

Expanding the Coverage of the Metabolome with Nitrogen-Based NMR

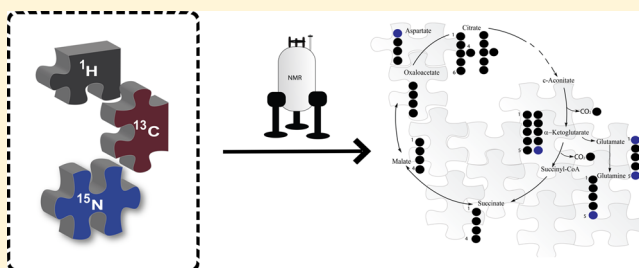
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S Supporting Information

ABSTRACT: Isotopically labeling a metabolite and tracing its metabolic fate has provided invaluable insights about the role of metabolism in human diseases in addition to a variety of other issues. ¹³C-labeled metabolite tracers or unlabeled ¹H-based NMR experiments are currently the most common application of NMR to metabolomics studies. Unfortunately, the coverage of the metabolome has been consequently limited to the most abundant carbon-containing metabolites. To expand the coverage of the metabolome and enhance the impact of metabolomics studies, we present a protocol for ¹⁵N-labeled metabolite tracer experiments that may also be combined with routine ¹³C tracer experiments to simultaneously detect both ¹⁵N- and ¹³C-labeled metabolites in metabolic samples. A database consisting of 2D ¹H–¹⁵N HSQC natural-abundance spectra of 50 nitrogen-containing metabolites are also presented to facilitate the assignment of ¹⁵N-labeled metabolites. The methodology is demonstrated by labeling *Escherichia coli* and *Staphylococcus aureus* metabolomes with ¹⁵N₁-ammonium chloride, ¹⁵N₄-arginine, and ¹³C₂-acetate. Efficient ¹⁵N and ¹³C metabolite labeling and identification were achieved utilizing standard cell culture and sample preparation protocols.



Over the last half century, a variety of OMICS techniques have become an integral component of most biological research projects. Accordingly, metabolomics, the newest member of the OMICS field, has evolved to becoming a fundamental contributor to systems biology.¹ In this regard, metabolomics has been applied to a wide range of research areas including drug discovery, disease diagnosis, biomarker discovery, plant biology, environmental issues, and nutrition.^{2–8} Metabolome profiling techniques have undergone tremendous advancements from their early historical application of smell and taste tests to the utilization of modern state-of-the-art analytical instrumentation.⁹ To date, the majority of metabolomics studies rely on mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR), or Fourier transform infrared spectroscopy (FTIR) to characterize a metabolome.¹⁰ While the application of metabolomics has grown exponentially over the past decade,¹¹ like all new technologies, metabolomics is still undergoing an iterative series of optimization and methodology development steps that are necessary to improve the technology. In essence, metabolomics is still maturing as a technology, and while it is becoming widely adopted by the scientific community, it is essential that metabolomics provides highly reliable and reproducible results.

Metabolomics currently faces a number of significant challenges such as the rapid and reliable assignment of known metabolites, the identification of unknown metabolites, and the

establishment of protocols to detect the “dark metabolome.”^{12–14} Metabolomics relies heavily on the availability of electronic databases that provide standard spectral information for individual metabolites in order to identify metabolites in biological samples. In fact, there is a growing proliferation of databases [Biological Magnetic Resonance Data Bank (BMRB), Birmingham Metabolite Library-NMR (BML-NMR), Complex Mixture Analysis by NMR and TOCSY customized Carbon Trace Archive (COLMAR-TOCCATA), Golm Database (GMD), Human Metabolome Database (HMDB), Madison Metabolomics Consortium Database (MMCD), NMR Shift Database (NMRShiftDB), Mass Bank, and MetLin] that contain reference NMR and mass spectral data routinely used to assist in the assignment of metabolites.^{15–25} A more recent development has been the emergence of specialized databases such as the Cerebrospinal Fluid (CSF) and Serum Metabolome Databases, the *Escherichia coli* Metabolome Database (ECMDB), and the ReSpec database for phytochemicals.^{5,6,19,26–28} Not surprisingly, a significant amount of redundancy occurs across these databases, while some data are unique to a specific database. Consequently, the distribution of reference data across multiple

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databases presents a practical daily challenge to the routine identification of known metabolites. Simply, an investigator needs to interrogate multiple databases to make sure a proper metabolite assignment is made. Therefore, there is a clear need to establish a concerted and coordinated effort to eliminate this unnecessary redundancy, consolidate databases and, more importantly, to expand the unique information within databases. Focusing effort on increasing the number of reference spectra is essential to expanding the coverage of the metabolome.

For example, the human metabolome database currently lists only 1551 compounds with an associated NMR spectrum, but the lower limit estimate for the number of endogenous and exogenous human metabolites is around 150 000.²⁹ The situation is significantly improved for mass spectral data where there are over 325 000 compounds with an MS spectrum. Nevertheless, the number of metabolites routinely detected in targeted or untargeted metabolomics studies is typically less than 200 or occasionally upward of 1500 metabolites.²⁹ So, especially in the case of NMR, additional reference spectra are needed to facilitate metabolite assignments. But, as evident by the abundance of mass spectral data, additional reference information is not sufficient to improve the coverage of the metabolome. Additional detection methods are also needed.

Both NMR and MS routinely utilize stable isotope-resolved metabolomics (SIRM) techniques¹⁰ to expand the coverage of the metabolome and facilitate metabolite assignments. The SIRM approach typically uses a readily available ¹³C-labeled nutrient source (e.g., ¹³C₆ glucose) to incorporate an isotope label into the metabolome. SIRM are also routinely used to monitor metabolic fluxes. In this manner, NMR and MS are able to readily detect the ¹³C-labeled metabolites derived from the nutrient source. SIRM effectively highlights a specific subset of the metabolome, which may improve the detection of metabolites not typically observed without the isotope label. Again, this is especially true for NMR, since the incorporation of the ¹³C isotope label greatly enhances NMR sensitivity. ¹²C nuclei are not NMR active, and consequently, not observable in an NMR spectrum. Since the natural abundance of the NMR-active ¹³C nuclei is only 1.1%, the incorporation of “100%” ¹³C label may easily lead to a 100-fold increase in sensitivity. Similarly, the incorporation of the ¹³C label is evident in an MS spectrum by a proportional change in the intensity of the monoisotopic peak profile. Accordingly, an MS spectrum can identify the number of ¹³C carbons incorporated into the metabolite. Conversely, NMR can effectively trace the exact atom position for each ¹³C carbon incorporated into the metabolite from the labeled nutrient. Thus, NMR and MS allows for a simple and elegant ability to trace each ¹³C atom across multiple metabolic pathways and to identify enriched and upregulated metabolic pathways. Again, the SIRM method allows for the detection of metabolites that may not be readily observable without the incorporation of an isotope label. Importantly, alternative regions of the metabolome can be emphasized by simply using a different ¹³C-labeled nutrient such as alanine, acetate, glutamine, lactate, ketone body, palmitic acid, etc. in order to follow the flow of ¹³C atoms through a specific set of metabolic pathways. To date, the vast majority of SIRM studies have relied on ¹³C-labeled metabolites, which of course, significantly limits the coverage of the metabolome to only carbon-containing metabolites. There are other nuclei of biological importance (e.g., ¹⁵N, ³¹P, and ³³S) that are NMR active, but with variable natural abundance. Specifically, while ¹⁵N isotope labeling has been extensively used in NMR protein structure determination, it has been rarely applied to metabolomics

experiments. A major road block for such experiments is a lack of established and standardized protocols and the lack of available ¹⁵N NMR reference spectra to assign ¹⁵N-containing metabolites.

Cellular metabolism is rich in nitrogen-containing metabolites (e.g., adenine, biotin, thiamine, etc.), which are key components of the building blocks of life, from genetic information to proteins. Nitrogen-containing metabolites may also function as regulators of various biological processes. Accordingly, nitrogen-containing metabolites and nitrogen metabolism is important across all organisms.^{30–34} For example, nitrogen metabolism in Gram-positive bacteria (e.g., *Bacillus subtilis*, *Staphylococcus aureus*) is controlled through CodY, a highly conserved repressor that responds to changes in amino acid concentrations.³⁵ As a result, CodY controls amino acid transport and, accordingly, regulates virulence factors. Similarly, for *Mycobacterium tuberculosis*, various amino acids, such as arginine, aspartate, and tryptophan, have been identified to play a role in the development of resistance to hypoxic stress, acid stress, and immune-system-driven starvation.³⁶ Other examples of the importance of nitrogen metabolism to cellular processes include the recent observation that mTORC, a major metabolic regulator associated with cell division and cancer, is controlled through nutrient sensing of amino acids.^{37,38} Similarly, glutamine addiction in cancer cells has been linked to drug resistance (i.e., gemcitabine resistance in pancreatic cancers).³⁹ Metabolic recycling of ammonia has been attributed to breast cancer proliferation and reprogramming of branch chained amino acids to myeloid leukemia progression.^{38,40,41} As these examples illustrate, nitrogen-containing metabolites, nitrogen metabolism, and maintaining an overall nitrogen balance are all critical factors in human diseases and for preserving proper cellular processes. Consequently, nitrogen-containing metabolites comprise a significant component of the cellular metabolome. The overall contribution of nitrogen-containing metabolites to some common metabolome databases is highlighted in Figure 1. On average, ~40% of the metabolites (15 to 73%) within these databases are a nitrogen-containing metabolite and comprise chemically diverse classes of compounds. Therefore, being able to expand the coverage of the metabolome by detecting these nitrogen-containing metabolites and being able to follow nitrogen flow through the metabolome is critical to an accurate and thorough analysis of a metabolome.

In addition to the lack of ¹⁵N NMR reference spectra, nitrogen-based metabolomics has seen limited usage because of the low natural abundance of ¹⁵N (0.365%), the intrinsically low sensitivity of the ¹⁵N nuclei (0.104% vs ¹H), and a further decrease in sensitivity due to exchange broadening. NH, NH₂, and NH₃ protons tend to be very labile and exchange readily with solvent, especially at biologically relevant pH values of 6.8 to 7.4. As a result, nitrogen protons may not be detectable by NMR under typical experimental conditions for metabolomics studies. To resolve this issue, we have developed experimental protocols and assembled reference NMR spectra to facilitate the detection of nitrogen-containing metabolites. Herein, we describe our protocol to expand NMR metabolomics databases to include two-dimensional (2D) ¹H–¹⁵N heteronuclear single quantum coherence (HSQC) spectra for a range of common nitrogen-containing metabolites. We demonstrate the utility of our methodology by characterizing ¹⁵N-labeled and ¹³C-, ¹⁵N-labeled metabolomics samples obtained from *E. coli* and *S. aureus* cell lysates.

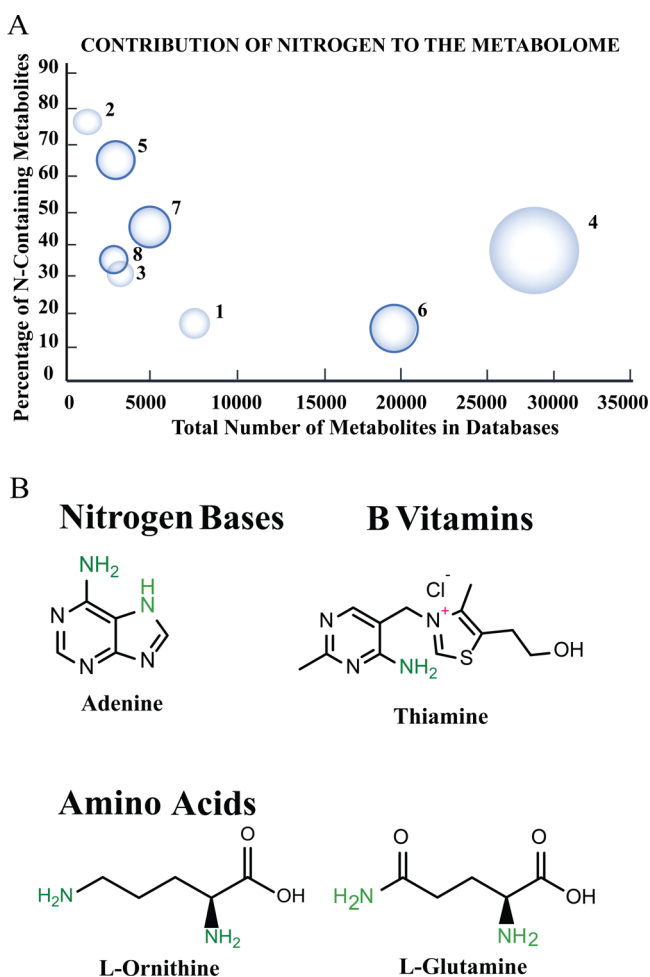


Figure 1. (A) Bubble plot showing the contribution of nitrogen-containing metabolites to each of the metabolome databases: (1) BMDB, (2) BMRB, (3) ECMDB, (4) foodDB, (5) HMDB, (6) MMCD, (7) plantcyc, and (8) RikenPRIME. Each database was searched for compounds containing at least one nitrogen atom. The number of nitrogen-containing metabolites listed in each database is as of May 15th 2017. (B) Examples of common nitrogen-containing metabolites illustrating the chemical diversity of the metabolome.

METHODS

Preparation of Standard NMR Samples of Nitrogen-Containing Metabolites. An initial standard NMR sample consisting of a small mixture of nitrogen-containing compounds was prepared to optimize sample conditions and experimental protocols. Please see [Supporting Information](#) for the list of chemicals used in this study. The metabolites were selected based on a relative importance for central nitrogen metabolism and/or as a routine target of metabolomics studies and consisted of glutamine, glutamate, arginine, and urea. Uniformly ^{15}N -labeled samples for the four compounds were combined to prepare a single standard NMR sample. Each compound (1 mM) was dissolved in 500 μL of 25 mM phosphate buffer at pH 7, 4, or 2 (uncorrected) in NANOPure water (Barnstead, Dubuque, IA, U.S.A.) with the addition of 50 μL of D_2O (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, U.S.A.) and 500 μM TMSP. The samples were transferred to a 5 mm NMR tube and used to collect 2D ^1H - ^{15}N HSQC spectra.

Singleton NMR samples were prepared for 46 other nitrogen-containing metabolites listed in [Table S1](#). Specifically, an unlabeled sample of each metabolite was dissolved to saturation

in 500 μL of 25 mM phosphate buffer at pH 2 (uncorrected) in NANOPure water with the addition of 50 μL of D_2O . The samples were transferred to a 5 mm NMR tube and used to collect 2D ^1H - ^{15}N HSQC spectra.^{42,43}

Preparation of ^{13}C - ^{15}N -Labeled Metabolomic Samples from Cell Lysates. *E. coli* (strain MG1655) was cultured in M9 minimal media containing $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source and $^{13}\text{C}_2$ acetate (Isotec, Sigma-Aldrich, Miamisburg, OH, U.S.A.) as the only source of carbon. Triplicate 25 mL cell cultures were grown aerobically until a final O.D.₆₀₀ of 36. Cells were harvested at the 12 h time point upon reaching stationary phase. Each cell culture was centrifuged at 5000 rpm for 20 min at 4 $^\circ\text{C}$ to pellet the cell suspension. Pellets were resuspended in 1.5 mL of 1:1 water/methanol solution. Cells were mechanically lysed by sonication using five 30 s intervals. The lysed cells were then centrifuged at 13 000 rpm for 20 min at 4 $^\circ\text{C}$, and 1 mL of the supernatant was transferred to a microcentrifuge tube. The metabolome cell extraction protocol was repeated, and the two supernatants were combined. The samples were kept on ice during the entire extraction process. Methanol was removed using a SpeedVac Plus SC110A system (Savant, Thermo Scientific, Asheville, NC, U.S.A.), and the samples were then lyophilized using a FreeZone freeze dryer (Labconco, Kansas City, MO, U.S.A.).

S. aureus (strain JE2) were cultured in a chemically defined medium (CDM) comprising 18 amino acids, where $^{14}\text{N}_4$ arginine was replaced with 0.1 g/L of $^{15}\text{N}_4$ arginine (Isotec, Sigma-Aldrich, Miamisburg, OH, U.S.A.).⁴⁴ Five replicates of 50 mL cultures of *S. aureus* were grown aerobically to an optical density (O.D.₆₀₀) of 1.9. Cultures were normalized to an O.D.₆₀₀ of 40 and pelleted by centrifugation (4000 rpm at 4 $^\circ\text{C}$ for 5 min). The resulting cell pellets were kept on ice throughout the entire metabolome extraction process. Cell pellets were washed twice with 10 mL of sterile ice-cold double distilled water (ddH_2O) and then centrifuged at 4000 rpm for 5 min at 4 $^\circ\text{C}$. Each pellet was then resuspended in 1 mL of sterile ice-cold ddH_2O , transferred to a 2 mL FastPrep Lysing Matrix B tube, and then mechanically lysed twice at speed 6 for 40 s with a 5 min interval. The cell lysates were then centrifuged at 13 200 rpm for 15 min at 4 $^\circ\text{C}$ and 800 μL of the supernatant was transferred to a 2 mL tube. The cell pellets were resuspended with 1 mL of ice-cold sterile ddH_2O and centrifuged at 13 200 rpm for 15 min at 4 $^\circ\text{C}$. Then, 1 mL of the supernatant was pooled together with the previous 800 μL cell extract and centrifuged at 13 200 rpm for 10 min at 4 $^\circ\text{C}$. The metabolome extracts were snap frozen in liquid nitrogen and lyophilized using FreeZone freeze dryer.

The *E. coli* and *S. aureus* metabolome extracts were dissolved in 500 μL of 25 mM phosphate buffer at pH 2 (uncorrected) in NanoPure water with the addition of 50 μL of D_2O and 500 μM TMSP. The samples were transferred to a 5 mm NMR tube and used to collect the 2D ^1H - ^{15}N HSQC and 2D ^1H - ^{13}C HSQC spectra.^{42,43}

NMR Data Collection and Processing. All NMR experiments were conducted at 277.15 K using a Bruker AVANCE III-HD 700 MHz spectrometer equipped with a 5 mm quadruple resonance QCI-P cryoprobe (^1H , ^{13}C , ^{15}N , and ^{31}P) with z -axis gradients. A SampleJet automated sample changer system with Bruker ICON-NMR software was used to automate the NMR data collection. Please see the [Supporting Information](#) for further details on the routine protocols used for NMR data collection and processing. A natural-abundance 2D ^1H - ^{15}N HSQC reference spectrum was collected for each of the standard nitrogen-containing metabolites listed in [Table S1](#). The 2D ^1H - ^{15}N HSQC were collected with nonuniform sampling

(25% sampling sparsity)⁴⁵ and were Fourier transformed, phased, and reconstructed using Topspin 3.5. The 2D ^1H – ^{15}N HSQC reference spectra were used to annotate the NMR spectra obtained for the *E. coli* and *S. aureus* metabolome extracts.

RESULTS AND DISCUSSIONS

Optimization of Sample Conditions. Nitrogen protons tend to be labile and to exchange rapidly with solvent as a function of pH, and correspondingly, are difficult to observe by NMR in aqueous solvents. Simply, the NMR resonance is severely line-broadened into the baseline due to the solvent exchange. Direct detection of ^{15}N NMR resonance is equally challenging because of the low natural abundance (0.365%) and low sensitivity (0.104% vs ^1H) of the ^{15}N nuclei. Thus, direct detection of a 1D ^{15}N spectrum, even with enrichment of the ^{15}N signal with ^{15}N labeling, is not practical for a metabolomics study

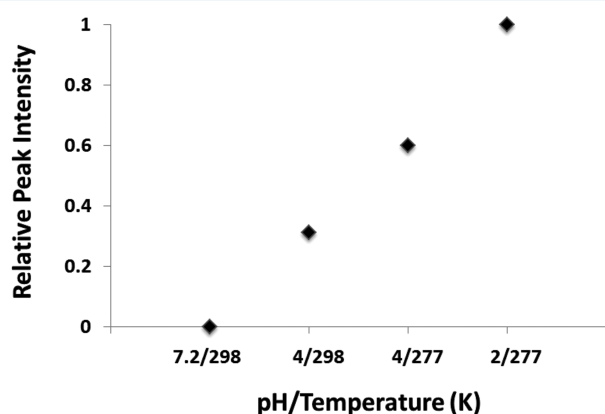


Figure 2. Plot of the sum of intensities for all peaks observed in the 2D ^1H – ^{15}N HSQC spectra that were collected at different conditions of pH and temperature.

because of the long acquisition times (hours) and large data sets. As a result, nitrogen-based NMR experiments and the detection of nitrogen-containing metabolites have seen limited applications in metabolomics. To circumvent these issues, a variety of experimental conditions were investigated to optimize the routine and reliable observation of nitrogen-containing metabolites by NMR. The two key aspects of the optimization process were sample preparation and NMR data collection, which was accomplished using a standard metabolomics sample containing four nitrogen-containing metabolites.

2D ^1H – ^{15}N HSQC NMR spectra were collected for the standard metabolite sample at pH 7, 4, and 2. Not surprisingly, lowering the pH of the sample lead to a significant improvement in signal-to-noise and in the overall quality of the NMR spectrum. At pH 7, only one metabolite, urea, was detectable. Lowering the pH to 4 resulted in observing three of the four metabolites, with only glutamate being undetected. At pH 2, all of the metabolites were observable, and the overall quality of the spectrum was acceptable, but significant line-broadening was still apparent. Simply, observing a nitrogen proton by NMR is dictated by its pK_a and the pH of the solution. Consequently, a low pH was deemed necessary for observing the majority of nitrogen metabolites. Again, most N–H bonds are easily exchangeable with a deuterium-labeled solvent such as D_2O , but in addition to pH, the exchange rate can also be reduced by lowering the sample temperature. Accordingly, a 2D ^1H – ^{15}N HSQC NMR spectrum was also collected for the standard metabolite sample at 298, 283, and 277.15 K. The impact of lowering the temperature on the quality of the NMR spectra was significantly less dramatic than the impact of pH. Overall, lowering the temperature to 277.15 K resulted in only a 1.6-fold increase in signal-to-noise. More importantly, combining a low pH (pH 2) with a low sample temperature (277.15 K) allowed for the detection of all of the nitrogen-containing metabolites

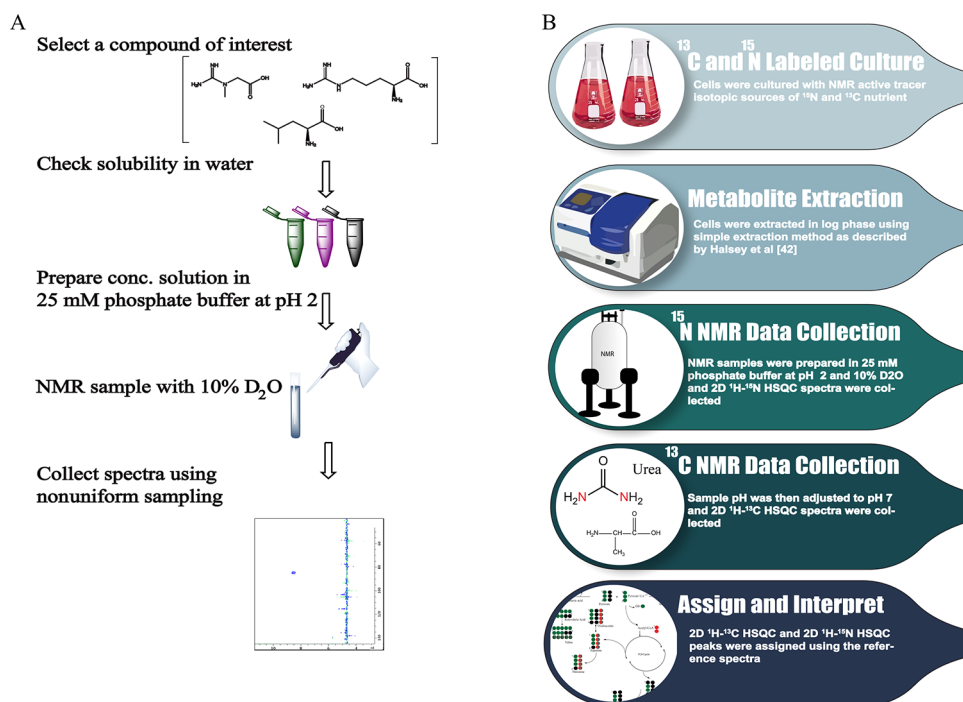


Figure 3. (A) Flowchart of protocol for collecting natural-abundance 2D ^1H – ^{15}N HSQC reference spectra for nitrogen-containing metabolites. (B) Flowchart illustrating the process of simultaneously analyzing ^{13}C - and ^{15}N -labeled metabolites by NMR. The figure was created with ChemDraw Professional 15.0 (PerkinElmer Informatics, Waltham, MA, U.S.A.).

tested (Table S1). Collecting the 2D ^1H – ^{15}N HSQC experiment at pH 2 and 277.15 K resulted in a significant improvement in the quality of the spectrum compared to spectra collected at higher pH or temperatures (Figure 2). The high pH dependency of NH , NH_2 , and NH_3 NMR resonances raises a legitimate concern regarding the reliability of metabolite quantification. Accordingly, great care is required to minimize pH variance between NMR metabolomics samples, which is achievable by optimizing buffer concentration, using an internal pH standard,⁴⁶ or manually verifying sample pH values.

2D ^1H – ^{15}N HSQC Spectral Database for Nitrogen-Containing Metabolites. A set of nitrogen-containing metabolites were identified to compile a 2D ^1H – ^{15}N HSQC spectral database. The set of reference spectra and chemical shifts will enable the routine identification of nitrogen-containing metabolites as part of any metabolomics study. Metabolites included in the database were first identified by general chemical classes, for example, amino acids, urea-cycle-related metabolites, amino-sugars, and nucleotides. We then used our existing FAST-NMR chemical library⁴⁷ to identify additional metabolites (e.g., cofactors and vitamins) to also include in the NMR reference

database. The remaining metabolites populating the database were identified based on general knowledge of metabolic processes and/or corresponded to compounds routinely encountered in cell extracts. In total, an initial set of 65 metabolites were identified.

A natural-abundance 2D ^1H – ^{15}N HSQC spectrum was collected for each individual compound at pH 2 and 277.15 K. We had to rely on natural-abundance HSQC spectra because of the limited availability of ^{15}N -labeled compounds from chemical vendors. Accordingly, a concentrated stock solution was required in order to obtain a high-quality 2D ^1H – ^{15}N HSQC spectrum of a natural abundant metabolite. To achieve this, NMR samples were prepared at concentrations in the range of 100–300 mM. In most cases, sample concentrations were close to saturation. Due to low-solubility issues, a 2D ^1H – ^{15}N HSQC spectrum was not obtainable for 15 of the selected compounds (Table S2). Non-uniform sampling (NUS) was used to reduce the total experimental time and maximize the signal averaging to further improve the overall quality of the natural-abundance HSQC spectra. The HSQC spectra were collected at 25% sparsity using a deterministic gap sampling method⁴⁵ and reconstructed with

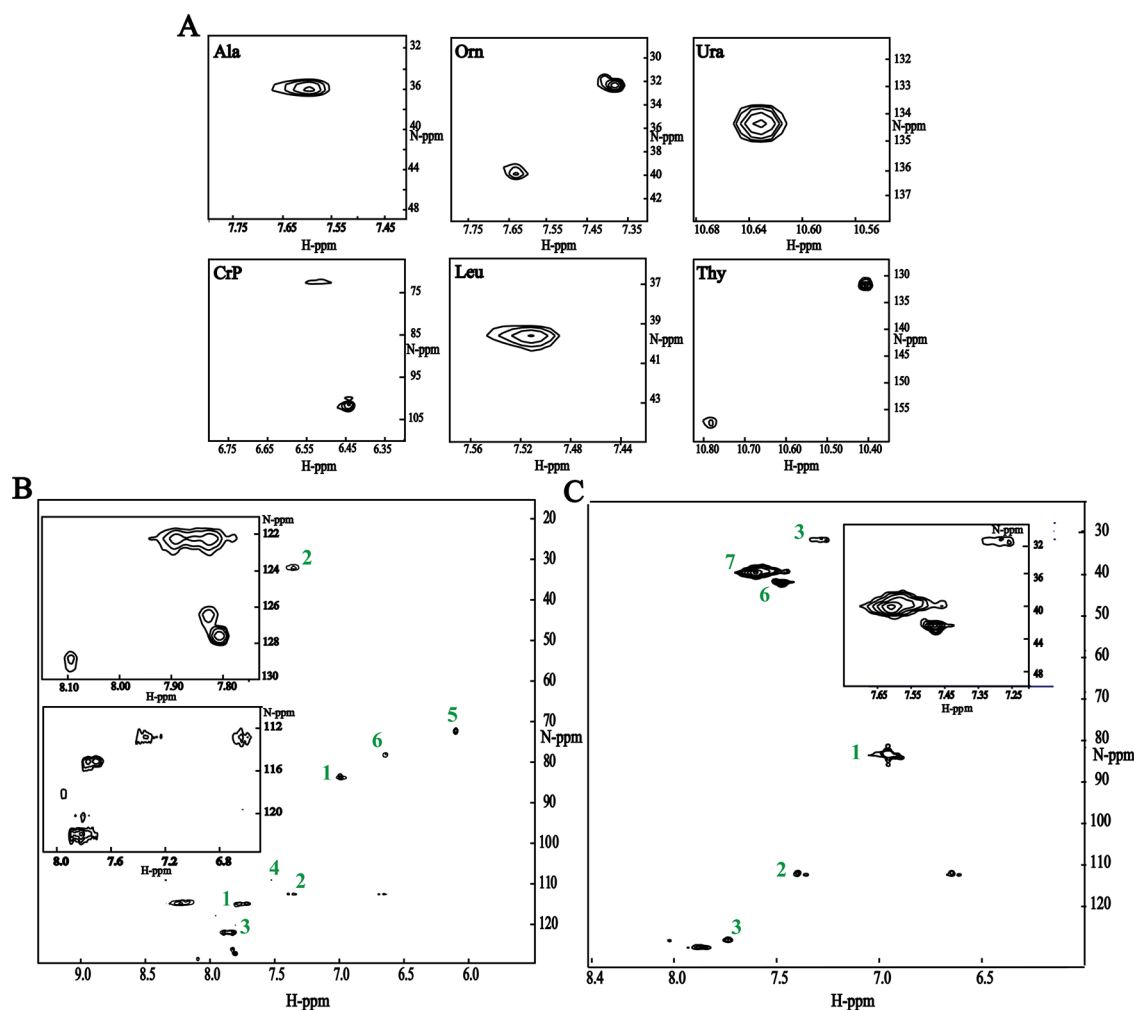


Figure 4. (A) Representative examples of 2D ^1H – ^{15}N HSQC spectra obtained for natural-abundance nitrogen-containing metabolites. [L-alanine (Ala), ornithine (Orn), uracil (Ura), creatine phosphate (CrP), L-leucine (Leu), thymine (Thy)]. (B) 2D ^1H – ^{15}N HSQC spectra obtained for *E. coli* cell lysate. *E. coli* was cultured in minimal M9 media with the addition of $^{15}\text{NH}_4\text{Cl}$. Cells were harvested during stationary phase. 1: arginine, 2: glutamine, 3: asparagine, 4: NAD, 5: ornithine, 6: 5-azacytidine. (C) 2D ^1H – ^{15}N HSQC spectra obtained for *S. aureus* cell lysate. *S. aureus* was cultured in chemically defined media with the addition of $^{15}\text{N}_4$ L-arginine. Cells were harvested during stationary phase. 1: arginine, 2: glutamine, 3: asparagine, 4: NAD, 6: aminobutyrate, 7: glutamate.

the compressed sensing algorithm. The final protocol used to obtain the reference database of 2D ^1H – ^{15}N HSQC spectra is summarized in Figure 3A. In total, 2D ^1H – ^{15}N HSQC spectra and ^1H and ^{15}N chemical shift assignments were obtained for 50 common metabolites, which are summarized in Table S1. Representative examples of a number of the experimental 2D ^1H – ^{15}N HSQC spectra obtained for nitrogen-containing metabolites are shown in Figure 4A.

^{15}N -Labeled and ^{13}C - and ^{15}N -Labeled Metabolomics in Bacterial Systems. The utility of our protocol for obtaining 2D ^1H – ^{15}N HSQC spectra of nitrogen-containing metabolites was demonstrated on metabolomics samples extracted from *E. coli* and *S. aureus* cell lysates (Figure 3B). Furthermore, two different nitrogen sources (e.g., $^{15}\text{NH}_4\text{Cl}$, $^{15}\text{N}_4$ arginine) were

used to incorporate ^{15}N labels into the metabolomes. Additionally, $^{15}\text{N}_1$ ammonium chloride was combined with $^{13}\text{C}_2$ acetate to demonstrate the simultaneous incorporation of both ^{15}N and ^{13}C labels into the metabolomics pool (Figure 3B). This illustrates the overall versatility of the approach and the inherent flexibility of utilizing a variety of nitrogen and carbon sources. The 2D ^1H – ^{15}N HSQC spectrum of the *E. coli* cell lysate following ^{15}N labeling with $^{15}\text{NH}_4\text{Cl}$ is shown in Figure 4B. A total of five ^{15}N -labeled metabolites were identified. Since the *E. coli* cells were stressed due to nutrient limitation, these five metabolites likely represent the major *E. coli* nitrogen pool necessary for cell survival during stationary phase. Presumably, the remainder of the ^{15}N nitrogens is distributed to other metabolites and biomolecules at concentrations levels below detection by NMR.

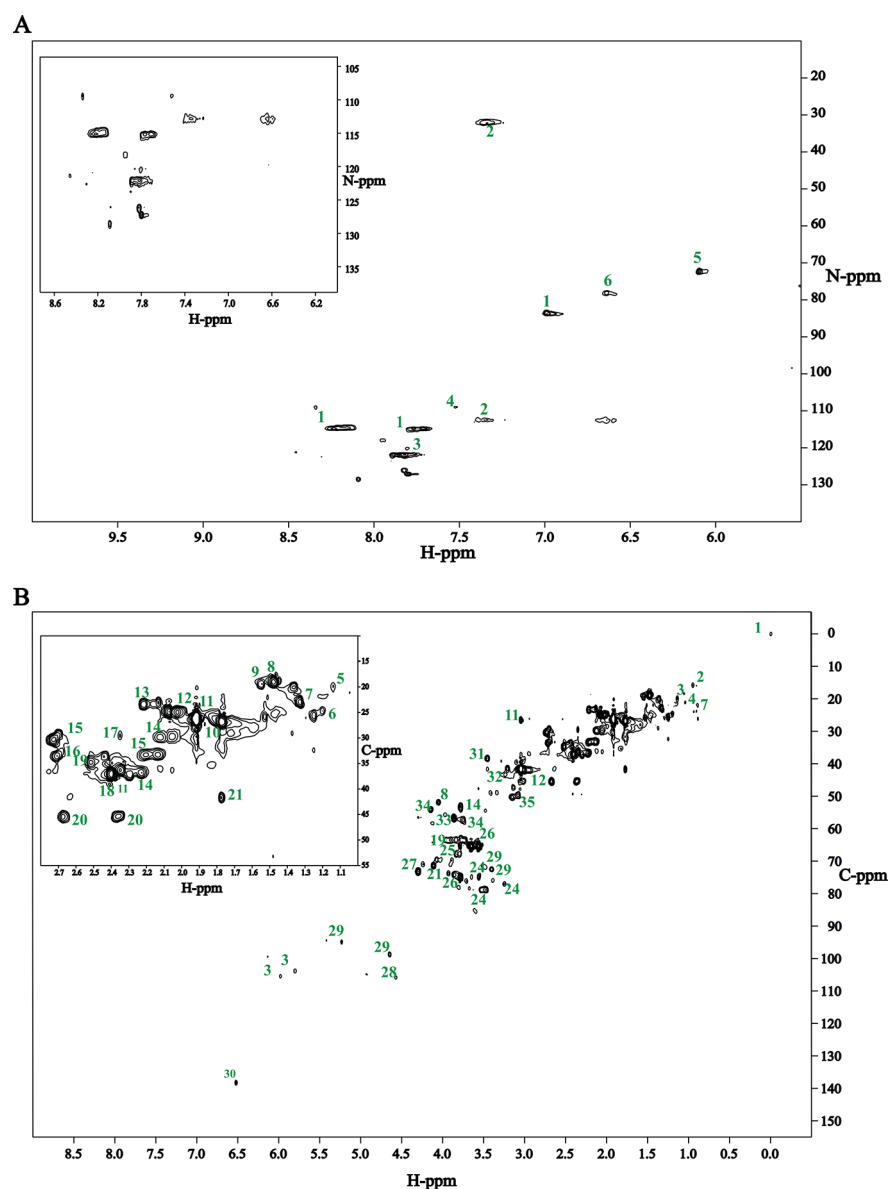


Figure 5. (A) 2D ^1H – ^{15}N HSQC spectrum and (B) 2D ^1H – ^{13}C HSQC spectrum obtained for *E. coli* cell lysate. *E. coli* was cultured in minimal M9 media with the addition of $^{15}\text{NH}_4\text{Cl}$ and $^{13}\text{C}_2$ acetate. Cells were harvested during stationary phase. Assignments for peaks in 2D ^1H – ^{15}N HSQC spectra can be found in Figure 4. Assignments for peaks in the 2D ^1H – ^{13}C HSQC spectrum are 1: Internal standard TMSP, 2: Butyrate, 3: Uridine diphosphate glucose, 4: 2-methyl glutarate, 5: 2-methyl glutarate, 6: 3-hydroxybutyrate, 7: valerate, 8: alanine, 9: butanone, 10: acetate, 11: 2-aminobutyric acid, 12: ornithine, 13: acetyl carnithine, 14: glutamate, 15: cystathionine, 16: methionine, 17: pyruvate, 18: glutamyl-folate, 19: serine, 20: malate, 21: *N*-acetylmannosamine, 24: glucose, 25: ascorbate, 26: glycerol, 27: ribose, 28: celliobiose, 29: glucuronic acid, 30: fumarate, 31: uracil, 32: *N*-acetyl lysine, 33: *N*-acetyl glucosamine, 34: glutamine, 35: malonic acid.

Similarly, the 2D ^1H – ^{15}N HSQC spectrum of the *S. aureus* cell lysate following ^{15}N labeling with $^{15}\text{N}_4$ arginine is shown in Figure 4C. A total of seven ^{15}N -labeled metabolites were identified. Since $^{15}\text{N}_4$ arginine was added to a standard culture medium, it represents only about 2% of the total nitrogen pool. Accordingly, the seven detected metabolites correspond to metabolites that were only derived from arginine metabolism. Thus, the ^{15}N -labeled metabolomes from *E. coli* and *S. aureus* are highlighting distinctly different cellular and metabolic processes. It is important to note that since only the *E. coli* and *S. aureus* cell lysates were analyzed, additional ^{15}N -labeled metabolites may have been excreted into the culture media. Significantly, both HSQC spectra were completely assigned using our reference 2D ^1H – ^{15}N HSQC spectral database and chemical shift assignments (Table S1). Furthermore, these results demonstrate that it is readily achievable to ^{15}N label metabolites with either ^{15}N -labeled salts or ^{15}N -labeled amino acids. In effect, the metabolome can be globally or specifically targeted based on the choice of nitrogen source.

The *E. coli* metabolomics pool was also successfully labeled with both ^{15}N and ^{13}C . The ^{15}N - and ^{13}C -labeled metabolites were identified by collecting a 2D ^1H – ^{15}N HSQC spectrum and a 2D ^1H – ^{13}C HSQC spectrum on the same metabolomics sample (Figure 5). The 2D ^1H – ^{15}N HSQC spectrum was collected first using a sample pH of 2 and a sample temperature of 277.15 K. The sample pH was then adjusted to pH 7 to collect the 2D ^1H – ^{13}C HSQC spectrum at room temperature. This was necessary, since most metabolomics databases contain reference 2D ^1H – ^{13}C HSQC spectra that have been collected at pH 7 and 25 °C. Again, both HSQC spectra were completely assigned using the combination of our 2D ^1H – ^{15}N HSQC spectral database and the BMRB/HMDB databases. In this example, all of the ^{15}N -labeled metabolites (five in total) were also observed as ^{13}C -labeled metabolites (34 in total). Nevertheless, even though no new metabolites were identified, the differently labeled metabolites monitor distinctly different metabolic pathways. In essence, the specific isotopic label within the metabolite conveys the expanded coverage of the metabolome. Consider the fact that the incorporation of ^{13}C carbons from $^{13}\text{C}_2$ acetate is monitoring carbon flow through the tricarboxylic acid (among other pathways), whereas the incorporation of ^{15}N nitrogen from $^{15}\text{NH}_4\text{Cl}$ occurs through nitrogen metabolism (including arginine biosynthesis, glutamate metabolism, etc.). Thus, the same ^{15}N - or ^{13}C -labeled metabolite provides fundamentally distinct information regarding changes in cellular processes that may be missed if only one label was used. For example, a study interested in amino acid metabolism may benefit from obtaining reliable Glu/Gln or Asp/Asn ratios, which would be an obvious use of ^{15}N labels. These results clearly demonstrate that the simultaneous incorporation of ^{15}N and ^{13}C labels is a viable option for NMR metabolomics. Furthermore, being able to expand the coverage of the metabolome by enabling the routine detection and identification of nitrogen-containing metabolites will be a valuable addition to any metabolomics study.

CONCLUSIONS

A standard protocol for the collection of 2D ^1H – ^{15}N HSQC spectra for the detection of nitrogen-containing metabolites has been presented. Furthermore, a reference database of 2D ^1H – ^{15}N HSQC spectra and the associated chemical shift assignments for 50 commonly encountered nitrogen-containing metabolites has been assembled to facilitate the rapid and easy

identification of these metabolites. Taken together, these results expand the coverage of the metabolome, especially since this tool gives a way to probe nitrogen metabolism with an atom-specific understanding of the metabolome. As nitrogen-containing metabolites comprise more than 30% of the known metabolome, it is likely that ^{15}N incorporation can highlight distinct metabolic pathways. Additionally, we also demonstrate that the simultaneous incorporation of both ^{15}N and ^{13}C labels into a metabolomics sample should be a routine choice for most metabolomics studies. Accordingly, the ^{15}N - and ^{13}C -labeled metabolites are readily assigned by the sequential collection of 2D ^1H – ^{15}N / ^{13}C HSQC spectra. It is worth noting that ^{13}C and ^{15}N labeling of metabolites in prokaryotic^{35,36} and mammalian^{37–40} cell cultures is routinely achievable. As recently demonstrated,⁴¹ 2D ^{15}N heteronuclear multibond correlation (HMBC) experiments were able to monitor cancer processes involving ^{15}N -labeled metabolites, which further highlights the overall versatility of NMR and the viability of nitrogen-based metabolomics studies. An outstanding challenge in SIRM techniques is obtaining similar results in animal models or human clinical studies. An alternative approach would be to collect natural-abundance HSQC spectra by combining NUS with fast data acquisition techniques⁴⁸ and by maximizing the sample size and the number of scans within practical limits.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04922.

List of chemicals used in this study; NMR data collection and processing methods; table of chemical shifts for nitrogen-containing metabolites from 2D ^1H – ^{15}N HSQC spectra; table of nitrogen-containing metabolites with low aqueous solubility (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

NMR nuclear magnetic resonance
E. coli *Escherichia coli*
S. aureus *Staphylococcus aureus*
HSQC heteronuclear single quantum coherence

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