cancer	Esophageal patients vs controls	Urea Acetate Pantothenate 3-hydroxyisovalerate Acetone Formate	[77]
Gestational Diabetes (GDM)	GDM patients vs controls	3-hydroxyisovalerate 2-hydroxyisobutyrate	[78]
Neonatal Health	Small vs appropriate for gestational age	Glycine Threonine	[65]
Neonatal Health	intrauterine growth retardation vs controls	myo-inositol sarcosine creatine creatinine	[64]

Table 3. Experimental conditions for precise quantitation of urine samples using NMR spectroscopy.

Sample Preparation	Parameters and recommended Values	Comments
Sampling	Overnight fasting urine collection	Ensures more stable homeostatic concentrations of metabolites
	Mid Stream urine collection	Avoids unwanted contamination from urinary tract
	Collecting urine sample in labled tube containing sodium azide (NaN_3)	To stop bacterial growth in samples. Final concentration of 0.05% wt/vol.
	Store immediately in to -40 to -80 °C until NMR experiments are performed	Helps arrest metabolic activities and sample degradation
Sample processing	Centrifugation/filtration	Centrifuge at 1000 rpm to remove the turbidity from unwanted particulates, or filter using 0.22µ filter to remove any macromolecular content in the sample.
	Phosphate buffer	Phosphate buffer helps in avoid chemical shift drift that occurs due to pH variations
	Internal Reference Standard; e.g., TSP or DSS	In protein/lipid free urine sample, TSP and DSS are a good choices as internal standards for quantification and normalization
	Use of deuterated EDTA	Only recommended when variation of ionic concentration urine is very large and drift in the chemical shifts is causing quantitative errors.
Acquisition Parameters	One-dimensional gradient NOESY with water presaturation experiment.	
	Time domain points (TD): 64K Line broadening (lb): 0.1- 0.5 Hz	Increased resolution
	Relaxation Delay >5.0 sec	Relaxation delay depends on longitudinal relaxation time (T_1) of metabolite resonances. It should be five times T_1 for absolute quantitation or matched to the T_1 of the reference spectra used for deconvolution.
	Acquisition Time: 2.5 sec	Increased resolution
	Spectral width (sw): 12 ppm	
	Number of Scan (ns): 64 Dummy Scan (ds): 8	For desired S/N, more are required for diluted samples To achieve steady state prior to acquisition
	Excitation Pulse: 90 degree	Shorter pulse widths can be used for single pulse NMR analysis

	Receiver Gain (rg): optimal	Either a constant RG for all or auto optimized for every sample
	Mixing time (t_m): 100 ms for standard experiment 10 ms for gradient experiment	Pulse sequence requirements for NOESY. Minor loss in signal intensity due to transverse relaxation.
	Sample Temperature: 300 K	Kept constant throughout the study
	Shimming, tune & match: for every sample	Increased accuracy, precision and reproducibility
Processing Parameters	Windowing: Exponential window function with line broadening of 0.3-1.0 Hz	
	Zero filling: A factor of 2 of TD	Increased resolution
	Phase correction: manual phasing is preferred	Optimal for accurate integration of peaks area
	Baseline correction: automatic/manual	Increased accuracy of peak integration
	Chemical shift referencing	Both TSP and DSS can be used for chemical shift referencing (δ=0.0 ppm), although DSS is the IUPAC standard (80).

Figure. 1 - Demonstration of the biomarker prediction test with two Gaussian curves indicating the distributions of measured values, with positive cases on the right side and negative cases on the left. The dashed lines indicate the cut-off threshold of hypothetical biomarker concentration that can be used to separate positive from negative tests. The overlap between the biomarker concentrations of the two populations represents the misclassification ratio between the left-hand side of the positive cases and the right-hand side of the negative cases. TP the number of true positives, TN the number of true negatives, FP the number of false positives, and FN the number of false negatives respectively [23].

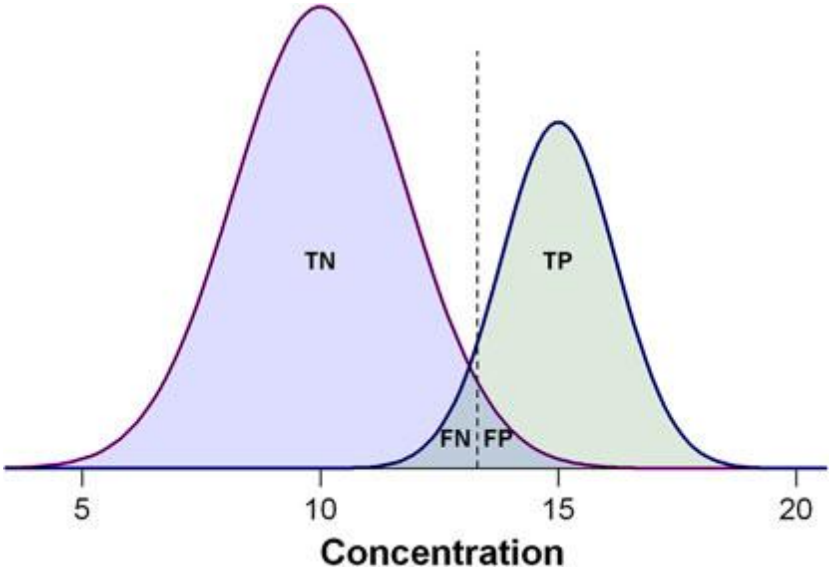
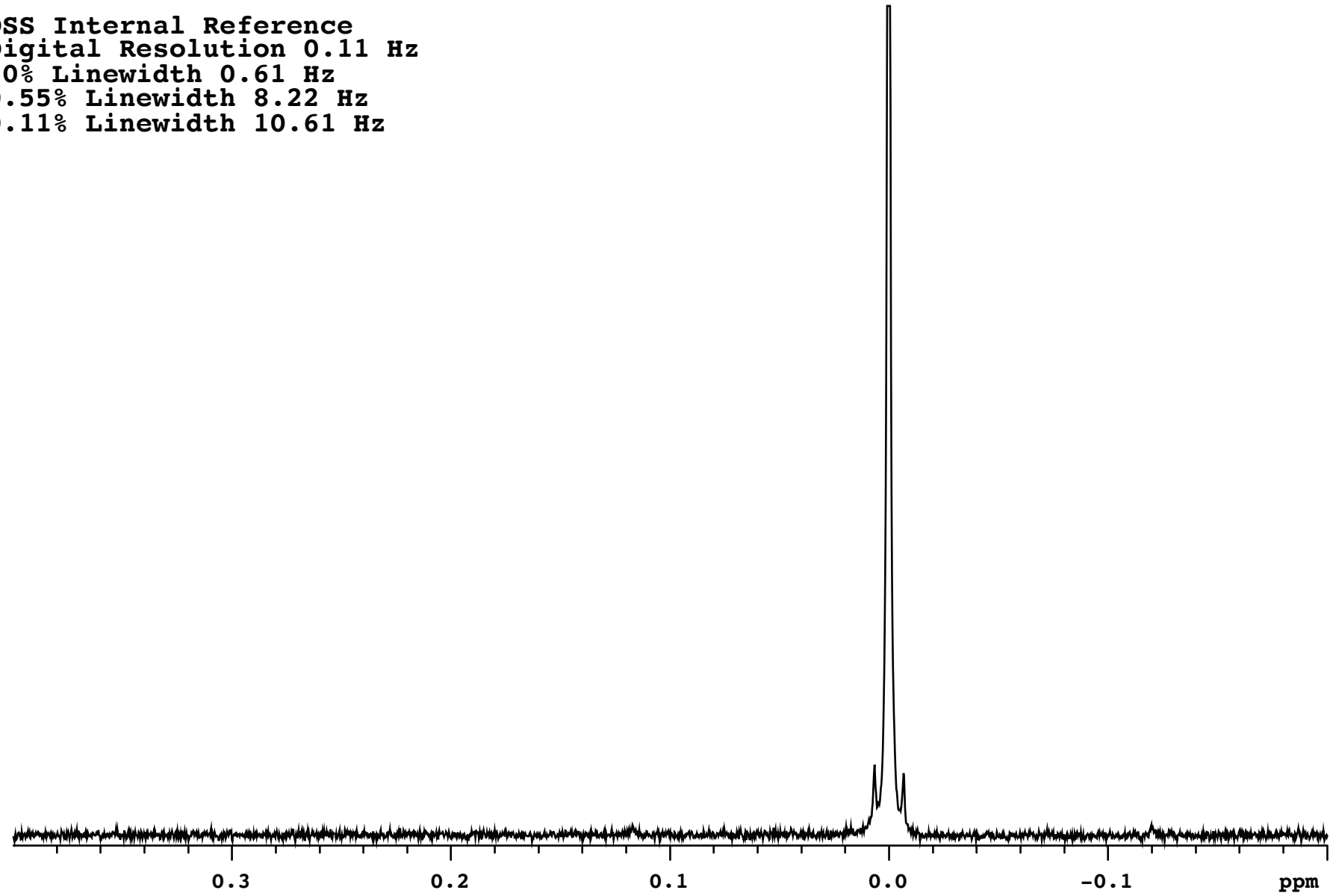


Figure 1

For TOC only


DSS Internal Reference
Digital Resolution 0.11 Hz
50% Linewidth 0.61 Hz
0.55% Linewidth 8.22 Hz
0.11% Linewidth 10.61 Hz
0.3
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ppm