CHAPTER

18

Mass Spectrometry and NMR Spectroscopy–Based Quantitative Metabolomics

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OUTLINE

METABOLOMICS

Metabolomics (also known as metabonomics or metabolic profiling) deals with the quantitative analysis of multicomponent mixtures of biological samples and is focused on establishing metabolic responses of living systems to pathophysiological stimuli or genetic alterations.[1,2] Although vast progress in the fields of genomics[3] and proteomics[4] has provided a wealth of information for understanding the factors that regulate cell physiology and pathology, great insight

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into the functions of biological systems is also gained through the study of concentrations and fluxes of metabolites. The quantitative measurement of small molecule metabolites that are the end products of genes, transcripts, and proteins provides vital information for understanding the composition and function of biochemical networks and promises solutions to many important questions related to human disease diagnosis, prognosis, and therapeutic development.[5–9] Significant interest in the application of metabolomics-based approaches stems from an ability to detect and quantitatively analyze up to many hundreds of metabolites in parallel, which provides an efficient method for unraveling altered biochemistry.[10,11] Because subtle changes in gene expression or protein levels can cause substantial changes in metabolite concentrations, analysis of metabolites represents a very sensitive measure of biological status. More important, metabolomics—through the fingerprinting of biological variations of individuals—offers avenues to achieve the goal of "personalized medicine."[12]

Numerous advanced analytical methods are used for the analysis of complex mixtures of biological samples including blood serum/plasma, urine, cerebrospinal fluid, bile, saliva, seminal fluid, amniotic fluid, synovial fluid, gut aspirate, and cells and tissue.[2] The two most commonly used methods are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Numerous methods within these two major techniques provide both complementary and supplementary information on the identity and concentration of metabolites. MS is highly sensitive and can detect more than a thousand metabolic features. Currently, methods that use MS are almost always combined with separation techniques such as liquid chromatography (LC) and gas chromatography (GC). Liquid chromatography enables separation of intact molecules; gas chromatography requires chemical derivatization of biological mixtures, often with silylating agents to make the metabolites volatile. NMR spectroscopy, on the other hand, detects relatively high concentration metabolites (typically >5 μM) and is highly reproducible and quantitative. The latest technological advancements in NMR—including strong magnetic fields, cryogenic probes and microcoils, and their combination with isotope labeling—promise routine access to more than a hundred metabolites in biological mixtures.[13]

The data obtained from MS or NMR spectroscopy are complex and require analysis using multivariate statistical methods. A large variety of statistical methods are used currently for metabolomics applications, with the most frequently used methods being principal component analysis (PCA), logistic regression, and partial least squares discriminant analysis (PLS-DA) and its variants. PCA is an unsupervised method that is often used as a starting point in the data analysis.[14,15] Subsequently, predictive models are often constructed using supervised methods. PLS-DA is commonly used to build a model based on the regression of data matrix X against matrix Y that contains class information for each sample.[16] Logistic regression, which is widely used in biomedicine, is helpful for the selection of metabolites that contribute most to the classification of patients and controls. To avoid overfitting, PLS-DA models are usually cross-validated, first internally using the same data set and later externally using independent data sets.

COMPARATIVE CHEMOMETRIC ANALYSIS VERSUS QUANTITATIVE METABOLOMICS

Metabolomics experiments fall into one of two somewhat broad categories: One category uses chemometric methods for comparative data analysis and the other uses a quantitative approach.[17,18] In the traditional chemometric approach, metabolites are not identified initially from the spectral data. The complex data are directly used for global multivariate statistical

analysis after subjecting the data to preprocessing steps such as baseline correction, peak alignment, and solvent peak removal. Subsequently, metabolite features that distinguish sample classes are identified and then the structures of distinguishing metabolic features are established.[19] A major drawback is that this approach often differentiates sample classes based on a long list of minor metabolite features that make small contributions. Possible solutions to this issue often involve scaling the data or filtering (feature selection, or targeted analysis as discussed below) based on a set of criteria such as univariate analysis. Another challenge is that errors due to imperfect spectral baselines and peak alignments and strong uneven solvent backgrounds can cause significant problems for the analysis. Metabolite peaks from both MS and NMR spectra are sensitive to sample conditions. Positions of NMR signals, for example, can be sensitive to subtle differences between samples such as pH, ionic strength, temperature, and concentration of macromolecules. Sensitivity to these parameters is more pronounced for biofluids such as urine.[20,21] Spectral binning, in which spectra are divided into several regions and the data points within each region are integrated, has been suggested to alleviate the deleterious effects of small peak shifts.[13,22,23] Nevertheless, peak shifts combined with baseline distortions can still translate into spectral bins that do not represent true peak intensity and pose a significant challenge to the accuracy of the outcome. The problem becomes more severe when the metabolite peaks involved are of low intensity.

Quantitative metabolomics, on the other hand, follows a targeted approach wherein the metabolites are first identified and quantitated.[24] The identities of metabolites are established generally based on the available databases of standard compounds; the identified metabolite peaks are then quantified based on internal or external reference compounds. The resulting data can then be used as input variables for statistical analysis using a variety of methods as described previously for global chemometric analysis. Because of the reliable peak identification and measurement of metabolite integrals, quantitative metabolomics can provide greater insights into the dynamics and fluxes of metabolites and promises robust statistical models for distinguishing classes with better classification accuracy.

MASS SPECTROMETRY

Due to its high sensitivity (typically pg level) and fast data acquisition speed, mass spectrometry (MS) is one of the most commonly employed analytical tools in metabolomics. Since early 2000, there has been tremendous growth in MS-based methods, including chromatography separation, ionization, and detection strategies.[2,25,26] Advanced software combined with rich databases have enabled automatic peak alignment, identification, and quantitation of metabolites. Because of the complexity of biological matrices, it is often necessary to separate metabolites of interest prior to MS acquisition, especially in case of metabolite quantitation. Thus, hyphenated analytical platforms that combine chromatography with MS have proved effective for metabolomics applications. Common separation techniques used include liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE).[27] Common MS techniques include quadrupole, triple-quadrupole, ion-trap, time-of-flight (TOF), and Orbitrap™ mass analyzers, which have been described in detail.[28] LC- and GC-based MS methods are particularly widespread, and the latest advances enable improved quantitation by canceling errors arising from sample complexity (i.e., matrix) effects.

Liquid Chromatography Resolved MS (LC-MS) Methods

Among the MS methods, LC-MS is by far the most widely used for metabolomics applications.[29] It allows direct detection of metabolites

from biological samples with no requirement for chemical modification such as derivatization. The latest LC-MS technological advances allow absolute quantitation of more than 200 metabolites in rat plasma[30] and relative quantitation of more than 250 metabolic species in bodily fluids, cells, and tissue.[31]

LC-based MS methods commonly use "soft" ionization techniques such as electrospray ionization (ESI), as it ionizes a wide range of metabolites without inducing metabolite fragmentation. Ion suppression is a major problem associated with ESI, as it affects both the detection of metabolites as well as their reliable quantitation.[32] Ion suppression, in which analytes do not become efficiently ionized because of competition with other ionizable species, is caused by numerous endogenous and exogenous factors including the presence of salts, macromolecules, or highly abundant interfering metabolites. The addition of volatile buffers such as ammonium acetate or ammonium formate in LC solvents can help alleviate ion suppression due to the salt effect. Ion suppression can also be reduced by proper choice of sample preparation and LC and MS parameters. Most biological samples contain macromolecules such as proteins, which need to be precipitated using organic solvents such as methanol or acetonitrile. Further purification by solid-phase extraction (SPE) can also help reduce matrix effects. However, SPE is more labor intensive and often requires a recovery test for each metabolite. Therefore, protein precipitation using an organic solvent is typically a better choice for large-scale studies. Another important step to alleviate ion suppression is optimization of chromatographic parameters for better peak resolution and minimization of co-eluting metabolites. In order to improve the separation, recently a number of researchers used multiplexed LC methods on different analytical columns. This approach allowed Wei et al. to perform absolute quantitation of over 200 metabolites in biological samples.[30] Regular cleaning of the ion source and optimization of the MS acquisition parameters are also critical steps for minimizing ion suppression.

Metabolite Quantitation Using LC-MS

A number of LC-MS approaches have been proposed to compensate for the effect of ion suppression and provide reliable metabolite quantitation. The most common approaches involve spiking biological specimens with stable isotope (^{2}H, ^{13}C, or ^{15}N)—labeled internal standards (SILISs) or structural analogues as internal standards. Because these materials can often be purchased commercially and can be obtained in high purity, they can serve as relatively reliable standards. Quantitation utilizing SILISs represents a very reliable approach, as such standards possess nearly identical chemical and physical characteristics as the analyte of interest. Each SILIS compound is eluted and ionized nearly identically to its corresponding metabolite in the biological sample, and the increased mass provides a peak offset by the mass difference between the two isotopic forms.

In practice, biological samples are spiked with a standard solution of a single or multiple SILISs, often prior to the sample preparation step to compensate for any inaccuracies caused by recovery loss.[30,33] Accurate concentrations of metabolites are then determined by directly comparing peak areas of metabolites and their isotope-labeled internal standard. The peak area comparison, however, is reliable only if the peak areas for both the metabolite and its internal standard are similar, which is often not the case for all metabolites. Some metabolites vary in concentration or may have unknown concentrations. In such cases, calibration curves need to be obtained using mixtures of standard compounds (calibrants) of different concentrations. All calibrant mixtures are spiked with SILISs at the same concentration as used in the analysis of actual biological samples. The calibrants' concentrations are chosen to cover the whole linear dynamic range (LDR) for each

target metabolite. A typical calibration curve is shown in Figure 1.

Using this calibration curve, for example, the actual concentration of the target metabolite in a biological sample is determined by inverting the equation for the fitted calibration curve as:

$$\text{Metabolite Concentration} = \frac{[\text{Park Area (Metabolite)} \div \text{Peak Area (SILIS)}] - 0.0332}{0.2474} \quad (1)$$

where Peak Area (SILIS) is the peak area, obtained from the multiple reaction mode detection, of the stable isotope-labeled internal standard spiked in the biological sample and 0.0332 and 0.2474 are the y-intercept and slope, respectively, as shown in Figure 1.

A major drawback of this approach is the often prohibitively high cost of stable SILIS compounds and/or commercial unavailability of isotope-labeled standards for many metabolites. To circumvent this problem, many studies have utilized a small set of representative SILIS compounds and used them to analyze up to 200 or more metabolites with coefficients of variation (CVs) of less than 15% for 80% or more of the metabolites (Figure 2).[30,34] Such approaches assume that the chosen SILIS compounds have similar ionization behavior to the metabolites under investigation. Another approach uses structural analogues as internal standards that have similar structures and chemico/physical properties as the targeted metabolite class and are not a part of the endogenous compounds. Tubercidin, for example, is thus used as a structural analogue for the analysis of nucleosides.[35,36] Quantitation using such structural analogues is performed by spiking biological specimens with one or more structural analogues as internal standards, and metabolite concentrations are determined by comparison of mass peak area with that of structural analogue or by using a calibration curve similar to that shown in Figure 1. CVs of 5% to 20% have been obtained by this approach in the analysis of

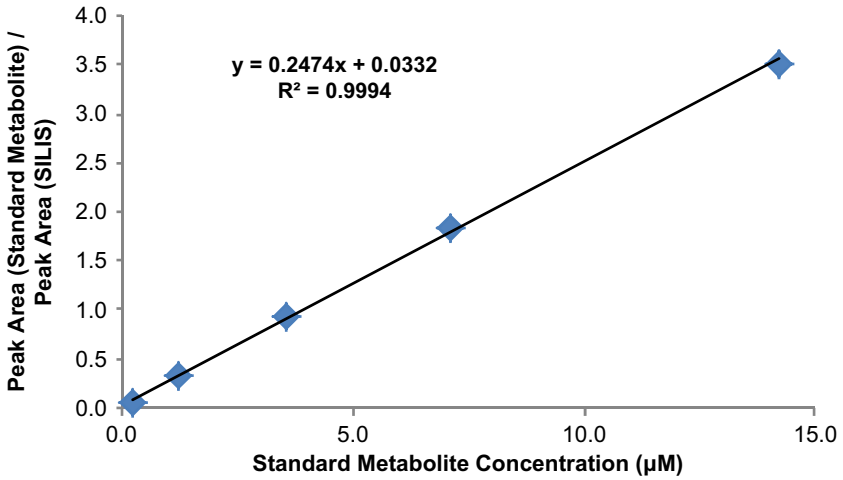

FIGURE 1 Typical calibration curve obtained using solutions with different standard metabolite concentration and fixed internal standard (SILIS) concentration.

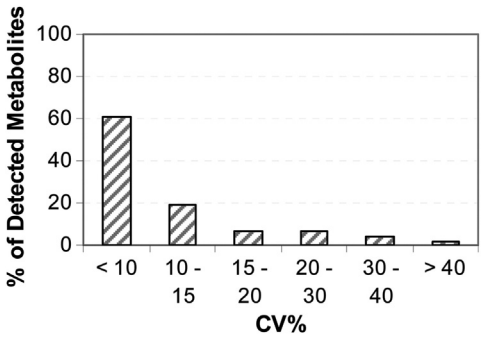

FIGURE 2 Distribution of CV values for metabolites in rat plasma obtained using a small set of representative internal standards (SILIS).[30]

modified nucleosides in biological samples.[35,36] The use of structural analogues or a small number of representative SILIS compounds can be cost-effective in targeted metabolite quantitation. However, spiking with a SILIS for each metabolite provides more reliable results (intra- and interday CVs <10%), as only the labeled counterpart can truly mimic the response of an individual metabolite as compared to a structurally similar internal standard or representative labeled metabolite.[37,38]

An approach that does not depend on the commercially available isotope-labeled compounds and eliminates a major drawback of LC-MS-based metabolite quantification utilizes isotope-labeled metabolites produced *in vivo*.[39,40] A mixture of hundreds of ^{13}C-isotope-labeled metabolites, for example, can be produced within a few hours by feeding microorganisms such as yeast or bacteria with U-^{13}C-labeled medium. This approach is significantly less expensive and enables access to isotope-labeled standards for the majority of metabolites of interest in biological samples. As with commercially available SILIS compounds, metabolite quantitation is performed by mixing biological specimens with *in vivo* isotope-labeled mixture and comparing mass peak intensities of each metabolite with its labeled counterpart or using the calibration plots (Figure 1). A one-time determination of metabolite concentrations of isotope-labeled metabolites in algal or bacterial extracts, however, is needed, utilizing unlabeled standard mixtures of metabolites, and one needs to be careful about SILIS sample stability. Nevertheless, using this *in vivo*—derived SILIS compound approach, more than 90% of measured metabolites were shown to exhibit CVs of less than 15%.[33] This method has fostered increased interest for determining absolute concentrations of cellular metabolites,[18,40–45] and it will be interesting to see whether it promises a robust method for mass spectrometry—based quantitative metabolomics using commonly used biological samples such as blood, urine, and tissue. Detailed step-by-step experimental protocols for quantitation of intracellular metabolites and measurement of cellular fluxes have been described using examples of *Escherichia coli* and primary human fibroblasts fed with U-^{13}C-labeled carbon sources and labeled metabolite detection using LC-MS/MS.[46,47]

In the previously described quantitation methods using a limited number of labeled internal standards, external calibration curves are usually used to calculate the metabolic concentrations. These calibration curves are obtained from calibrant samples that were spiked with labeled internal standards. However, some studies utilized external calibration curves without spiking the calibrants with labeled internal standards, and the metabolic concentrations were determined from a plot of the MS peak intensity versus standard metabolite concentration. The advantage of this approach is that the calibrant samples do not require spiking with labeled standards. The disadvantage of this approach is that the calibration curves are based on the series of standard dilutions with significantly lower matrix effects than those exhibited in the biological samples. Without the presence of labeled internal standards to offset the ion suppression effect, one risks underreporting the actual metabolic concentrations. In addition, the MS signal can drift over time due to the fluctuations in the ionization efficiency, and as a result

the linearity of the predetermined calibration curves can no longer be valid.

Gas Chromatography–Resolved MS (GC-MS) Methods

GC-MS is a robust method for metabolomics applications. It combines high separation efficiency and sensitive detection following electron ionization.[27,48] In particular, it is the preferred method for the analysis of metabolites with low molecular weight, as they can be quite effectively analyzed with good sensitivity and reproducibility. Although the volatile, low-molecular-weight metabolites can be directly analyzed, nonvolatile, polar metabolites require chemical derivatization—often silylation—before analysis. There is a large literature on derivatization methods in GC-MS.[49] To improve resolution in GC-MS, the development of two-column approaches (2D GC-MS) offers further enhancement and is well suited for the analysis of complex biological mixtures.[50–52]

The quantitative accuracy obtainable from GC-MS critically depends on numerous factors including sample collection, storage, extraction, derivatization, stability, and analysis. By comparison, instrumental stability reproducibility is much less problematic. In addition, the ionization method of choice—electron ionization—avoids complications such as ion suppression. Factors that are of most concern for GC-MS are the efficiency of derivatization and the stability of derivatized metabolites. Regarding the derivatization process, metabolites can be broadly grouped into three classes: Class 1 metabolites, which contain hydroxyl or carboxyl groups, exhibit the highest efficiency towards derivatization and stability of the derivatized metabolite; Class 2 metabolites contain amine or phosphoric groups and are very sensitive to experimental conditions and are measured with intermediate precision; and Class 3 compounds, with amide, thiol, or sulfonic functional groups, are more difficult to derivatize and analyze. To account for these differences, use of representative compounds from all three groups as internal references has been proposed.[26]

Nevertheless, with proper care regarding both the sample processing and experimental conditions, precise results can be obtained to compare relative metabolite concentrations. In general, quantitative methods for GC-MS are less popular than for LC-MS. External calibration methods are sometimes used for quantitation; however, literature-reported applications of SILIS to GC-MS are quite limited. In contrast, *in vivo*–labeling methods attract more attention. One study reported the use of ^{13}C-labeled yeast cell extracts as a source for internal standards to quantify intracellular metabolites in *S. cerevisiae* cells. This study focused on measuring nonoxidative pentose phosphate pathway intermediates using GC-MS.[53] More recently, a simplified GC-MS method for absolute quantitation of metabolites using commercially available ^{13}C-labeled algal extract for use as internal standards has been reported.[54] ^{13}C isotope labeling was also used to estimate metabolic fluxes and concentrations in mammalian (hepatic) cells using a combination of GC-MS and LC-MS methods.[45,55] Results of these studies promise more widespread use of *in vivo*–labeled metabolites as internal standards for routine quantitative metabolomics applications.

NMR SPECTROSCOPY

NMR spectroscopy is one of the most information-rich techniques in the field of metabolomics. It exhibits a number of important characteristics for both identification and quantitative analysis of metabolites in complex biological samples. In particular, the high resolution and superb reproducibility enable access to a large number of metabolites and their accurate concentrations. Unlike MS, NMR allows structural verification/identification of both known and

unknown metabolites as well as quantitation, from the same measurement. Because the same nuclei, ^{1}H for example, are detected with the same sensitivity, a single internal standard is sufficient to determine the absolute concentrations of all detected metabolites in a single experiment. In addition, the ratios between peaks for a specific metabolite are fixed and depend on the number of equivalent nuclei that contribute to the peak and hence the integrated peak area for any one isolated peak is sufficient to determine a metabolite's concentration.

Solvent Suppression

A critical requirement for quantification by NMR is the efficient suppression of the water signal. Owing to its high natural abundance, sensitivity, and ubiquitous nature, ^{1}H is the most preferred nucleus for NMR-based metabolomics. Biofluids are aqueous in nature and the concentration of water in these samples is four orders of magnitude or more higher than the typical concentrations of metabolites. To date, a large number of water suppression methods exist, each with its own advantages and disadvantages. Generally, these methods use weak radio frequency (RF) pulses, pulse field gradients, or their combination to suppress water signal.[56–59] Numerous improvements have been made that circumvent many challenges associated with water suppression and provide spectra without distortions in phase or peak intensity.[60–65] A recently developed water suppression pulse technique, WET180, efficiently suppresses faraway water that experiences significantly reduced RF fields relative to bulk water within the RF coil redgion and enables sensitive detection of metabolite signals (even those very close to the water signal).[66]

Suppression of Macromolecular Signals

NMR spectra of biofluids such as blood serum and plasma obtained using the single pulse or 1D NOESY (nuclear Overhauser effect spectroscopy) sequence are complicated due to the overwhelming macromolecular signals from lipids and proteins. It is very difficult to derive quantitative information on metabolites from such spectra, although spectral fitting approaches can provide limited solutions. Considering that blood is the most important medium and is widely used in metabolomics applications, numerous developments have been made to avoid the interference of macromolecular signals. The spin-echo pulse sequence, specifically its improved version, the Carr-Purcell-Mieboom-Gill (CPMG) pulse sequence, which exploits the large difference in the nuclear spin relaxation properties between metabolites and macromolecules, is often used to eliminate or reduce macromolecular signals.[2,67–73] Currently, the CPMG pulse sequence is commonly used for the analysis of blood serum and plasma samples. However, caution should be exercised when using this sequence for quantitative analysis because the metabolite signals are attenuated somewhat due to T_2 relaxation. Further, many physiologically important metabolites including lactate, ketone bodies, and aromatic amino acids such as tyrosine, phenylalanine, and histidine bind to protein molecules in blood serum/plasma. The ^{1}H nuclei from such bound metabolites experience a substantial decrease in their transverse relaxation (T_2) times and make such metabolites substantially invisible in the ^{1}H NMR spectra[74,75]; due to the line broadening caused by such binding, some metabolite signals can even altogether disappear from NMR spectra. Thus, use of the CPMG sequence can underestimate concentrations of all endogenous and exogenous compounds that bind to proteins.[74,76]

An altogether different approach that completely separates metabolites and macromolecular signals in blood plasma is shown in Figure 3.[77] This approach utilizes diffusion-sensitized ^{1}H NMR spectroscopy and exploits a large difference in the translational diffusion coefficients between blood plasma metabolites

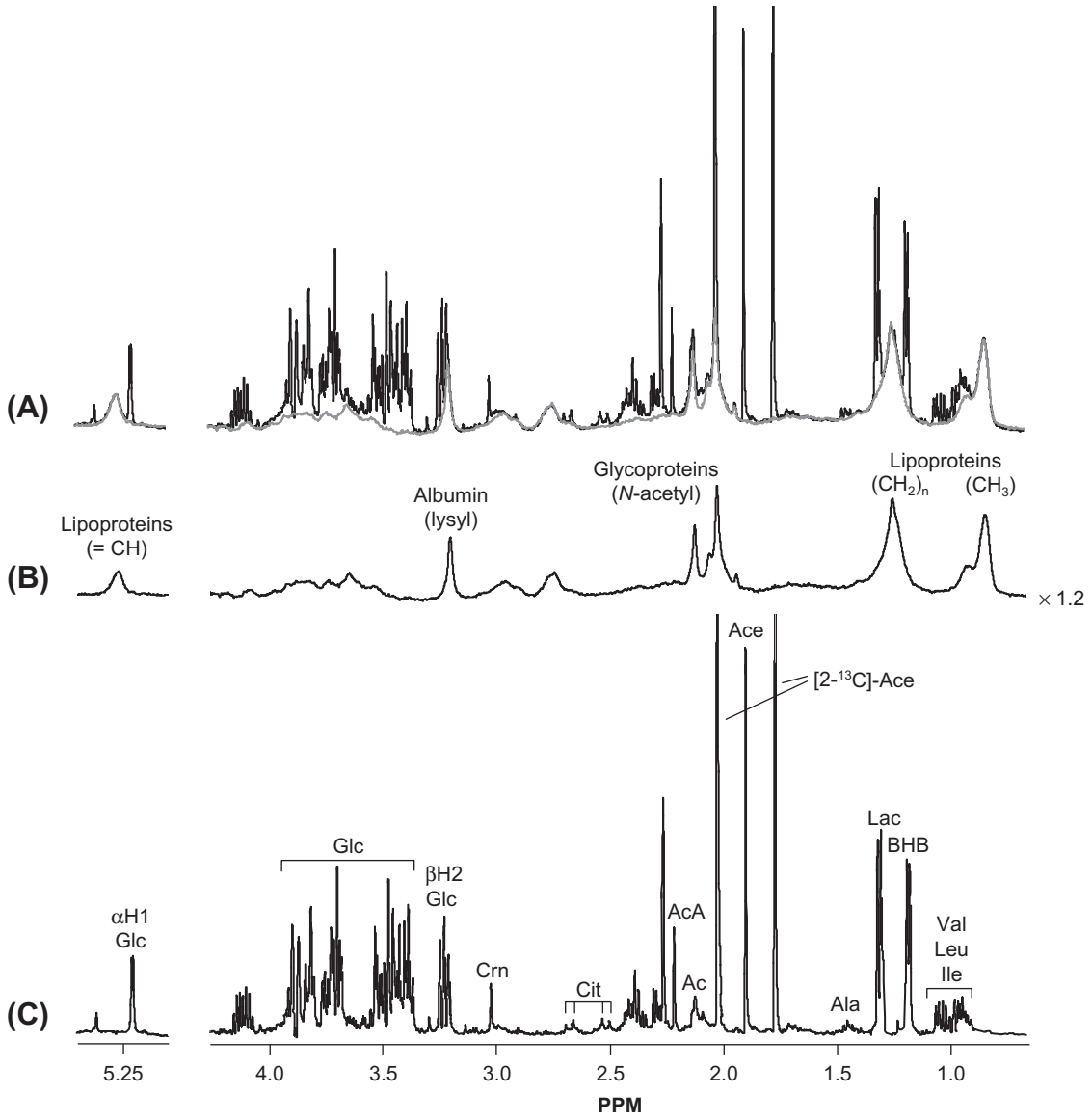

FIGURE 3 (A) ^{1}H NMR spectrum of blood plasma acquired with a low sensitivity toward diffusion (diffusion factor, b = 4.1 s/mm^2), overlaid with a spectrum obtained with high diffusion sensitivity (diffusion factor, b = 10,000 s/mm^2, gray). (B) ^{1}H NMR spectrum of blood plasma acquired with a high sensitivity toward diffusion (diffusion factor, b = 10,000 s/mm^2). The macromolecule spectra in (A) and (B) are identical. (C) Difference spectrum between (A) and (B). Abbreviations: acetate (Ace), acetoacetate (AcA), aceton (Ac), alanine (Ala), β-hydroxybutyrate (BHB), citrate (Cit), creatinine (Crn), glucose (Glc), isoleucine (Ile), lactate (Lac), leucine (Leu), and valine (Val). *(Reproduced with permission from reference #77.)*

and macromolecules to separate signals from the two groups. Two separate NMR spectra are obtained: one that is minimally affected by relaxation and diffusion and another in which all metabolite signals are removed based on their faster diffusion, which causes the NMR signal to attenuate. Subtraction of the latter spectrum from the first provides a spectrum for blood plasma metabolites, which is devoid of broad peaks as well as baseline distortions from macromolecules. Another important method for macromolecular signal suppression includes physically removing proteins from the biological mixtures. There are numerous ways for such removal of proteins from blood plasma and serum including precipitation by adding organic solvents, salts, or altering pH, or by ultrafiltration.[78] The performance of such deproteinization methods is shown to vary significantly, and hence caution should be exercised while optimizing the deproteinization protocol.[79] Owing to their superior performance, deproteinization by ultracentrifugation using 3kDa cutoff filters, for example,[79,80] and precipitation using methanol solvent[81] are more commonly used for quantitative applications.

Quantitative Referencing

Adding a known amount of TSP [sodium d_4-3- (trimethylsilyl)-propionate] or DSS (sodium d_6-2, 2-dimethyl-2-silapentane-5-sulfonate) can provide a signal that can be used as both a quantitative and chemical shift reference. However, the binding of these standards with macromolecules such as proteins and their sensitivity to sample conditions such as pH can lead to severe signal attenuation or peak shifting; hence, addition of these compounds directly into biological mixtures is generally not preferred. To circumvent this problem, formate was explored as a quantitative reference, which reportedly does not interact with macromolecules.[82] Its utility as a reference for quantitation is limited because formate is present generally in most biological mixtures. The use of a coaxial capillary tube containing a solution of TSP or DSS in deuterium oxide (D_2O) offers a number of advantages. The reference compound does not interact with the sample matrix; it serves as a chemical shift as well as a quantitative reference, and the D_2O solvent serves as a field-frequency locking solvent. Further, the same capillary tube can be used for all samples, which adds to minimizing the quantitation errors. Nevertheless, this approach adds some additional steps and is hard to automate.

Somewhat recently, considering that water is highly concentrated in biological mixtures, a method that uses water itself as a concentration reference was proposed.[83] Here, the very large solvent (water) signal is obtained in a separate experiment with reduced receiver gain. The integral of the solvent signal is then compared with those of metabolites using the two spectra to obtain absolute concentrations of the metabolites of interest. It was shown that quantitation of metabolites can be made with errors less than 2% over a wide range of concentrations. This method does not require additional compounds for concentration referencing and is indifferent to probe tuning. For accurate quantitation, however, it assumes a linear response to radiofrequency pulse length and transmitter power, and receiver gain settings, which is generally true. Several following reports discuss the influence of such parameters on the accuracy of metabolite quantitation.[84–87] A method known as ERETIC (Electronic REference To access In vivo Concentrations) that does not require either a reference compound or a solvent signal for quantitation has also been proposed.[88] It involves synthesis of a reference signal by an electronic device, and its position in the spectrum can be conveniently chosen so as to not interfere with metabolite signals. For accurate quantitation, however, the ERETIC method requires occasional calibration using a standard compound. Although this approach is very simple, the results are reliable only when the

same sample load and probe tuning is maintained between samples.

Spectral Simplification Methods

In a typical ^{1}H NMR spectrum, signals are observed between 0 ppm and 9 ppm, and most are crowded into two spectral regions that roughly span 5 ppm (0.8−4.4 ppm, 6.8−8.0 ppm). Because of the signal overlap, the identification and quantification of many metabolites of interest often becomes impossible. This problem is compounded because it is almost always the case that a given biofluid will contain a relatively few species present at high concentrations that will dominate the NMR spectra. Numerous studies have been focused on alleviating this challenge. One such approach uses selective total correlation spectroscopy (TOCSY) methodology, which can detect metabolites quantitatively even if they are found at concentrations 10 to 100 times below those of the major components and provides improved data inputs for principal component analysis.[89,90] Recently, quantitative aspects were examined by optimizing the 1D TOCSY experiment and comparing integrations of 1D TOCSY read peaks to the bucket integration of 1D proton NMR spectra.[91] An important aspect of this approach is that selective TOCSY, apart from metabolite quantitation, enables unknown peak identification in complex fluids.[92] More recently, a new method called Add to Subtract for efficient suppression of background signals from highly concentrated metabolites in biofluids such as strong glucose background in serum and diabetic urine spectra was shown.[93] This method is simple to perform, as it requires only obtaining a second spectrum after the addition of a small drop of concentrated glucose solution. It can reduce the glucose signals by 98% and allow retrieval of the hidden metabolic information. This spectral simplification approach enables identification of distinguishing low concentrated metabolites by multivariate statistical analysis and shows promise for a number of quantitative metabolomics applications.

Metabolite Quantitation Using 1D NMR

Unambiguous peak identification is a critical step in quantitative metabolomics. To aid such identification, databases that contain NMR chemical shifts and spectra for several hundreds of metabolites have been developed using standard compounds that are publicly available.[94,95] For applications involving large sample sets, automation of metabolite identification and quantitation is often sought. Numerous method development efforts have been focused on automated peak identification and/or metabolite quantitation. Automated integration of defined spectral regions is the simplest approach for metabolite quantitation. It reduces the number of variables and, at the same time, provides integrals for the reduced variables (metabolite signals). However, this approach assumes that each variable contains the same chemical information, which is often not the case because many metabolites—for example, citrate, histidine, and taurine—exhibit significant peak shifts due to altered pH or ion concentrations. The severity of their peak shifts is more prominent for biofluids such as urine.[96,97] Moreover, baseline distortions deleteriously affect the quantitative measurement of metabolites using this approach.[98]

A number of curve fitting methods have been proposed to focus on metabolite identification and quantitative analysis. A frequency domain data fitting approach was proposed to identify metabolite peaks from overlapped spectral regions.[99] It uses a semiautomated approach and can therefore be time consuming for the analysis of large sample sets. A different approach, which is insensitive to the variation of peak shifts because it makes use of prior knowledge of the spectra of pure compounds, was suggested.[100] This method assumes that at least one peak for the metabolite is isolated, without overlap from other compounds, such

that it can be used as a reference. A major improvement to peak identification and accurate quantitative analysis of metabolites using 1D NMR spectra is provided by Chenomx Inc.[101,102] Metabolite identification is facilitated by modeling of the spectral features using spectra of pure compounds from pH and spectrometer frequency–dependent chemical shift databases. Accurate metabolite concentrations are then obtained by comparing modeled metabolite peak integrals with that of an internal reference compound such as DSS. This method allows identification of peaks, even if they are shifted due to altered conditions, such as pH, and provides accurate concentration values that can then be inputted for further multivariate statistical analysis. The approach is semiautomated and needs manual selection of appropriate metabolites from the library and manual feedback in the fitting of peaks. More recently, a method that performs automated spectral deconvolution of 1D NMR spectra was presented.[80] It uses an algorithm, AutoFit, to reconstruct experimental spectra using a reference compound library. The algorithm optimizes a number of parameters, including a recalibration of reference spectral library based on position, intensity, and the peak line width from a reference compound such as DSS, from the experimental spectrum. An alternative approach, based on a Bayesian fitting algorithm, was recently proposed.[103] Although this approach is currently limited to small spectral regions due to its computational requirements, it operates in a fully automated mode.

Metabolite Quantitation Using 2D NMR

In 1D ^{1}H NMR, the relatively narrow spectral region in which all metabolite signals are observed results in peak overlap and limits the number of metabolites that can be identified and quantified accurately. This problem can be greatly alleviated by making use of two-dimensional (2D) NMR spectra, in which peaks are spread along two dimensions to enable both unambiguous identification and accurate quantitation of an enhanced pool of metabolites. Various 2D NMR methods that provide complementary information on metabolite identity and metabolite concentrations are used. 2D *J*-resolved spectroscopy, correlation spectroscopy (COSY), TOCSY, heteronuclear single quantum-coherence (HSQC) spectroscopy, and heteronuclear multiple-bond correlation (HMQC) spectroscopy experiments are the most commonly used. Projections of 2D *J*-resolved spectra are much simpler as each multiplet that arises due to spin–spin couplings (*J*-coupling) is collapsed to a single peak.[104,105] However, care should be exercised in quantitating metabolites using this experiment since, as for the 1D CPMG experiment, peak integrals are affected by transverse relaxation during the long evolution period. The 2D HSQC experiment is particularly attractive due to its much higher resolving power, which arises from the wider chemical shift ranges of heteronuclei such as ^{13}C and ^{15}N. Lower sensitivity due to the low gyromagnetic ratios of ^{13}C and ^{15}N, combined with their low natural abundances is, however, a major challenge that limits wider utility of such 2D experiments. Nevertheless, the latest advances in NMR instrumentation, including the development of strong field magnets, cryogenic and microcoil probes, and isotope labeling approaches, are contributing to the increased utility of these experiments for metabolomics applications.

A protocol for the determination of metabolite concentrations was evaluated using the ^{1}H-^{13}C HSQC spectrum.[106] Here, the 2D peak integrals are compared with calibration curves obtained from 2D spectra of three equimolar mixtures of standard compounds to predict the metabolite concentrations. Improving on this quantitation strategy, an algorithm to deconvolute 2D NMR peaks was developed.[107] It constructs simple time domain models with the fewest number of peaks, whose frequency

domain spectra match with target regions of the experimental spectrum. Focusing on enhancing the accuracy for the derived metabolite concentrations, three time-zero ^{1}H-^{13}C HSQC series spectra with incremented repetition times were used. From this series of spectra, an extrapolated time-zero HSQC spectrum ($HSQC_0$) was obtained.[108,109] Peak intensities in the $HSQC_0$ spectra represent true concentrations of individual metabolites, as they are not influenced by delays during the pulse sequence that could cause substantial loss of coherence due to relaxation. However, this method is somewhat time consuming because of the need for multiple 2D spectrum acquisitions. A method that does not require standard compounds for determining metabolite concentrations using ^{1}H-^{13}C HSQC spectra was also reported.[110] This approach is based on applying a correction factor to the 2D peak integrals, calculated from the solution of the Bloch equations and analysis of product operator formalism utilizing longitudinal (T_1) and transverse (T_2) relaxation parameters, ^{1}H-^{13}C heteronuclear *J*-coupling and the delays used in the 2D pulse sequence. Applying a correction factor eliminates the effects of T_1 and T_2 relaxation, heteronuclear couplings, and experimental parameters and provides peak integrals that represent true metabolite concentrations.

Isotope-Labeled NMR

Another approach is the use of isotope labeling using heteronuclei such as ^{13}C and ^{15}N, which provides a number of benefits for quantitative metabolomics. Currently, isotope labeling is used in two major applications. One area focuses on tracing metabolic pathways and flux analysis using *in vivo* isotope labeling[111–117] and the other on increasing the pool of detected metabolites by enhancing resolution and sensitivity using *ex vivo* labeling. Because the first area is not used primarily for quantitation, we focus on the second.

Ex Vivo Isotope Labeling

Owing to the extremely high complexity of biological mixtures, only a small fraction of metabolites can be accurately analyzed, and the information derived from such a small number of metabolite is insufficient to gain insights into altered cellular biochemistry. Targeting different metabolite classes (acids, amines, etc.) using chemoselective isotope tags reduces molecular complexity and improves the detection of low-concentration metabolites by reducing the contribution of less interesting chemical signals. Stable isotopes such as ^{13}C and ^{15}N and abundant heteronuclei such as ^{31}P have been used to tag metabolites with specific functional groups and thereby significantly enhance the resolution and sensitivity of NMR experiments.[118–122]

Isotope labeling of metabolites using a ^{13}C-acetylation tag results in selective labeling of amine containing metabolites, and using the ^{1}H-^{13}C HSQC experiment allows the detection of the ^{13}C-tagged metabolites with improved resolution and sensitivity.[118] This labeling approach is quantitative and the tagging reaction can be carried out directly in aqueous solution at ambient temperature. More recently, tagging using formylation using ^{13}C-formic acid was shown to improve the detection of amine containing metabolites.[119] The large, 200 Hz, one-bond *J*-coupling between the labeled ^{13}C and ^{1}H facilitates efficient transfer of polarization between the two nuclei in HSQC experiments. Carboxyl functional group containing metabolites represent another major class of molecules in biological systems. They can be chemically tagged with ^{15}N-ethanolamine and detected using a 2D ^{1}H-^{15}N HSQC experiment.[120] The enhanced sensitivity and resolution from this approach enables detection of metabolites at concentrations as low as a few micromolar, quantitatively and reproducibly. Using this approach, nearly 200 well-resolved signals corresponding to well over 100 carboxyl-containing metabolites can be routinely detected in biological mixtures. A method to detect lipid metabolites with

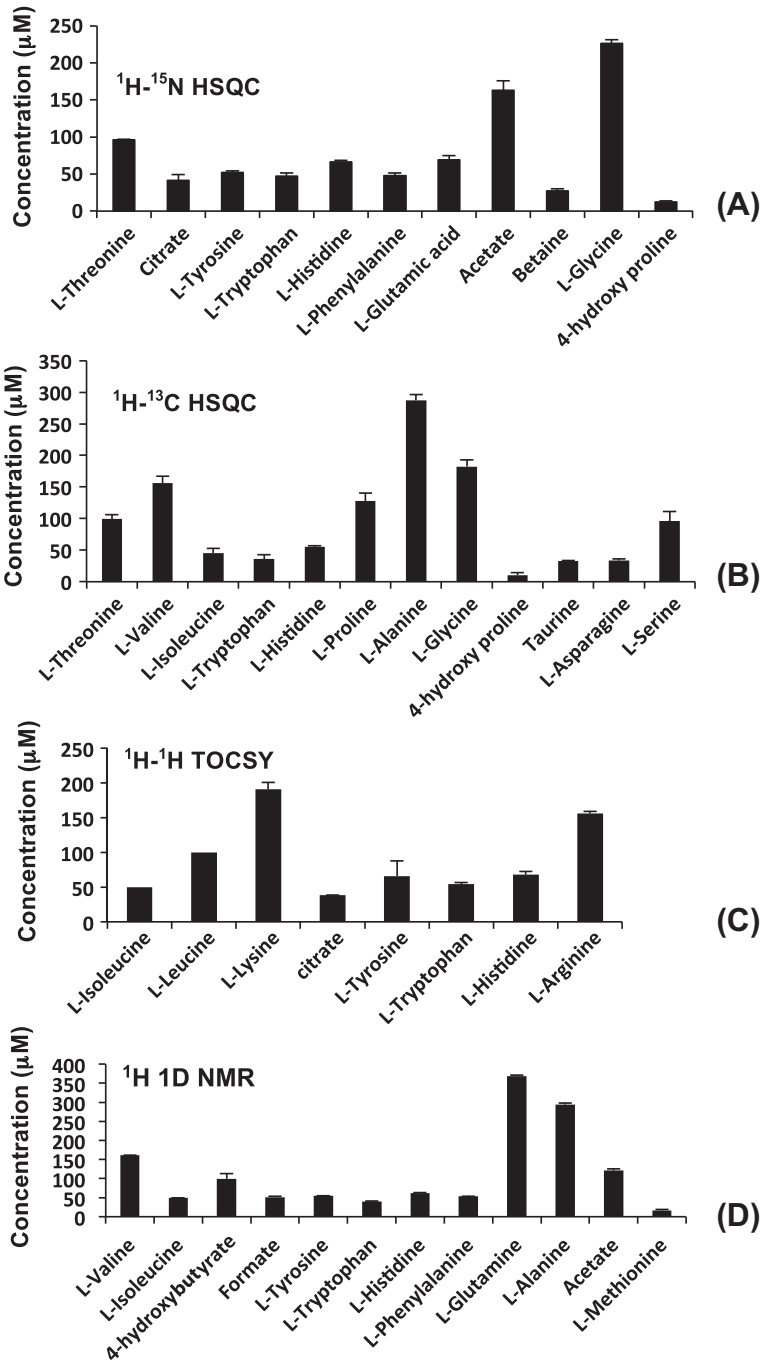

FIGURE 4 Concentration of some of the metabolites in the NIST plasma obtained using 1D/2D NMR experiments with and without isotope tagging: (A) obtained from ^{1}H-^{15}N HSQC NMR after ^{15}N tagging; (B) obtained from ^{1}H-^{13}C HSQC NMR after ^{13}C tagging; (C) obtained from ^{1}H-^{1}H TOCSY NMR of neat plasma; and (D) obtained from 1D NMR of the neat plasma sample. *(Reproduced with permission from reference #122.)*

hydroxyl, carboxyl, or aldehyde groups was recently developed using a ^{31}P isotope label.[121] It uses a ^{31}P containing reagent, 2-chloro-4,4,5,5-tetramethyldioxaphospholane (CTMDP), to tag the lipid metabolites. Derivatized metabolites are then detected with enhanced resolution using one-dimensional ^{31}P NMR. The method provides sufficient sensitivity and spectral resolution, and derivatized species have unique and well-resolved resonances in the ^{31}P NMR spectrum.

A single peak, devoid of multiplicity, for each tagged metabolite and effective filtering of non-tagged metabolites significantly add to the sensitivity and background suppression. Identification of tagged metabolites requires the knowledge of chemical shifts of isotope-tagged standard compounds. In view of this, a chemical shift library for nearly 150 isotope-tagged metabolites is developed and using this library, more than 50 metabolites in human serum and urine have been identified.[119–121] More recently, using a combination of NMR methods—including isotope tagging—metabolites in human plasma from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD) were quantified.[122] ^{1}H-^{15}N HSQC and ^{1}H-^{13}C HSQC experiments on the isotope-tagged samples combined with the conventional ^{1}H one-dimensional and ^{1}H-^{1}H TOCSY experiments provide quantitative information on a large number of metabolites in plasma (Figure 4). The methods were first tested on a mixture of synthetic analogues of metabolites commonly present in human blood and then metabolites in a standard NIST plasma were identified and quantified with an average coefficient of variation of less than 5.6%.

CONCLUSIONS

Quantitative metabolomics has progressed quickly on a number of fronts. For mass spectrometry, the most accurate and quantitative approach is to use SILISs to minimize ion suppression effects. This technique is capable of performing metabolic measurements with intra- and interday CVs in the single digit range. Another, less accurate approach, is to use structural analogues as internal standards, and finally the use of external calibration curves is cost effective, relatively straightforward, and gaining popularity in the metabolomics field. NMR spectroscopy, with its ability to provide both the identities and accurate concentrations of a large number of metabolites using a single internal reference, represents an important method for quantitative metabolomics. NMR's ability to profile metabolites in intact biological samples, including cells and tissue, is unique and offers important avenues to identify and quantify new metabolites, as well as to translate the findings of biomarker discovery research to *in vivo* clinical applications. New methods that provide enhanced resolution and better sensitivity for both NMR and MS platforms will continue to propel quantitative metabolomics.

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