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Perspective

Securing the Future of NMR Metabolomics Reproducibility: A Call for Standardized Reporting

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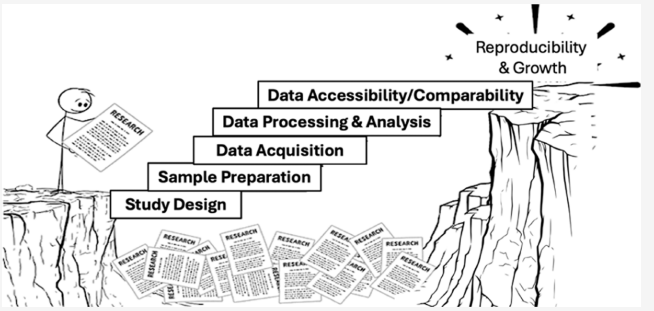
ABSTRACT: Metabolomics is a rapidly growing multidisciplinary field with ever increasing demand and usability, which is attracting a surge of new researchers. While their varied skill sets, scientific questions, and approaches enrich the field with fresh perspectives and innovation, individual investigators also bring wide-ranging levels of metabolomics-specific experience and diverse areas of interest. These factors introduce considerable variability and inconsistency in both the methodology and reporting. A recent comparative literature review of nuclear magnetic resonance (NMR) metabolomics from studies published in 2010 and 2020 revealed significant shortcomings in the reporting of experimental details necessary for evaluating both the scientific rigor and the reproducibility of NMR-based metabolomics experiments. Each stage of metabolomics research contains multiple methodological choices and various optimization parameters, all of which can introduce experimental bias and alter the study results. This emphasizes the need for proper reporting to enhance reproducibility, data reusability, and study comparability. To address these concerns, the NMR Special Interest Group within the Metabolomics Association of North America presents reporting recommendations focused on fundamental aspects of NMR metabolomics research identified from the detailed literature review report. These include specifics with respect to study design, sample preparation, data acquisition, data processing and analysis, data accessibility, and comparability to previous studies. Also presented is a complementary list of seminal papers in the field to guide the study design and implementation of NMR metabolomics experiments. This initiative seeks to enhance the long-term impact of NMR metabolomics by supporting high-quality, reproducible, and impactful data collected from well-executed and thoroughly reported studies.



1. INTRODUCTION

Over the course of the past 25 years, the field of metabolomics has expanded in conjunction with many analytical and methodological advancements.[1] These advancements, along with the establishment of numerous academic societies and consortia,[2−4] and metabolomics databases and repositories[5] have led to the rapid growth of metabolomics-based research. Although this growth underscores the broad utility of metabolomics, it has also brought together researchers of various subdisciplines and training backgrounds, increasing methodological variability within the field.[3,6] These factors necessitate the establishment of standardized best practices, particularly for the standardization of reporting metrics, to improve interpretability and comparability across the field of metabolomics.[7]

Efforts to establish reporting standards in both nuclear magnetic resonance (NMR)- and mass spectrometry (MS)-based metabolomics have been initiated by several organizations, including Metabolomics Standards Initiative (MSI), Coordination of Standards in Metabolomics (COSMOS), Metabolomics Association of North America (MANA), and Metabolomics Quality Assurance & Quality Control (QC) Consortium (mQACC).[6,8−15] The adaptation of recommen-

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ded reporting standards, however, has been inconsistent.[16,17] For instance, a large-scale, NMR-specific literature review that quantitatively assessed methodological reporting patterns in the metabolomics literature revealed minimal or near-absent reporting of many fundamental parameters needed to properly describe NMR-based metabolomic studies.[18] The challenge lies in the fact that metabolomics is a rapidly evolving field that requires a diversity of technical expertise and analytical skills. These are often difficult to acquire within a single research group. Further, the low barrier to entry in the field and the absence of widely distributed and well-vetted best practices have contributed to laboratory-specific methodologies, which limit the reproducibility and reuse of metabolomics data for other investigators. Metabolomics necessitates continuous, collaborative communication among researchers to promote and adopt best practices including reporting norms embraced by the research community. For example, enforcement of specific reporting criteria (e.g., by journals, funding agencies, or academic societies) has proven difficult in such a dynamic field where the diversity of metabolomics applications that span various biological contexts require standard practices tailored to individual uses. The inconvenience of detailed reporting leads to over-reliance on citing previous publications for methods without providing full experimental details. This practice often omits critical information, further challenging the widespread adoption of reporting guidelines.[7,17] The lack of properly validated and widely adopted best practices is likely contributing to the reproducibility crisis in metabolomics.[19−22] Thus, the establishment of community-adopted best practices and reporting standards would help to address reproducibility concerns and enhance the value of metabolomics studies.

NMR-based metabolomics data can be analyzed in two fundamentally different ways. The first approach is a purely quantitative method that uses spectral deconvolution to quantify a predefined set of metabolites in the sample prior to downstream statistical analysis. The second approach employs statistical methods (e.g., principal component analysis, partial least squares discriminant analysis (PLS-DA), and statistical total correlation spectroscopy) to identify spectral features and utilizes relative quantification to highlight metabolites that exhibit distinct differences between classes or cases, which may be considered semiquantitative. Quantitative studies typically produce lists of positively identified and accurately quantified compounds and are usually done using commercial programs or company services (e.g., Chenomx,[23] FoodScreener,[24] In Vitro Diagnostics Research (IVDr),[25] and Nightingale Health[26]). These methods use internally added standards and strictly defined sample preparation/acquisition protocols and are limited to a select number of sample types (serum, plasma, urine, and wine).

The term "profiling" has been used variably in the metabolomics literature, which highlights the further need for standardized terminology usage in the field. For example, profiling and "fingerprinting" are routinely and incorrectly used interchangeably.[27] To avoid ambiguity and promote consistent usage of terminology, profiling is defined herein as an NMR workflow that uses a single internal standard for chemical shift reference and then applies various multivariate statistical techniques for feature selection and individual metabolite identification, which is commonly followed by relative or absolute quantification and comparison of these individual metabolites through univariate analysis. Conversely, fingerprinting simply relies on the entire NMR spectral data set with multivariate statistical analysis to discriminate between two or more biological groups without the identification of individual metabolite changes. As quantitative profiling and semi-quantitative profiling or fingerprinting approaches follow different workflows, their reporting requirements also differ. Therefore, our reporting recommendations consider the specific methodologies used in the NMR-based metabolomic studies.

This manuscript serves as a direct follow-up to a previously published review of the NMR metabolomics literature that evaluated both differences and scope in reporting in 2010 versus 2020.[18] The text of this paper highlights and discusses some significant reporting deficiencies identified from that 2024 literature review and expands on additional topics that are insufficiently reported in the literature. Accompanying this paper is a reporting recommendations table that can be utilized as a guide for designing experiments as well as for writing and reviewing metabolomics manuscripts. We also provide a list of seminal references in the Supporting Information that demonstrates the current consensus on the implementation and reporting of experimental parameters in a metabolomics study. The table, text, and Supporting Information have been arranged into five reporting categories that we believe are integral for evaluating study integrity and repeatability: (1) study design, (2) sample preparation, (3) data acquisition, (4) data processing and analysis, and (5) data accessibility and comparability to previous studies. With these reporting categories in mind, researchers can use the table and the references as a template for developing a thorough, well-planned, and properly implemented NMR metabolomics experiment. The overarching goal of this work is to establish minimum reporting criteria in NMR-based metabolomics studies, which will help generate well-executed studies that have the potential to significantly enhance the long-term value of reproducible and meaningful NMR-based metabolomic data. Ultimately, this will help propel progress and discovery in the field of metabolomics research.

2. REPORTING RECOMMENDATIONS AND GUIDELINES

2.1. Study Design. Comprehensive reporting of the experimental design is crucial for providing the necessary context that allows readers to accurately interpret a study, making it essential for the dissemination of research results. A complete description of the overarching goals and specific objectives should form the foundation of a properly reported study, yet fewer than 50% of studies published in 2010 and 2020 reported a clearly stated research hypothesis.[18] This low reporting rate stems in part from field-specific factors, highlighting the ongoing challenges in establishing hypothesis-driven research in NMR metabolomics.

The nature of hypotheses that can be reported in NMR metabolomics studies is determined by the goals set during the planning and development of the study, which occurs prior to the setup of experiments. For example, when experimental questions focus on predetermined sets of metabolites or metabolic pathways (i.e., targeted design), all research hypotheses to be tested should be reported. In contrast, when well-defined phenotypes of interest are compared without prior knowledge of relevant metabolic interactions, a general metabolome assessment can be conducted to form broad conclusions (e.g., compare metabolomes of clinical phenotypes) with the goal of generating specific, testable

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Table 1. Reporting Recommendations for NMR Metabolomics

Section	Category	Subcategory	Information to report
Introduction	1. study design	objectives	scientific hypothesis and study goals clearly stated
Methods	2. sample preparation	sample information	appropriate metadata for reproducing study
		sample pretreatment	collection and storage conditions
		sample processing	extraction or treatment of samples clearly detailed
		sample scheme	order in which samples were extracted; number of batches; how treatments were divided among batches
		QC samples	use and treatment of blanks, technical replicates, pooled QCs, and SRMs
		experimental parameters	sample pH; sample temperature; buffer; number of samples, groups, and replicates per group (biological and analytical); reference compound; spectrometer vendor, model, and frequency; probe type/vendor; experiment conducted with specific pulse sequence; inclusion of presaturation pulse; sample order and acquisition on any QC samples (buffer blanks, extraction blanks, pooled QC, SRMs)
	3. data acquisition	spectral data processing parameters	software used for processing spectra; baseline correction method; phasing (automated or manual); spectral region removal of solvent/buffer peaks; choice of window function; application of zero-filling; line broadening (or any other raw data manipulation); bucketing
	4. data processing and analysis	data manipulation	normalization; centering; transformation; scaling
		statistical methods	statistical software(s) used; unsupervised and supervised multivariate statistics utilized; univariate statistics utilized
		annotation	type of NMR experiments used for metabolite identification and validation, especially in complex mixtures; samples used for metabolite assignment
		spectrometer performance	stability of 90° pulses; water suppression efficiency; signal-to-noise values on standard samples; system suitability test on standard sample (i.e., sucrose)
Results	3. data acquisition	metabolite assignment and quantification	number and list of identified and quantified metabolites; level of assignment for each metabolite
	4. data analysis	statistical validation	minimal fold change; Student's t statistic; p-value; degrees of freedom; false discovery rate or other multiple hypothesis correction; quality values R^2 and Q^2 of supervised multivariate models; multivariate model validation (e.g., CV-ANOVA, permutation test); receiver operating characteristic (ROC) curve-cross validation, sensitivity, and specificity
		QA/QC validation	performance results of the system suitability test; QC precision within and across batches (spectral median RSD; RSDs for each compound); SRM performance metrics; variability
	5. data accessibility	data accessibility	metabolomics data deposition; metadata availability
Discussion	6. data comparability	study comparison and reporting	compared identified metabolites with prior literature, where applicable

hypotheses (e.g., about cellular mechanisms), which were not previously feasible (i.e., untargeted design).[28−31] In such cases, these exploratory aims should be clearly reported in the absence of more traditional hypotheses to avoid any ambiguity.

Due to their complementary nature, untargeted studies should ideally be followed by targeted studies in sequence as a study system becomes more resolved.[28,30,31] Although exploratory studies bring about identification and discovery of undescribed metabolites, researchers should be cautious about designing exploratory studies that simply aim to report detectable metabolites in understudied systems. Such efforts do not necessarily lead to insightful research and often provide minimal utility to the wider research community, especially as the field transitions from hypothesis generation to mechanistic insights into metabolic functions.[1,32] Quantitative NMR studies almost exclusively utilize targeted designs due to methodological constraints (e.g., selection of internally added standards), while profile NMR metabolomics studies can be either targeted or untargeted depending on study design. Additional study designs that may lack traditional hypotheses, such as method and technical papers, should clearly state their objectives similar to the recommendations for untargeted designs described above.

Sample size is a critical factor in a study design, representing the total number of data points in the evaluation of the study objective. Sample numbers greatly impact the ensuing protocols. For example, there is a vast difference between processing a few dozen samples compared with processing hundreds or thousands of samples. A larger number of samples are always desirable to achieve the required statistical power and to obtain meaningful outcomes, especially considering the high-dimensionality of metabolomics data (i.e., observation of more experimental features than samples).[33−35] The type of sample also dictates the number required; therefore, there are no clear rules; only guidelines exist. Researchers should assess the expected biological variability of their samples before determining the appropriate sample size. For example, well-defined samples with modest biological variability (e.g., cell cultures, plant tissues, food and beverage products) require fewer biological replicates compared to more complex biological samples (e.g., animal- and human-derived materials), especially in cases where confounding factors (e.g., local environment, diet, age) cannot be rigorously controlled (see Supporting Information Table S2).

Despite its intrinsic value, the use of analytical replicates was rarely reported in the NMR metabolomics studies previously reviewed,[18] likely due to the high innate reproducibility (i.e., coefficients of variance, CVs $\leq$ 5%) of NMR data, yet analytical replicates can assess variance in sample preparation and instrumental analysis. It is difficult to justify the value of

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including analytical replicates due to the added cost and time, especially for studies involving hundreds to thousands of biological replicates. Of course, numerous practical considerations tend to limit the number of samples that are either available for a metabolomics study or can be properly handled and processed. Based on the total number of samples used (median of 40) and the number of biological replicates per group (median of 10) reported in the 2024 literature review, most metabolomics studies examined, especially clinical studies, seemed to rely on too few biological replicates to obtain statistical significance. This lack of statistical power may contribute to a perceived reproducibility problem in the field.[19−22,36] In response, the metabolomics community is working toward establishing recommendations on the minimum number of biological replicate samples for a reliable metabolomics study (Supporting Information Table S2). These minimum recommendations must take into account the type of samples being analyzed and the study goals.

The experimental design itself largely determines the overall quality of a study. It is thus crucial to clearly report the study objectives and/or hypotheses, and to critically assess these goals to ensure that the proposed work and subsequent methods not only support one another but are also achievable.[37,38] The rapid response of the metabolome to both internal and external factors is part of the widespread allure of metabolomics; however, as a fundamentally comparative experimental design, it also necessitates appropriate and well-defined biological controls and a randomized sample-run order to minimize bias in both sample preparation and data acquisition steps, regardless of the study size. The most rigorous checkpoint for the experimental design should occur prior to the initiation of the study, as afterward, many controllable study variables become entrenched. When reporting findings, it is important for researchers to explain their experimental design in detail (see Tables 1 and Supporting Information S1) so readers can accurately assess the ability of the study to achieve the stated experimental goals.

2.2. Sample Preparation. Due to the labile nature of metabolites, the pretreatment of samples, including sample collection and storage protocols, must be appropriately designed and implemented to minimize technical variability and to avoid nonbiologically relevant changes in metabolite patterns. These considerations remain true across the metabolomics field and are not analytical platform specific. Rather, the type and number of unique specimens and the research question should drive sample collection, storage, and handling approaches. It is well-known that a combination of collection, handling, and storage parameters will impact the chemical composition of the sample. Pretreatment and postprocessing considerations range from spatial location of the specimen to time of day for collection and include various sample transfer and storage options, such as temperature, duration, and chemical treatment. Failure to disclose such vital information is a barrier to successfully reproducing metabolomics experiments. For example, metabolites in blood, which is the most widely collected biofluid,[39] are influenced by the type of collection tube (e.g., no additive, 3.2% sodium citrate, potassium EDTA, lithium-heparin, etc.) used during sampling,[40,41] and the sample processing method (e.g., centrifugation, mixing, temperature, standing time, number of freeze−thaw cycles, etc.) postcollection.[39,42] Challenges also arise with spatial and temporal sample collections. For example, variations may occur among different cells within a leaf or across the whole plant (stem, root, leaf) depending on the time of day and photosynthesis stage.[43]

Thoroughly documenting and reporting the pretreatment of samples is critical for repeatability, study comparison, and future growth in the field because sample collection and handling protocols are not universally agreed upon and remain undefined for many nonmodel species. Recommendations on sample collection and handling protocols for each type of biospecimen are beyond the scope of this article and will require additional efforts to recognize and disseminate best practices for specific sample types. We refer readers to a preanalytical and biobanking review[44] and other relevant studies.[45−47] for detailed insights into sample collection and biobanking. Additional references to protocols regularly utilized by the community for the collection and handling of various biofluids and tissue samples are also provided in Supporting Information Table S2.

A lack of unified protocols also holds true for sample processing and metabolite extraction steps, which must be carefully defined prior to undertaking the project since the study goals and research questions should define the methodological approach. It is well-known that the method used for metabolite extraction can greatly influence the results by modulating the metabolomes of interest via the chemical environment. For example, the type, number, and polarity of the solvent(s) used for metabolite extraction can affect both metabolome coverage and data quality.[48] When multistep extractions and multiple solvents are used, the order and steps in which the solvents are added can affect metabolite partitioning between solvent phases, ultimately affecting both reproducibility and overall extraction yield.[49] There are many metabolite extraction protocols reported in the scientific literature that cover a variety of biological sample types commonly encountered in metabolomics studies (Supporting Information Table S2). Of course, the availability of several distinct extraction protocols per sample type can contribute to the confusion and lack of standardized methods. Implementing metabolite extraction protocols across laboratories may enable harmonization of metabolomics data.

Methodological details of the protocol used should be clearly defined within the paper rather than referencing a protocol for the whole methodology. In the 2024 review of NMR metabolomics papers,[18] a common outcome was an inability to locate the original protocols. Instead, it was found that readers were often directed to references within references, which led to ambiguities or the complete absence of knowledge about the methodology used. Software tools, such as protocols.io,[50] can greatly simplify the reporting, sharing, and updating of protocols by associating a protocol with a digital object identifier. Protocols can then be used directly by other researchers or "forked" to modify a given protocol while maintaining its history. We encourage metabolomics researchers to adopt a method to help standardize NMR metabolomics sample preparation, study design, and even data collection and processing steps.

The inclusion of QCs, especially in MS-based metabolomics workflows, is widely recognized as important, as evident by a majority of the community (83%) self-reporting the use of pooled materials in their metabolomics projects in a 2017 questionnaire.[28] Despite their importance in MS-based metabolomics, QC methods for NMR were reported at an extremely low rate in the literature (2% of 2010 papers, 17% of 2020 papers), including multiple specific categories (e.g.,

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pooled QCs, buffer, and extraction blanks) that were nearly entirely absent from the 2010 literature. It is likely that the discrepancy between the 2017 questionnaire and the 2024 NMR literature review can partly be explained by the fact that most NMR facilities perform routine quality checks (temperature calibration, shimming, signal-to-noise ratio (S/N) testing, and tuning on external reference materials) that are not included in the final manuscripts. The quantitative approach does not require QC samples due to the strict protocols and inclusion of specific internal standards, so laboratories that use quantitative NMR methods are unlikely to report QC data. Given that approximately 50% or more of NMR metabolomic studies fall into this category, this alone may explain the dearth of QC reporting for NMR as noted in the 2024 literature review.[18] On the other hand, profile NMR metabolomics studies do require QC samples to help address variation from experimental (e.g., identification of contaminants, alignment of peaks) and instrumental (e.g., correct for instrument drift and changes in instrument sensitivity or probe tuning) sources. Therefore, these types of NMR studies require rigorous QA/QC and should report QC methods and data. Certainly, if this was done more frequently, then the quality of data being published by the metabolite profiling NMR community would be greatly enhanced.

For profile NMR metabolomics studies, reporting the use of QCs during sample pretreatment demonstrates that measures were taken to ensure the quality of the study and resulting data. Better yet, if these types of NMR studies reported the scope of use of their QCs (e.g., extraction efficiency, contamination, batch monitoring/correcting, two-dimensional (2D) annotation, etc.) based on definitions accepted by the community, it would prevent readers and reviewers from having to interpret and assume how the QC samples were being used in the study. Members of the metabolomics Quality Assurance and QC Consortium (mQACC) have defined the roles for each QC in a profile NMR metabolomics workflow, especially for MS-based metabolomics. These include the minimal and best practices on the reporting of QCs, and how to predefine the scope of use for all QC samples. This includes analysis of blanks, buffer blanks, pooled QCs, and certified reference materials (CRMs).[14,51] To this point, the validity of a metabolite profiling study can be determined only if QC methods and data are demonstrated or presented in the paper or associated repository. Reporting QC results is discussed further in the Section 2.4 Data Processing and Analysis.

2.3. Data Acquisition. NMR spectrometer specifications, reconstitution buffers, and experimental parameters largely influence the quality of the resulting NMR spectra. Thus, these variables must be prominently defined to allow for proper interpretation and comparison of metabolomics data between studies.[52] In fact, properly describing the data acquisition parameters is so fundamental to disseminating metabolomics research results that any reporting rates below 100% are a cause for concern. Yet, even a straightforward parameter like sample temperature was severely underreported across the literature in the 2024 literature review[18] (reported in <75% of 2020 papers) despite its importance in influencing the chemical shift values of NMR signals. Again, some of this underreporting may be due to the widespread use of quantitative NMR metabolomics methods, where the protocols are strictly defined and largely identical from one study to the next.

Reporting rates for the inclusion of one-dimensional (1D) or 2D NMR experimental parameters were more encouraging (97.5% and 89%, respectively, in 2010 papers; 96% and 93%, respectively, in 2020 papers); however, these metrics do not describe the extent to which experimental details were included. For example, specific NMR pulse sequences used for 1D and 2D NMR experiments were reported less frequently than reported inclusion rates in both 2010 papers (86% and 76%, respectively) and 2020 papers (85% and 79%, respectively), which is of concern for profile NMR metabolomics studies. Due to the wide array of potentially suitable 1D and 2D NMR experiments for metabolite profiling, reporting specific and complete experimental details is required to fully convey the proper and reproducible detection of spectral data.[53] On the other hand, because quantitative NMR studies use only a small number of very specific pulse sequences (1D nuclear Overhauser effect spectroscopy or variants thereof), the issue of pulse sequence variability is less of a concern. Reported compositions of the NMR buffers presented a similar issue. For example, the chemical shift reference compound included in the NMR buffer was very frequently reported (99% in 2010 papers; 92% in 2020 papers), while notably fewer studies reported the buffer type (81% in 2010 papers; 74% in 2020 papers), pH (56% in 2010 and 2020 papers), and concentration of buffer components (49% in 2010 papers; 52% in 2020 papers). Sample pH, osmolality, buffer and solvent type, and temperature all influence NMR chemical shifts, line widths, and spectral splitting patterns (especially for compounds with ionizable functional groups). Thus, providing a complete description of the chemical shift standard, buffer composition and sample conditions is necessary to achieve accurate annotations, harmonize data, and to generate reliable results enabling comparisons across studies.[54,55]

The reviewed metabolomics papers well reported the total number of samples analyzed (96% in 2010 papers; 83% in 2020 papers) but was not as effective in fully describing the complete set of samples used (e.g., number of replicates per group, QC samples, etc.). Based on these findings, we reinforce and concur with previous recommendations[9,10] and urge NMR metabolomics studies to include a table or a figure that describes the complete set of experimental and QC samples (if required) and the cohort composition. Simply put, it should not be the responsibility of the reader to ascertain the overall study design and sample size by counting the number of symbols in the data plots, for example. In addition to the low reporting rates of QC samples (described in detail in Section 2.2), the results necessary for demonstrating optimal spectral quality for metabolite profiling studies were also underreported. For example, metrics related to spectrometer performance and reproducibility (e.g., water suppression efficiency, spectral relative standard deviation of pooled QCs, etc.) were not assessed in the literature review, nevertheless, they should be reported to provide transparency regarding data quality.[56,57]

Some parameters (e.g., buffer type, chemical shift reference, number of samples, etc.) were reported less frequently in 2020 compared to 2010 papers. This decrease over time suggests that reporting expectations for basic data acquisition parameters may have eased in the past decade, perhaps from a tendency for methods sections to focus on the increasingly elaborate and diverse data processing and analysis procedures used in NMR metabolomics. In this context, we urge the field

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of NMR metabolomics to re-emphasize the importance of reporting of foundational data acquisition parameters outlined in Tables 1 and Supporting Information S1, thus allowing readers to confidently interpret the presented results. In specific instances where one of the recommended parameters listed in Tables 1 and Supporting Information S1 may not be relevant or not applicable (NA) (e.g., sample pH when using organic solvents), it should be explicitly stated as such in the text and/or in relevant tables and figures rather than being omitted. It is also important to recognize that a growing amount of metabolomics research is being generated by commercial vendors, instead of individual investigators. In such cases, we recommend that the companies that provide such services clearly report all relevant experimental details needed to accurately and reliably reproduce results, such as the types of NMR experiments performed and the acquisition and processing parameters.

2.4. Data Processing and Analysis. NMR data collected from an instrument can be processed and manipulated using various mathematical functions to enhance S/N, resolution, and sensitivity and to correct baselines and phase spectra. For quantitative NMR studies, these spectral transformations are strictly defined and are usually part of a well-defined protocol or menu of functions offered by data processing software, such as Chenomx NMRSuite,[23] IVDr,[25] and FoodScreener;[24] as well as academic programs such as BATMAN,[58] Bayesil,[59] and SAND.[60] Ideally these processing details should be provided in the method descriptions to ensure broad reproducibility. However, for some programs and certain commercial providers, this information is not available to the user, thereby limiting the reproducibility of the result.

For NMR metabolite profiling studies, the variability of data processing methods is much greater, and therefore much more information about data processing must be reported. For example, ensembles of spectra must be aligned and integrated, and solvent or buffer peaks are often removed from spectral regions to avoid their impact on downstream data analysis. When this is done, it is crucial to report the type of mathematical functions used in transforming the NMR spectra into a format employed to quantify and identify the metabolites. Failure to do so might compromise reproducibility, data utilization, and data preservation. For instance, studies show the approaches used in processing NMR spectra, such as line broadening factors,[61] zero filling and window functions,[62] baseline correction methods,[63] and removal of noise[64] affecting data interpretation. Therefore, it is important to report all of the processing methods that have been applied to the NMR spectra.

Regardless of whether the data are acquired from a quantitative NMR study or a profiling NMR metabolomics study, normalization, transformation, and scaling are critical data processing steps to prepare metabolomics data for statistical analysis. However, the 2024 literature review found that only about 60% of papers published in 2010 and 2020 reported a normalization method, a critical step for making data comparable across samples.[65] Reporting on data scaling was even lower, appearing in only about 45% of papers.[18] This may stem from confusion regarding the need for both data normalization and data scaling and the inherent difference between these two methods. To clarify, normalization corrects for differences in overall concentration (e.g., dilution factor) between samples. On the other hand, data scaling accounts for large dynamic range differences between individual spectral signals and prevents intense signals from dominating statistical models and negating biologically important changes in lower intensity peaks. There are various approaches available for the manipulation of metabolomics data to enhance feature selection. Different methods are recommended depending on the specific sample types. For instance, probabilistic quotient normalization, which estimates the most probable dilution factor by comparing the distribution of intensity quotients to a reference spectrum, is considered robust for analyzing urine samples.[66] Similarly, different scaling approaches have been shown to affect the outcome of the analysis.[67] The primary consideration in choosing an appropriate scaling or normalization technique is to ensure an accurate classification of the specific sample type into different metabophenotypes, a distinct pattern of metabolite levels and types in a sample that correlates with a particular phenotype, and to achieve the reproducible identification of spectral features that distinguish sample groups. Since different combinations of scaling and normalization methods can yield varied results,[68] it is essential for researchers to report each step in the data processing pipeline to facilitate the harmonization of data retrieved from public databases and to ensure study reproducibility and reliability.

Many researchers in the NMR metabolomics community do not have extensive experience in biostatistics or statistical modeling, which can result in an overreliance on default software settings or adopting incorrect literature protocols because of a false impression of an established precedent. Thus, a common occurrence across many metabolomics studies is the failure to thoroughly validate models. For instance, supervised analysis, such as PLS-DA and orthogonal PLS-DA (OPLS-DA), is widely used for multivariate analysis of metabolomics data sets. These techniques, however, require validation before interpreting the variables that contribute to the observed group differences. Encouragingly, there has been a positive trend in reporting R^2 and Q^2 values, with reporting rates increasing from 30% in 2010 to 63% in 2020.[18] Unfortunately, this increase may have been driven, in part, by a common misunderstanding of the meaning of R^2 and Q^2 values. These parameters are often incorrectly used to infer validity of a model. Instead, R^2 only provides a measure of the quality of fit, and Q^2 identifies the inherent variability or stability of the model following a leave-one-out or, preferably, leave-*n*-out analysis. For model validation and to avoid an overfitted model, it is essential to report metrics such as a permutation test or cross-validation analysis of variance (CV-ANOVA) *p*-values. Adhering to best practices also includes ensuring $R^2 > Q^2$, $Q^2 \geq 0.4$, and Q^2 is within 20% of R^2 to minimize an overfitted model. Further, negative R^2 and Q^2 values indicate an invalid model, and the resulting model should never be presented as useful or analyzed for biological significance. Multivariate statistical models are routinely interpreted by a visual inspection of the relative group clustering in a scores plot, which can be inaccurate and lead to bias outcomes. Instead, a few simple quantitative methods can be applied to scores plots to avoid any such errors. Group membership can be defined by ellipses that correspond to the 95% confidence limit of the normal distribution of each cluster. Group overlap or separation can then be statistically defined by within-group and between-group Mahalanobis distances and a univariate analysis (i.e., *p*-value) to assess the relative similarity of these distances.[69]

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Univariate analyses, including *t*-tests and ANOVA, are also frequently applied to compare group means and variances. Given the large number of metabolites analyzed in metabolomics studies, corrections for multiple hypothesis testing are vital to control the false discovery rate (FDR). Findings from the 2024 literature review paper indicate that FDR reporting remains inadequate, with only 14% of studies in 2010 and 34% in 2020 addressing this crucial requirement.[18] Researchers should routinely report FDR-adjusted *p*-values to minimize false positives (type I errors). It is also important for researchers to explicitly note the method used for such corrections to improve the reliability and reproducibility of their results.[33,70] Reporting both uncorrected and FDR-corrected *p*-values to address concerns with false negative rates (type II errors) is acceptable.

In addition to reporting proper metrics and correcting for multiple hypothesis testing, NMR metabolomics studies that rely on metabolite profiling must also report QC results. These must be assessed to appropriately define the technical variability within a study and to validate the biological significance of a model. For QCs, reporting their use alone is not sufficient. Kirwan et al. (2022), on behalf of mQACC, provided valuable guidance on acceptance criteria and precision of QC analysis for NMR metabolite profiling studies.[14] Specifically for these types of studies, reporting should ideally include the spectral median standard deviation[56] for CRMs, pooled QCs, and experimental groups. Any assessment between batch precision and batch precision, especially if corrections are applied, should also be reported. Demonstrating the extent of reproducibility through reporting is necessary for all NMR metabolite profiling studies, especially when conclusions are drawn about the relative concentration changes of specific metabolites in different phenotypic or health states.

The manner in which metabolite assignment is achieved and reported can also impact accuracy, rigor, translation to other studies, and adoption of new methods. A recent human urine metabolome study clearly illustrated this ongoing metabolite annotation challenge when it demonstrated assignment errors that resulted in metabolites being mistaken for other compounds.[71] These inaccuracies were evident as the reported human concentrations were well below the detection limit of NMR. In addition, some of these inaccurate annotations were apparent due to metabolite insolubility in water. Reporting the extent of metabolite identification and describing previously cited examples in the literature may help to prevent compound misidentifications. Unfortunately, the level of assignment for each metabolite proposed by the MSI Chemical Analysis Working Group[6,9] does not appear to be well-documented in the literature and was also not recorded in the previous literature review, likely due to too few instances of reporting. Along these lines, the 2D NMR experiments necessary to reach the optimal annotation level (MSI level 1) were only utilized about 45% of the time and did not improve from 2010 to 2020.[18] This low level of reporting may also reflect the frequency of quantitative NMR studies, which do not require 2D NMR experiments to confirm their metabolite identifications. Journals requiring information concerning the level of metabolite assignments combined with a clearer definition of MSI levels specific to NMR (especially for NMR metabolite profiling studies) would likely encourage wider adoption of the practice by metabolomics investigators. More importantly, it would lead to a greater level of metabolite annotation confidence.

While quantitative NMR studies follow very consistent practices in compound nomenclature (because of the uniformity of the software), the same nomenclature consistency is not seen in NMR metabolite profiling studies. Nomenclature standardization is currently an urgent need that requires serious consideration by the metabolomics community. Broad use of a standard nomenclature would facilitate the cross-referencing of results across multiple NMR metabolite profiling studies. Unfortunately, the 2024 literature review did not record whether the studies reported more than one name per metabolite or if the study leveraged standard metabolite identifiers. Ambiguous nomenclature continues to occur in interlaboratory studies and greatly impedes attempts to compile and harmonize metabolomics data.[72]

As previously stated, efforts in nomenclature standardization suggest a minimum of two types of naming conventions to be included in a metabolite annotation list.[6,9] Ideally, this would include one structural code (e.g., InChI, SMILES) and one chemical name (e.g., IUPAC, common name). Among identifiers derived from structural information, the InChIKey is generally the preferred form for translation purposes due to its length and suitability for indexing and searching as it is a unique ID.[73] Canonical SMILES is also often used to both define structure and act as a unique ID. In contrast, InChI and SMILES strings can be written in numerous ways to represent the same molecule. However, none provide unique naming for protons, which is essential for NMR studies.[74] D-Glucose alone has 25 synonyms for common names in the Human Metabolomics Database (HMDB, https://hmdb.ca/), clearly illustrating the challenge and need for more accurate structural identifiers. Chemical formula and database ID numbers (e.g., HMDB, Kyoto Encyclopedia of Genes and Genomes [KEGG, https://www.genome.jp/kegg/], etc.) provide additional sources of information that can improve data searching and harmonization. The inclusion of database ID numbers can not only aid in quick database searches but also provide further resolution of potentially ambiguous chemical structures and names. Chemical taxonomy-based tools, such as ClassyFire and ChemFOnt, enable standardized chemical structure and function classification, and this information also helps with automated comparisons with previous studies.[75,76]

An additional challenge in nomenclature is the reporting of stereochemistry without conducting further experiments to confirm the specific conformation of the metabolite as there are currently no clear guidelines for the NMR metabolomics community. NMR can identify and distinguish structural isomers, but depending on the complexity of the molecular structure, additional experiments are often necessary to confirm stereochemistry. Metabolite stereochemistry is often assumed without verification, leading to inaccurate reporting and challenges for data harmonization. In such cases, we recommend avoiding differentiating between stereoisomers and instead recommend the reporting of the nonstereoisomeric form. The NMR metabolomics community would greatly benefit from clearly defining standardized criteria for confidently reporting structural isomers.

Of all parameters examined in the literature, including study design, sample preparation, data acquisition, and data processing/analysis, the choice of statistical software and tools for data analysis showed the most notable variation in NMR metabolomics research. Researchers used a wide range

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Anal. Chem. 2025, 97, 20655−20666

of software and databases to process and analyze NMR metabolomics data sets, with over 110 distinct programs being used by the community, together with in-house R or Python scripts. However, many publications reviewed in the 2024 paper failed to provide essential details such as software names, versions, and availability.[18]

To improve reproducibility, researchers are encouraged to include detailed reports of their data analysis methods. For instance, when using MetaboAnalyst[77] for statistical analysis, the R code generated by the platform can be shared in data repositories (e.g., GitHub) or as Supporting Information, which could enable others to replicate the analysis and support the metabolomics field's progress toward standardization. At a minimum, reporting of the software and version used to process, analyze, and annotate NMR data is needed for data reuse and study reproducibility. Further, to improve transparency and confidence in published metabolomics data, especially results generated by groups with limited prior involvement in the field, it is strongly recommended that reproducibility assessments be reported for a given project. For guidance, several studies have provided practical approaches to include reproducibility assessments in NMR metabolomics that should be broadly followed by the community.[78−85] This would include intra- and/or interlaboratory validation efforts, overall instrument and biological variability measurements, and, critically, acknowledgment and comparison to previous results reported in the literature. Unfortunately, it is all too common for metabolomics manuscripts to exclude a proper comparison of their results to prior studies, even though such an analysis would be critical to assess the true reliability and reproducibility of metabolomics data.

2.5. Data Accessibility and Comparability. Ideally, acquired spectra along with sufficient metadata would be deposited in a publicly accessible repository appropriate for NMR metabolomics data to enable the reproducibility of results. The inclusion of QC and reference material spectra allows for data quality assessment, especially in consideration of data reuse and comparability across laboratories even as technology advances. Most commonly used repositories for metabolomics data sets are currently Metabolights (https://www.ebi.ac.uk/metabolights/), the NIH Common Fund's National Metabolomics Data Repository, formerly known as Metabolomics Workbench (https://www.metabolomicsworkbench.org/), and the Biological Magnetic Resonance Data Bank (http://bmrb.io).[86] According to the literature review, there were no reports of metabolomics data being deposited in an appropriate repository in 2010. This figure increased to a mere 11% by 2020.[18]

The landscape of data sharing has shifted dramatically since 2010, when few journals enforced data deposition. Presently, journals such as Metabolites and Metabolomics strongly encourage this crucial step, and the lack of deposition requires a statement regarding why the data have not been deposited. Despite an overall shift toward FAIR (findability, accessibility, interoperability, and reusability) data principles (https://www.go-fair.org/fair-principles/), the 2020 11% deposition rate leaves vast room for improvement. The lack of data deposition, except in cases of proprietary information and health insurance portability and accountability act regulations, may be attributed to several factors, including the length of time required to gather and deposit the associated metadata, the challenges in learning how to deposit data (or new data format required) into a new repository system, the loss of experimental details, or limitations of currently available repositories. There is also a significant discrepancy between what is expected and required for the deposition of quantitative NMR metabolomics data versus profiling NMR metabolomics data. Technically, quantitative NMR metabolomics produces lists of unambiguously identified compounds and accurately measured concentrations, which are publicly shared upon publication. Most quantitative NMR metabolomics methods are rigorously validated (externally and internally) and require adherence to strict operational protocols. Therefore, for most analytical and clinical chemistry journals, lists of compounds and their concentrations are all that is normally required for public, FAIR-compliant reporting. It is still an open question as to whether the raw data from a quantitative NMR metabolomics study is of general use given the strong dependency on commercial software providers or commercial entities to generate the quantitative data sets. Clearly, the availability of free, open source, or open access deconvolution software would help address this issue and would make the deposition of raw metabolomics data from quantitative studies more meaningful.

On the other hand, because NMR metabolite profiling data is so variable in nature, the need to access raw data for secondary confirmation and validation is a near-absolute requirement, and these types of data must be deposited in the public repositories. To address these challenges and ease data deposition, the NMR metabolite profiling community would greatly benefit from developing or enhancing data reporting tools that can be implemented with NMR metabolite profiling studies in real-time. Such tools would allow metadata to be systematically collected and organized during the study, thus, simplifying the deposition process. A recent perspective[15] outlined recommendations for the use and reuse of metabolomics data by highlighting both the progress in the field and the existing needs to achieve the laudable goal of routine and ease of data depositions.

Comparisons of study outcomes with the existing scientific literature and the reporting of previously identified metabolites, together with noting possible inconsistencies, should be included as a fundamental component of all published metabolomics manuscripts. These issues tend to be less significant for quantitative NMR metabolomics studies, which tend to be highly consistent and uniform, but can be quite significant for profiling NMR metabolomics studies given the extreme variability in sample processing, data analysis, and interpretation. Even when experimental designs differ, comparisons of results provide valuable insights, especially if the procedural differences are highlighted. Reporting a study as "groundbreaking" while failing to contextualize findings within the existing body of research can impede scientific progress. For instance, metabolomic studies that identify a significant change in the concentration of a certain metabolite without acknowledging the prior findings could lead to redundancy or missed opportunities to explore novel aspects of the metabolite(s) of interest. Doing so will also neglect the opportunity to verify the reproducibility of outcomes across multiple laboratories and studies.

3. SUMMARY

First and foremost, the intent of this paper, particularly Table 1 and Supporting Information, is to serve as a useful template for reporting fundamental study parameters for the successful dissemination of NMR metabolomics data. Second, this paper

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underscores the importance of comprehensive reporting to ensure study reliability, reproducibility, and study comparability. We aim to encourage thoughtful reflection on the reasons why experimental reporting in NMR metabolomics, especially NMR metabolite profiling metabolomics, is currently inadequate, strategies to make metabolomics data reporting easier, areas where current guidelines fall short, and the need to establish best practices for reporting reliable findings to the scientific community.

We also provide Supporting Information which points to seminal papers in the field to serve as guidance on how to best design and implement quantitative and profiling NMR metabolomics studies. While this paper does not propose best practices for specific biological systems or NMR techniques, we suggest follow-up efforts to establish guidelines in collaboration with the respective research communities. Similarly, establishing best practices for study design, methodology, and data analysis should be a high priority and the logical next step for the NMR metabolomics community. These efforts should include the adoption of standardized formatting in conjunction with the mQACC best practices for profiling NMR metabolomics and a living guidance document to ensure consistency, accessibility, and interoperability as the field of NMR metabolomics grows. A link to the mQACC living guidance document, including a glossary of terms, is forthcoming, and mQACC efforts in establishing a framework for best practices are documented in a study by Mosley et al. (2024).[51] Additionally, the Best Practices for Non-Targeted Analysis (BP4NTA) working group developed a MS-based Non-Targeted Analysis Study Reporting Tool and a Study Planning Tool, which have aided study design, evaluation, and peer review, serving as excellent examples for our intended efforts.[87]

An extensive list of items to report in an NMR-based metabolomics study is provided in Tables 1 and Supporting Information S1, and some of the details necessary for a thorough report on a few critical topics are discussed throughout the text. We acknowledge that the efforts required to include this level of detail in a manuscript and the fact that journal word count limitations commonly lead to the exclusion of experimental details from the main text. Therefore, we recommend incorporating many of these important experimental details into Supporting Information text and tables.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.5c03274.

Reporting recommendations for NMR metabolomics and compilation of references for study design and implementation of robust NMR metabolomics studies (PDF)

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The manuscript draft was prepared by E.R.A., A.L.B., and T.G., and all authors commented on and edited the manuscript and approved submission. E.R.A. and A.L.B. contributed equally to this work and have the right to list their name first on their CV.

Notes

The authors declare no competing financial interest.

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