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Importance of Purity Evaluation and the Potential of Quantitative ^{1}H NMR as a Purity Assay

Miniperspective

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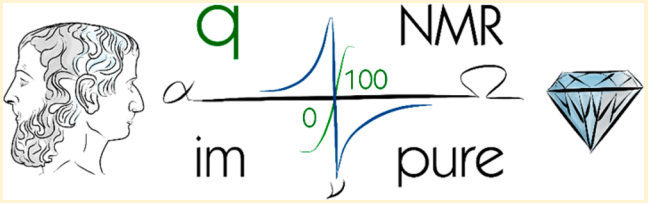
Supporting Information

ABSTRACT: In any biomedical and chemical context, a truthful description of chemical constitution requires coverage of both structure and purity. This qualification affects all drug molecules, regardless of development stage (early discovery to approved drug) and source (natural product or synthetic). Purity assessment is particularly critical in discovery programs and whenever chemistry is linked with biological and/or therapeutic outcome. Compared with chromatography and elemental analysis, quantitative NMR (qNMR) uses nearly universal detection and provides a versatile and orthogonal means of purity evaluation. Absolute qNMR with flexible calibration captures analytes that frequently escape detection (water, sorbents). Widely accepted structural NMR workflows require minimal or no adjustments to become practical ^{1}H qNMR (qHNMR) procedures with simultaneous qualitative and (absolute) quantitative capability. This study reviews underlying concepts, provides a framework for standard qHNMR purity assays, and shows how adequate accuracy and precision are achieved for the intended use of the material.



INTRODUCTION

Both the use and the purity of chemical substances are subject to the philosophic reflection by Werner Heisenberg: "What we observe is not nature itself, but nature exposed to our method of questioning."[1] The term "purity" (as in carbon in a diamond) ultimately refers to the complex question of the integrity of chemicals and is inevitably linked to the analytical method (of questioning) and any subsequent use of the material. This article challenges the current general practice of analytical purity determination and proposes the implementation of quantitative NMR (qNMR) as a nearly universal and practical method for purity assessment.

The Value of Purity. The designation of a substance as experimental material ("research grade") makes it clear that it differs from material intended for human use ("pharmaceutical grade"). Following the guidance of global pharmacopoeial and regulatory frameworks, materials for clinical use require a detailed characterization and need to fulfill certain criteria. For example, the purity of pharmaceutical grade materials is rigorously defined. Generally, purity assessment of pharmaceutical grade materials involves both the structural characterization and the quantification of the impurities, frequently down to the 0.1% w/w level. Analogous criteria for research grade materials are generally much less rigorous, partially incomplete, and/or poorly followed.

Research Is the Search for Truth. As purity is a key parameter of the *true* chemical constitution of a substance, purity assessment is the logical prerequisite for the accurate characterization of any research grade material. Consequently, the reproducibility and interpretability of research data always hinge on the accuracy of the chemical characterization to which it is assigned, regardless of whether the material is designated as a research or pharmaceutical grade material. For the purpose of producing new insight, it is important to apply identical or at least congruent standards to both experimental and clinical materials. These considerations particularly apply to organic substances of synthetic or natural origin, which can range from highly characterized reference materials to early stage experimental materials with assumed single chemical character.

Purity assessment is perhaps most critical in the case of novel compounds to which a biological activity is ascribed, because trace impurities of high potency can lead to false conclusions. Examples are the historic case of the lead compound sesbanamide "hidden" in sesbanin,[2,3] the more recent findings of inactive leads such as epiquinamide containing the β_2-selective nicotinic acetylcholine receptor agonist, epibatidine,[4] and the lack of in vitro anti-TB potency in high-purity ursolic

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acid.[5] The first two instances used synthetic rather than analytical approaches to uncover the problem, and while they would have required rather sensitive analysis (e.g., the authors estimated the presence of ~0.1% of epibatidine in the epiquinamide sample),[4] this demand most likely would not have been beyond the capabilities of qNMR. The third case actually was discovered by means of qNMR. While synthetic pathways in medicinal chemistry are typically more predictable than the combinatorial biosynthetic pathways of nature, similar considerations apply regarding the relevance of impurities in drug discovery and safety profiling. One such example is from a drug discovery program driven by high-throughput screening, aimed at finding new treatments of schizophrenia, where the N-hydroxylated impurity of the initial lead compound, an aminodihydroquinolone, turned out to be the active ligand and high-potency (nM) inhibitor of kynurenine amino-transferases, KAT II.[6] Another example is the selective nuclear factor κB inhibitor, NSC 676914: the structure of this ethanesulfonoperoxoic acid derivative was revised after validation of the initial hit by NMR and LC−MS exhibited a mismatch with the published structure, and HPLC was used to purify the active constituent.[7] A third example is the discovery of the antiviral (anti-Varicella zoster) lead, FV 100: the development precursor of this water-soluble prodrug, Cf1743, was derived from Cf1368, which in turn was the fluorescent impurity of the initial lead compound, Cf904.[8,9]

Intriguingly, in the experience of the authors, database-driven mining of the literature for examples of "significant impurities" is hampered by the infrequent use of the term "(im)purity" in titles and abstracts of relevant scientific publications, as this might reflect a general reluctance to disclose findings of impurities as agents of toxicological relevance or even the actual active principle(s) as constituent(s). A more prominent inclusion of such information into the primary literature has the potential to advance the search for the science behind active agents. Overall, purity is an essential physicochemical parameter in organic chemistry and should be addressed in the physicochemical characterization of any material. As such, purity assessment is as important as structural determination, a point often overlooked by virtue of the greater intellectual stimulation emerging from the latter.

In summary, as any scientific advancement requires a full commitment to the search for the truth, when projected on research involving chemical substances, this ethical mandate inevitably includes a closer examination of purity as an essential physicochemical parameter and its role in observed bioactivity. Ultimately, this mandate also protects the human and financial resources devoted to research.

The Role of Sensitivity. The increased availability of high-sensitivity analytical techniques has lowered the threshold of the amount of sample that is amenable to organic analysis, including evaluation by NMR. At the same time, this improvement has indirectly led to a decrease in the purity level required to generate spectroscopic data for unambiguous structural (qualitative) characterization. One indication of the changing landscape in the chemical sciences is the reduced use of combustion analysis as a method for establishing elemental composition, molecular weight, and molecular formula. Notably, combustion analysis represents an orthogonal analytical method (see "Independence and Orthogonality" below) for purity determination and complements results of hyphenated mass spectrometry, albeit requiring much more sample. For the limited sample quantities frequently encountered in, for example, natural products or metabolite research, combustion analysis, for any purpose, is for the most part precluded. In medicinal chemistry applications, the increased acceptance of HPLC as a purity assay has likely led to a reduced use of combustion analysis.

Another indication is the major reduction over time in the use of crystallization as a classic method for purification.[10] Crystallization also requires sufficient quantities of material, and even though small samples of materials may be crystallized, the use of this purification procedure may be precluded because of limitations in the handling of small sample volumes and the unavoidable material losses encountered during the process. While X-ray crystallographers employ low temperature techniques for obtaining small quantities of crystalline material, these methods are not practiced in a routine manner for small molecule purification. The role of purification methodology and its relationship to purity have been recently discussed in detail in the context of organic natural products.[11]

Finally, it is important to keep in mind that the acquisition of the majority of widely used NMR spectra such as 1D ^{13}C and the information-dense 2D $^{1}H-^{13}C$ correlation experiments is generally done at relatively low signal-to-noise (S/N) levels, at which impurities frequently remain undetected. While typically sufficient for structural analysis, this approach is driven by the optimum use of valuable spectrometer time, i.e., throughput oriented, rather than aimed at the comprehensive characterization of individual samples. As a net result, the enhanced spectroscopic equipment and development of high-sensitivity techniques have actually diverted attention away from purity/impurity considerations, despite the fact that enhanced sensitivity improves the ability to detect minor components. While the recent trend to include spectroscopic information, particularly spectral figures, as Supporting Information enables the reader to review original information and gain insights beyond the interpretation by the original authors, this system bears the partial risk of relegating essential information to portions of a manuscript which are not subject to the same level of scrutiny as the "main" text.

The Role of Versatility. The majority of analytical techniques have principal strengths as either qualitative *or* quantitative methods, with NMR being the notable exception. The predominance of methods with an exclusive qualitative outcome may have contributed to the perception that purity assessment requires substantial additional effort. Accordingly, because of the abundance of LC instrumentation, generic LC methods are widely employed for purity assessment using relative calculations that follow the 100% method. Another potential interpretation of the predominant use of LC approaches in purity determination is that there might be a perceived strong correlation between chromatography as a method, achievement of separation, and determination of purity. It should be noted that spectroscopic dispersion such as chemical shift dispersion in NMR can be equivalent to separation while not requiring physical fractionation of the analytes.

WHY IS qNMR PARTICULARLY SUITABLE FOR PURITY ASSAYS?

Independence and Orthogonality. A method used for purity assessment should be mechanistically different from the method used for a final step of purification. In keeping with this need for analytical independence[12] (i.e., orthogonality[13,14]), spectroscopic methods are by nature orthogonal and, thus, ideally suited to assess materials purified chromatographically.

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Relative and Absolute Purity. Quantitative analytical methods can be relative (100%) or absolute methods, yielding relative and absolute purity assignments, respectively. The choice of relative vs absolute methods with regard to the determination of purity should be made congruent with the subsequent use of the material. For example, if the material is used for quantitative experiments such as the determination of biological activity (potency) or chemical content, an absolute purity determination is most appropriate.

Precious Materials. For mass-limited materials, such as natural product isolates including endogenous markers in metabol(om)ic studies, quantitative NMR (qNMR) spectroscopy is available as a nondestructive method. Representing a primary ratio analytical method,[15] qNMR can perform both relative and absolute determinations and is capable of absolute quantitation, akin to (thermo)gravimetry, coulometry, and titrimetry.

Selectivity. Another important aspect is selective vs universal detection. The most extensively used chromatographic detectors such as UV/vis or MS are relatively selective, a property that results from the underlying physical mechanisms. This highlights the universal nature of a nuclear technique such as NMR, especially quantitative ^{1}H NMR (qHNMR): for the most part, protons are ubiquitous in organic compounds. It should still be noted that certain heterocyclic compounds, i.e., those with a H-to-C ratio of <2,[16] but also some common natural metabolites such as oxalic acid represent notable exceptions to the general *universality* advantage of qHNMR.

Rigor. In addition to its suitability as a universal, concurrently qualitative and quantitative method, recent validation studies have confirmed that qNMR methodology is fit for Good Laboratory and/or Manufacturing Practices (GLP/GMP)[17−19] and can fulfill metrological criteria. In this context, it is noteworthy that the pharmaceutical and chemical industries have been utilizing qHNMR in their GMP/GLP settings for decades, thereby providing substantial justification for both the validity and broader acceptance of this methodology. However, as the results of this extensive application rarely appear in the peer-reviewed literature, much of the scientific community may underrate both the validity and the practicability of qNMR and generally perceive it as being a rather specialized method. The specific[20] and broader[21] suitability of qHNMR protocols for the validation of reference materials has been documented. These concepts have recently been developed further in an industrial setting, aimed at producing highly accurate (<0.1% uncertainty) and traceable reference standards for GLP and GMP environments.[22] The achievable rigor of qHNMR analysis is also reflected by the recent practices of the inclusion of qHNMR analyses in dossiers submitted to drug regulatory authorities such as FDA, e.g., as part of the analysis of drug metabolites.[23]

■ PURITY ASSESSMENT TRUMPS PURITY THRESHOLDS

In general, purity values are closely linked to the method used for purity assessment. Accordingly, any declared purity values and purity thresholds have to refer to the method by which the results were obtained. For instance, depending on the detection method and properties of the impurities, purity determination by TLC might be either more or less sensitive than purity assessed by HPLC, and one or both will likely be different yet from the purity value determined by qHNMR.

Unless based on a specific rationale, purity thresholds of the general format >"*X*%" (e.g., "at least 99% pure" or "80% minimum purity") commonly found in journal, pharmacopoeial, and regulatory guidelines as well as certificates of analysis must be considered as arbitrary values. This applies in addition to the frequent lack of reference to a specific analytical method when defining the threshold and/or when making the purity determination. While declarations such as ">*X*% by HPLC" are common, it has to be kept in mind that without full disclosure of the HPLC method including detection parameters and quantitative calibration, the results likely will not be reproducible in other laboratories.

This leads to the soul-searching question: is it desirable to replace minimum purity thresholds "should be >*X*% pure" with the requirement to assess purity with a well-documented and orthogonal analytical method and disclose the actual values? Such a disclosure could be in the form of a statement such as "*Y*% pure by qHNMR using the 100% method described below/in Supporting Information/in ref no.", with *Y* being expressed with three significant figures. Taking this one step further, materials that exhibit relatively low purity but have been characterized by an orthogonal and well-documented method will likely lead to more reproducible and, therefore, meaningful experimental results than materials that meet an arbitrary threshold and, therefore, are *perceived* to be of high-purity but essentially lack a rigorous purity assessment. Importantly, the availability of actual measured purities enables the establishment of correlations between purity and biological responses, something that cannot be achieved when relying on purity threshold definitions alone.

In the event that the availability of analytical and/or other resources (e.g., human, funding, supply) limit the achievable purity, further studies with these experimental materials can still retain full integrity as long as the actual purity and preparation protocol are properly determined and documented. In fact, detailed purity assessments and documentation can be considered superior to the use of "inflexible" purity thresholds or goals for minimum purity. In addition, this approach offers new opportunities for subsequent studies such as optimization of purification or preparation protocols. That is, it can be more meaningful to work with well-defined materials of lesser purity than using arbitrary purity and/or impurity thresholds or unknown impurity profiles.

■ IMPURITY PROFILING

Purity assessment is a coin with two faces: on one face, purity reflects the proportion of the target compound, which is typically the main constituent and commonly designated as a single chemical entity. On the reverse side, all other components of the sample are the impurities. While purity and impurity values always add up to 100%, the purity value alone does not necessarily reflect the true composition and proportions of the material because both depend on the source of the material. As impurity profiles can be more or less complex, they are complementary to purity values and very important for understanding the biological activity of the material. The assessment of impurity profiles presents its own challenges associated with identifying their types (e.g., structurally related vs foreign impurities) and exact structures at the given dynamic range of their abundance in the sample.

One important consideration in the purity/impurity balance is the potential presence of "inert" materials which are invisible to the analytical method. Materials such as silica gel, sorbents,

and inorganic and polymeric matter frequently escape chromatographic analysis and require the use of an absolute method such as qHNMR or combustion analysis to be recognized,[24,25] adding further justification to the use of both qNMR and combustion analysis as orthogonal techniques.

Following the recently established concept of residual complexity (see S2 of Supporting Information in ref 26, http://go.uic.edu/residualcomplexity), the detailed interpretation of biological outcomes might actually require consideration of the role of the minor components rather than the single chemical entity, as documented for the exemplary cases cited above.[2−5] Therefore, the method of questioning purity is incomplete unless it also addresses the impurity profile in addition to the purity value. Accordingly, impurity profiling can become an essential parameter for the proper characterization of a material.

FREE AS IN qNMR

Provided that well-documented *quantitative conditions* are used for NMR acquisition (see below), the resulting spectra can be used *simultaneously* for qualitative and quantitative purposes. This means that implementation of *quantitative conditions* in standard ^{1}H NMR produces qHNMR spectra that are equally suitable to fulfill the requirements of structural characterization, as well as to simultaneously perform purity evaluation.

In fact, when used for *relative* quantitation by applying the 100% method (see calculation below), qHNMR spectra have the advantage of being a "free" add-on to basic 1D ^{1}H NMR. As such, standard qHNMR purity evaluation does not require any additional experimental effort beyond postacquisition processing and data evaluation. With the advent of NMR processing software tools, the former has already become standard in most laboratories, whereby quantitative measurements and calculations are natural components of any purity assay.

Notably, it is even feasible to re-evaluate previously acquired or even historic 1D ^{1}H NMR data sets by using the 100% method for *relative* quantitation, provided that the raw FIDs are available. The main limitations of such retrospective analyses are predetermined by the often limited *S*/*N* of the spectra and their possible deviation from *quantitative conditions*, which often can be reasonably assessed from the acquisition parameters stored along with the FIDs.

HOW TO PUT THE "q" INTO qNMR?

Quantitation by NMR requires two main components

- The *quantitative measurements* encompass the measure or indicator used to derive the quantitative information, the method of computation, and the quantitative calibration of the process.
- The *quantitative conditions* consist of key experimental considerations for the acquisition and processing of qNMR data.

There are three principal qNMR methods with regard to their approach to calibration (see below for details and S1, Supporting Information, for terminology): internal calibration (IC), external calibration (EC), and a hybrid of both (ECIC). Provided compatible calibration data are available for the chosen calibration method, all three methods can be applied to the same raw NMR data and, therefore, require no additional acquisition of qNMR data.

Considering that synthetic and especially natural molecules are frequently subjected to a laborious purification process, there is a need for qNMR methods that do not require the addition of an internal calibrant (IC). Methods that fulfill this requirement are available and described below.

QUANTITATIVE MEASUREMENTS

Measures. The application of qNMR methods depends on our ability to measure signal responses in the NMR spectra. Quantitative information (measures, syn indicators) can be extracted from the NMR spectra in three different forms:

- Integration areas (Int): Integrals are the most common measurements in qNMR analysis. The areas under "pure" signals (i.e., resonances that do not overlap with other resonances) are frequently chosen for this purpose. Alternatively, the integration areas can be determined using curve fitting procedures (deconvolution).
- Peak height (Height): Under specific processing conditions,[27] the NMR signal intensities represent a valid alternative to the measurement of integration areas.
- Resonance profiles: Computer-generated spectral profiles can be fitted to the experimental NMR spectrum in order to determine analyte content. Examples are libraries of metabolite profiles (e.g., NMR signatures in Chenomx [Chenomx Inc.], Adaptive Spectral Library in PERCH, PERCH Solutions Ltd.) and the QM-based approach of ^{1}H iterative full spin analysis (HiFSA), which uses NMR fingerprints of the target analytes.[26,28,29] The resonance profile approach enables quantitative analysis in spectral regions with extensive signal overlap.

Historically and in common practice, integrals (Int) are the most widely used quantitative measures in qNMR. Int values are linearly proportional to analyte concentration. The dynamic range is only limited by the signal-to-noise ratio and can easily surpass 10^4 within a few minutes of acquisition time. The dimension of Int values is arbitrary, reflecting the relative character of qNMR as a relative primary analytical method. This means that in postacquisition processing, Int values can be scaled (normalized, see S1 Supporting Information) to any desired value. This opens the opportunity to tailor the normalization to, for example, the specific requirements of a given qNMR application. In practice, when the 100% method is used, it can be useful to normalize a certain integral of a target analyte to "1.000" or "100%" for an integral equivalent to one proton (normalized integral, nInt [1H]; see below) in order to facilitate the interpretation. However, it has to be kept in mind that regardless of whether normalization was applied or not, Int values always reflect molarity. Therefore, any mass-related interpretation requires further calculation, specific to each integral, taking into account the molecular weight and number of protons of the analyte giving rise to the integrated signal.

As the most widely used quantitative measures, Int and Height, are derived from relatively few signals in the experimental spectra, signal purity should be considered as a limiting factor for both achievable precision and accuracy of qHNMR measurements. Practical ways to detect potential signal overlap and validate peak purity in 1D qHNMR include the following approaches: (a) employ 2D NMR experiments such as the particularly sensitive 2D ^{1}H−^{1}H COSY experiment to achieve additional signal dispersion via a second dimension; (b) the use of multiple measures of the same analyte enables a consistency crosscheck; (c) application of different magnetic field strengths, particularly higher fields for increased

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dispersion; (d) the elimination of the ^{13}C satellites via broadband ^{13}C decoupling (see below) is particularly useful in the case of high purity materials; (e) employment of resonance profiles such as HiFSA fingerprints to detect signal overlap.

Calculation (syn Computation). The signals in qHNMR spectra reflect molar responses of the protons in the analytes. In principle, in both absolute and relative quantification, the molarity of the response involves the exact amount (mass, *m*) of the molecule in the sample, its molecular weight (MW), and the number of protons (*n*), giving rise the each observed resonance. While the response is directly proportional to the mass, the relative quantification ("100%") qHNMR method still does not require knowledge of the exact mass of the sample, because it determines the relative abundance of the molecules within the sample to each other, not the absolute amounts (see below for further discussion). The present study concentrates on the use of integrals (Int) as the most widely used quantitative measure in qHNMR. This method can be readily applied to resonance profiles, such as HiFSA profiles[26,28,29] and other profiles generated by QM-based spin analysis,[30] all of which are, in fact, superior in terms of integration accuracy and reproducibility. While these general concepts apply in principle to peak height (Height) quantitation, it shall be noted that this quantitative measure requires different calculation protocols. The development of procedures for Height-based quantification has to consider the relevance of differences in the signal multiplicity and, thus, the intensities of the individual resonance lines of the different spin particles (^1H nuclei). While individual protocols will depend on specific applications, one caveat is that the generally accepted proportionality of integrals in Int based qHNMR cannot be assumed for Height based quantification.

In a nutshell, the determination of purity by qHNMR involves calculations that establish the proportionality between *m*, MW, *n*, and Int of the target analyte (t), its impurities (Imp), and the calibrant(s), such as an internal calibrant (IC), when performing absolute quantification. There are numerous ways to approach the calculations, and monographs on this topic have long been published.[31] The following provides concise descriptions of the two key calculations for relative (100% method) and absolute quantification. Step-by-step details of the workflow and calculations related to the examples discussed below are provided in S2-5, Supporting Information. Widely available and naturally omnipresent compounds were chosen as examples.

Calibration. There are four principal methods of handling quantitative calibration:

i. Relative (Rel) 100% method with no calibration: This approach is commonly applied in chromatography-based purity analysis and is particularly suitable for precious and/or mass-limited samples. It uses the grand total measure of all signals as the basis for the 100% calculation.
ii. Internal calibration (IC) is the most common method and conceptually most straightforward for absolute quantitation. It involves the addition of a known amount of a certified calibrant to the sample.
iii. External calibration (EC) includes different approaches such as the use of artificial signals (ERETIC,[32] QUANTAS[33]), concentric or coaxial NMR tubes, and the very versatile *Q*-factor correction method developed by Burton et al.,[34] which is found in software implementations such as PULCON.[35]
iv. External calibration of the internal solvent signal (ECIC): This hybrid approach[34,36−38] involves the calibration of the residual protonated solvent signal or its ^{13}C satellites using a certified calibrant. The solvent signal is then used as internal calibrant to assess the analyte content.

Relative Quantification (Normalization, 100% Method). The calculation process for the relative quantification method can be summarized as follows: The sum of the averaged one-proton integrals of all detected analyte species yields the number taken for "100%". The mole percentage of each species is the average normalized one-proton integral for that component relative to the "100%" number. Key to this approach is the ability of the analyst (a) to distinguish pure and impure NMR resonances; (b) to assign proton numbers to all the signals of the major and minor constituents accounted for in the calculation and, thereby, (c) to establish relative molarities for all observed resonances; (d) to account for all observed resonances by either including them into the 100% calculation or developing strong rationales for their exclusion, e.g., NMR solvent signals (not residual solvents!) and their ^{13}C satellites, and spectral or instrument artifacts such as quad detection or filtering images. Notably, the considerations (a)−(c) also apply to the absolute method described below.

Key to the purity calculation of the 100% method is the normalization of the integrals to the number of protons giving rise to them, leading to normalized integrals for one proton (nInt [per 1H]; see S2-5 Supporting Information for terminology and calculation). The general calculation of purity, *P*, is as follows:

$$P[\%] = \frac{\mathrm{nInt_t}\cdot \mathrm{MW_t}}{\mathrm{nInt_t}\cdot \mathrm{MW_t} + \sum_1^u (\mathrm{nInt_u}\cdot \mathrm{MW_u})} \times 100$$

where MW is the molecular weight, *u* is the number of impurities, and t is the target analyte.

Section S2 of the Supporting Information provides detailed step-by-step guidance and further explanation of the calculations of the 100% qHNMR method. The authors have practiced 100% qHNMR for >15 years and realize that the protocol appears rather complex at first sight. However, once implemented, it should actually be the most straightforward of the qHNMR protocols to perform. Moreover, in our experience, purities determined by the 100% method show excellent congruence with those assessed by absolute qHNMR (unpublished data). This is not entirely surprising, as HNMR uses universal detection. Accordingly, the main differences between the two methods are that 100% qHNMR does not consider contributions from water, inorganic and organic chemicals that contain no or only exchangeable protons, and sorbents, such as the commonly observed impurities resulting from the use of silica gel-based chromatography. However, when the 100% method is combined with internal and external calibration (ECIC) via the residual solvent signals (batch calibration of solvent), it offers a useful means of assessing the absolute content. Such an approach can be readily implemented to serve as an absolute quantitative confirmation of the results achieved with the 100% method (see ref 36 and references therein for further details).

The 100% qHNMR method has two important advantages. First, it does not require precise weighing of the sample. This eliminates analytical errors resulting from inconsistent weighing or inadequate balances. In the experience of the authors and depending on the particular laboratory environment,

establishment of accurate weighing and volumetric conditions can be more challenging than the establishment of quantitative conditions in qNMR, especially when using 3 mm or smaller NMR tubes.

The second advantage of the 100% method, at least in the perception of the authors, lies in the demand it places on the analyst to assign *all* signals of a qHNMR spectrum. While this can be a challenging task, it stimulates critical analysis and consideration of other analytical methods, e.g., for the identification of potential impurities and the determination of their MWs. This task benefits considerably from LC−MS or GC−MS analysis of the same sample, which is orthogonal to qNMR, by providing information about the number of constituents in a given sample and their MW. However, in instances where these challenges cannot be addressed for various reasons or when the emphasis is to be placed on determination of the target analyte only, absolute quantitation is often the method of choice.

Absolute Quantification. This method allows the determination of the mass of a compound with known structure in an accurately weighed sample. It involves the use of a calibrant of known exact weight and purity. When using an added internal calibrant (IC), the general calculation of purity (P) is as follows:

$$P[\%] = \frac{n_{IC}\cdot Int_t\cdot MW_t\cdot m_{IC}}{n_t\cdot Int_{IC}\cdot MW_{IC}\cdot m_s}\cdot P_{IC}$$

where Int is the integral, MW is the molecular weight, m is the mass, n is the number of protons, P is the purity (in %), IC is the internal calibrant, s is the sample, and t is the target analyte/molecule.

Section S3 of the Supporting Information provides detailed step-by-step guidelines for the workflow and calculation used for the absolute quantification method. The absolute method can be used to determine the purity of a target analyte without knowledge of the specifics of the impurities, although absolute quantification of more than one component within the same mixture can also be performed. This follows the general logic that the accuracy of the quantification of a target compound in qHNMR is inevitably connected to knowledge of its molecular weight, which again emphasizes the importance of orthogonal MS analysis.

■ BUILDING A STANDARDIZED qHNMR METHOD

While there are various ways to assemble a qHNMR workflow, it is useful for any given laboratory environment to standardize the procedures and eventually establish a calibrated NMR spectrometer. The following describes key considerations for this process.

Coupled vs Decoupled Acquisition. Depending on the aim of the qHNMR analysis, the spectra can be acquired without or with ^{13}C broad band decoupling. Decoupled spectra no longer exhibit the ^{13}C satellite resonances and, thus, are less crowded, especially in low-abundance resonances. An experimental protocol employing GARP ^{13}C broad band decoupling[39] is available for most NMR spectrometers and only requires appropriate choice of acquisition parameters to minimize heating effects arising from the decoupling duty cycles. The choice of ^{13}C coupled vs decoupled qHNMR acquisition depends on (a) the complexity of the sample, (b) the desired limits of quantitation (LOQ), and (c) the probe/hardware configuration. As a general rule, higher sample complexity and/or the lower desired LOQs (i.e., analysis of high purity materials) favor ^{13}C decoupled qHNMR methods.

The potential utility of ^{13}C satellites as internal threshold for low level impurities (~1% and below) has been described[12] and takes advantage of the highly conserved natural abundance of the ^{13}C isotope. Its abundance (1.091−1.110%, depending on the initial, carbon-fixating natural source) controls the occurrence and intensity of a pair of satellite signals centered at the proton signal of the ^{12}C isotopomer and split by the direct (^1J) $^1H,^{13}C$-coupling constant. This opens the opportunity to utilize the ^{13}C satellite signals for two purposes: (A) definition of a 0.5% threshold level (integral based, per one proton, and relative to MW); this allows the recognition of low level impurities that fall below this threshold and can be readily utilized to classify spectral components as falling "well below 1% (mol/mol)"; (B) use as low abundance signals in external calibration of the solvent signal for use as internal calibrants ("1:200 diluted solvent peak"; ECIC method, see below). While this approach is equivalent to the use of the main residual solvent signal (proton signal of the ^{12}C isotopomer), it can have merit in situations with very high dynamic range, e.g., when using NMR solvents with lower degrees of deuteration and/or low-volume, high sensitivity NMR probes. However, it shall be pointed out that approach B still requires external calibration, whereas method A can take full advantage of the natural internal calibration offered by the fixed $^{13}C/^{12}C$ isomer ratio. Therefore, in simplified terms of practical qHNMR, the ^{13}C satellites of the target analyte (not the solvent) can serve as an approximate level gauge to determine if/which impurities are present below or above 1% abundance.

Developing a Blueprint for Practical qHNMR. The following subsections describe the two most common methods of calculation and the two most widely used calibration methods for qHNMR analysis. All are compatible with the measurement of integration areas and peak heights, as well as the application of computer-generated ^1H NMR profiles.

Relative ("100%") qNMR (Figure 1). The method relies on the measurement and comparison of integral/intensity ratios, which can be transformed into a detailed composition profile on both a weight and/or a molar basis. However, the lack of calibration frequently results in an overestimation of purity, as this method does not consider the contribution of

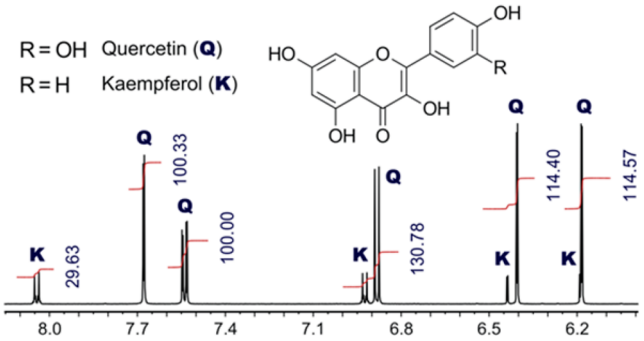

Figure 1. Application of the relative (100%) qHNMR method (see also S2, Supporting Information). A commercial sample of quercetin (Q; declared purity >99%; 24.67 mg/mL [not required for purity calculation] in DMSO-d_6, 600 MHz) was analyzed. A structurally related compound, kaempferol (K), was identified as an impurity. On the basis of the relative integral ratios, the content of quercetin and kaempferol in the sample was determined as 87.8% and 12.2% w/w, respectively.

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"nonobservables" (e.g., salts, silica) or ubiquitous species (e.g., water) to the overall sample weight.

Nevertheless, the relative 100% method represents a rapid and efficient methodology to perform an initial purity assessment of any given sample by qNMR. It can be very easily implemented as part of existing qualitative NMR workflows, without any need for additional experiments. In our experience, the 100% method is well suited to assess the presence and level of impurities that represent structurally related and/or unrelated compounds, as well as commonly known impurities such as solvents. While not suited by default to determine "nonobservables", the 100% approach still has proven to be very valuable in the authors' laboratory, in particular for the assessment of mass-limited, rare materials. Section S2 of the Supporting Information provides a detailed 10-step outline of the workflow and calculation for the 100% qHNMR method, using the data shown in Figure 1 as an example.

Absolute qNMR Using Internal Calibration (Figure 2). The addition of a well-characterized calibrant to the sample is the most common approach to absolute quantitation. General criteria for calibrant choice are high purity, low toxicity and cost, common availability (including traceable primary standard), stability, lack of signal overlap with analyte, few signals, and suitable chemical shift distribution. An overview of frequently employed calibrants and their NMR profiles has been published.[19,40] The authors have found dimethylsulfone ($DMSO_2$) to be a widely suitable qHNMR calibrant.[41] It is preferable that the calibrant be approximately equimolar with the analyte. Section S3 of the Supporting Information provides a detailed step-by-step outline of the workflow and calculation for the absolute qHNMR method, using the data shown in Figure 2 as an example.

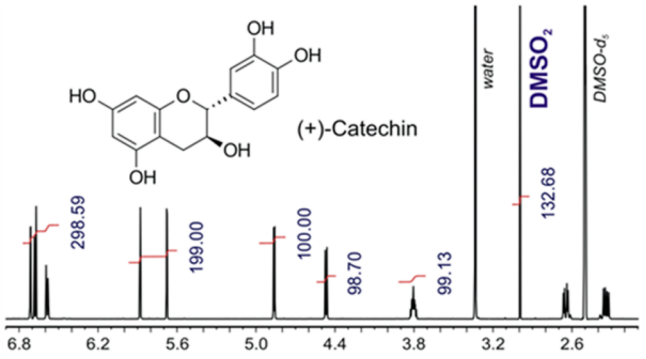

Figure 2. Application of the internal calibration method for absolute qHNMR analysis (see also S3, Supporting Information). A commercial sample of (+)-catechin (5.33 mg/mL in DMSO-d_6, 600 MHz; declared purity >96%) was analyzed. Dimethylsulfone ($DMSO_2$, 99.4% pure) was added to the sample as internal calibrant using a stock solution (2.28 mg/mL; final concentration in the sample 0.380 mg/mL). The content of (+)-catechin in the sample was established as 98.2% w/w.

Absolute qNMR Using External Calibration (Figure 3). The robustness and reproducibility of modern NMR spectrometers underpin this method, which relies on the comparison of two separate NMR spectra: one for the analyte and one for the calibrant. The two NMR spectra must be acquired under identical conditions (see section 2 for a summary of the factors to be considered). Ideally, this includes using the same number of transients (scans) and the same receiver gain, although postacquisition adjustments for these two parameters can be made.[42]

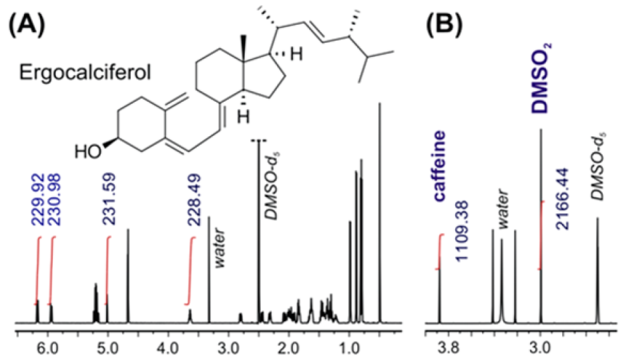

Figure 3. Application of the external calibration method for absolute qHNMR analysis, exemplified for a commercial sample of ergocalciferol (11.11 mg/mL in DMSO-d_6, 600 MHz; declared purity 99.8%; spectrum A). The qHNMR spectrum of an approximately equimolar mixture of two calibrants, caffeine (98.7% pure) and $DMSO_2$ (99.4% pure), was recorded under identical experimental conditions (spectrum B). The integral absolute values of the analyte and the standards were directly compared to establish the purity of ergocalciferol as 98.8% w/w, as the calculated average of both calibrations (see also S4, Supporting Information).

When two experiments are acquired and processed under identical conditions, the absolute values of their integrals can be directly compared. It is recommended to use similar concentrations in both samples to avoid shimming inconsistencies.[34] As the sample is not spiked with an internal calibrant, the sample can be recovered without additional purification steps to eliminate the added calibrant. This method is also suitable for the analysis of rare, valuable materials.

Absolute qNMR Using an Externally Calibrated Solvent Signal as Internal Calibrant (Figure 4). This method is based on the use of the residual protonated solvent

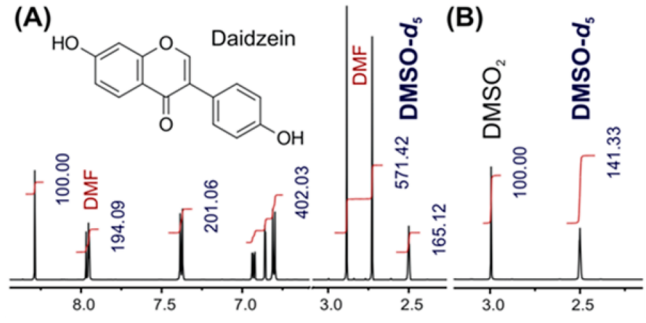

Figure 4. Application of an externally calibrated solvent signal as internal calibrant for absolute qHNMR analysis. A commercial sample of daidzein (17.78 mg/mL in DMSO-d_6, 600 MHz), which was marketed as 97% pure (by LC−MS), was analyzed. The content of residual protonated DMSO-d_5 in the DMSO-d_6 lot was established using the internal calibration qNMR method with a certified $DMSO_2$ standard as calibrant (see also S5, Supporting Information). The content of daidzein in the commercial sample was established as 78.4% w/w. Analysis of the qHNMR spectrum also revealed the presence of significant amounts of dimethylformamide (DMF) of 21.4% w/w. This translates into an almost equimolar ratio of daidzein/DMF (1.054:1.000), which is consistent with a daidzein solvate from a glycoside hydrolysis protocol (HCl/DMF), containing 51.3. mol % daidzein rather than the labeled pure aglycone.

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Table 1. Proposed Standardized Acquisition Parameters

temperature	Run experiments at 298 K.
flip angle	(a) Determine the requirements and best balance for quantitative accuracy and limits of detection (LOD) and quantitation (LOQ). As a general rule, smaller flip angles enable faster signal averaging for increased S/N, LOD, and LOQ, at the primary cost of precision.
	(b) If a 90° pulse experiment is used, determine the pulse width by defining the null at 360° and applying the equation: pw90 = $^1/_4$ × pw360.
acquisition time and spectral width	Choose an acquisition time of 4 s. The acquisition time, spectral width, and number of data points are highly related parameters. We recommend the use of a wide spectral window (20−30 ppm width; centered at ca. 5−10 ppm). As the acquisition time and spectral width are already defined, the actual number of data points will be automatically established by the spectrometer.
relaxation	Determine the longest longitudinal relaxation time (T_1) in the sample, e.g., by an inversion recovery experiment. Define the interpulse delay (often, D1) as (5−7) × T_1. In our experience, an interpulse delay of 60 s is sufficient to guarantee 5 × T_1 even for relatively slowly relaxing protons (e.g., solvents), that is, 99.3% recovery of the signal. Shorter interpulse delays can be justified when reduced accuracy is acceptable for the purpose of the analysis.

Table 2. Proposed Standardized Processing Parameters

window function	Our in-house optimized conditions consist of Lorentzian-to-Gaussian apodization, with line broadening of −0.3 and Gaussian factor of 0.05.
zero filling	2- to 3-fold zero filling, depending on resulting S/N. Typical final data sizes are 128 K for 400−500 MHz and 256 K for 600 MHz and above.
phasing	Careful manual phasing for best reproducibility.
baseline correction	Apply best available baseline correction method, depending on the software used. The goal is to obtain a visually flat baseline at zero intensity for accurate integration.
integration	(a) For pure compounds with nonoverlapping signals: integration areas based on at least 5 × fwhh. Peak heights may also be used, although different processing parameters are required to ensure a quantitative outcome.
	(b) For mixtures with extensive signal overlap: use curve fitting and, alternatively, computational approaches such as ^{1}H iterative full spin analysis (HiFSA) for unambiguous identification of chemical components.

signal as a concentration calibrant. It requires the calibration of the solvent signal using a well-characterized reference standard. This method provides absolute quantification without the need for adding a new substance (internal calibrant) to the sample. However, samples must be carefully prepared to guarantee that the same volume of solvent is added. The calibration process must be performed for every solvent batch. The method is not recommended for highly volatile solvents.

■ QUANTITATIVE CONDITIONS

Acquisition Parameters. The employment and documentation of quantitative conditions are essential when acquiring qNMR data. The key parameters include the following:

(a) The probe must be tuned and matched properly. Good shimming is also essential (manual or gradient shimming).
(b) The experiments must be acquired under temperature-controlled conditions for both the probe and the ambient temperature.
(c) The pulse width should be calibrated, and the pulse sequence and flip angle should be reported.
(d) The interpulse delay must match the desired level of accuracy. In general, for 90° flip angles, the delay must be at least 5 times the longest relaxation time (T_1) to guarantee full relaxation and recovery of the signal intensity. For shorter flip angles, the delay can be reduced.
(e) The receiver gain should be set to 30% of maximum nonattenuated response to achieve high dynamic range, which is important for low level impurities.
(f) The preacquisition delay must be optimized to improve the baseline.
(g) The number of transients (scans) needs to be set to reach a signal-to-noise ratio (S/N) that is sufficient for all signals to be used in quantitative assessment and for the desired level of accuracy. Documented S/N requirements for the limit of quantification are ≥150 for metrological work of reference materials[43−45] and $S/N \gtrsim 10$ for quantification of low-level impurities.[36]

^{1}H Spectral Characteristics. This refers to the type of 1D ^{1}H NMR experiment acquired.

(a) Standard mode: 1D ^{13}C-coupled ^{1}H NMR spectrum. This is the most common NMR experiment. Particular attention must be paid to the presence of ^{13}C satellites during the measurement and calculation part of the qHNMR analysis.
(b) Decoupled mode: for example, GARP ^{13}C-decoupled ^{1}H NMR spectrum. Eliminate ^{13}C satellites that might interfere with low intensity signals in the range of 0.5−1%. By elimination of the ^{13}C satellites, this method also ensures a more accurate integration and a substantial reduction of the spectral complexity, especially for the signals at lower abundance.

Standardized qHNMR acquisition and processing parameters are proposed in Tables 1 and 2. Depending on the intended precision and statistical significance of the measurement, multiple acquisitions of the same sample may be required, especially if only one batch of the sample is available. In practice, the use of at least duplicate determinations has proven helpful to determine outliers produced by inconsistent acquisition conditions or other deviations in the workflow.

Processing Parameters. In general, the NMR processing workflow must be clearly described and part of the documented experimental design. This includes the software and the processing conditions, as follows:

(a) Several software packages are currently available for NMR processing. The list includes, but it is not limited to, NMR manufacturer programs (e.g., Agilent VnmrJ, Bruker TopSpin and XWINNMR, Jeol Delta), third-party software (e.g., ACDLabs NMR Processor, AcornNMR NUTS, MestreLab Mnova, Nucleomatica iNMR), and open-source options (e.g., SpinWorks, NMRPipe).

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(b) Selection of window function for FID apodization: balance between resolution and *S*/*N*.
(c) Use of zero-filling. The resulting digital resolution (in hertz per point, Hz/pt) must be specified.
(d) Phasing strategy (e.g., manual, automatic).
(e) Baseline correction strategy.
(f) Spectral reference (e.g., TMS, TSP, DSS, residual solvent signal).
(g) Integration strategy (e.g., *n* times the full width at half height, $n \times$ fwhh).
(h) If curve fitting is used, specify the software and type of function used (e.g., Lorentzian, Gaussian).

Exemplary qHNMR Conditions for Common Spectrometers. The information compiled in Section S4 of the Supporting Information provides guidance for suitable qHNMR parameters as follows: (S4-A) sample preparation, (S4-B) instrument and software acquisition parameters, (S4-C) additional hardware parameters, and (S4-D) parameters for postacquisition processing and measurement of integrals. The information is generic and relevant for the majority of contemporary NMR spectrometers.

CONCLUDING REMARKS

The unambiguous assignment of structure and the purity evaluation of chemicals used for biological and/or analytical assessment are equally important. The case of the commercial reference material of daidzein in Figure 4 underscores both the need for a "method of questioning"[1] and the ability of qHNMR to provide answers: qHNMR has utility as a universal quantitative detector and offers relatively straightforward options for absolute calibration. As both quality and quantity are essential physicochemical parameters of therapeutic and experimental agents, ^{1}H NMR (HNMR) becomes an increasingly important tool for their characterization: both parameters can be determined using the same quantitative ^{1}H NMR (qHNMR) data set. The proposed qHNMR approach offers the great advantage of providing simultaneous qualitative and quantitative information. This means that qHNMR combines structure elucidation with purity assessment at insignificant extra cost and effort. Importantly, the broad use of qHNMR for simultaneous purity evaluation of organic molecules has great potential to advance the search for the truth behind their biological activity and to find explanations for problems that require consideration of unexpected chemical diversity due to residual complexity.

At nearly the same time when Werner Heisenberg published a critical view on the "method of questioning",[1] Henry Eyring, in his 1948 article on "purity and identity of organic compounds",[46] offered a thermodynamics-driven philosophic reflection on the identical topic discussed here. While the invention of NMR in 1946 by the groups of Edward Purcell[47] and Heisenberg's former graduate student, Felix Bloch,[48] was too young to produce supporting experimental evidence, Eyring interestingly described the occurrence of stable nuclear spin isomers of protons, among other known causes such as stereoisomerism and keto enol tautomerism, as a "complication" that fits the definition of impurities.[46] Eyring's conclusion from 1948 is as true today as ever: "It is the fashion to forget about such complications, with respect to purity, on the theory that 'what the eye doesn't see, the heart doesn't grieve.'"[46] NMR technology is certainly well developed to function as the "qHNMR eye" of the scientist that can provide new perspectives on organic molecules in medicinal chemistry and adjacent fields in the life sciences. The α and ω of any subsequent interpretation of experimental outcome is the consideration of the intrinsically janiform nature of "(im)-purity" and the notion that adamantine purity is evenly associated with magnificence, cost, and reflection (on relevance).

ASSOCIATED CONTENT

Supporting Information

Experimental parameters for qHNMR, description of the qHNMR workflow, and explanations and examples for the calculations in qHNMR. This material is available free of charge via the Internet at http://pubs.acs.org. Additional material is made available by the corresponding author at http://go.uic.edu/gfp and http://qnmr.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Shao-Nong Chen completed a B.S. degree in Organic Chemistry from Lanzhou University, China, and an assistantship in the Lanzhou Institute of Chemical Physics (CAS) and then pursued his interests in natural products chemistry, obtaining a Ph.D. under the joint mentorship of Drs. Yao-Zu Chen, Lanzhou University, and Han-Dong Sun, Kunming Institute of Botany (KIB, CAS). After a 2-year post-Ph.D. training with Dr. Guo-Wei Qin at Shanghai Institute of Materia Medica (SIMM), he joined Dr. Sydney Hecht's group at the University of Virginia. He joined University of Illinois at Chicago in 2000, where he currently is an Assistant Research Professor, working on botanical standardization and integrity in the UIC/NIH Botanical Center, as well as on method development for the analysis of bioactive natural products in interdisciplinary programs.

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David C. Lankin obtained his Ph.D. in Organic Chemistry from the University of Cincinnati, followed by postdoctoral work in organic photochemistry at the University of New Orleans. In 1974, he joined Borg-Warner Chemicals conducting work in phosphorus chemistry, subsequently assisting in establishing the NMR facility in Chicago. In 1985, he joined Varian Associates as an NMR applications chemist. In 1988, he moved to G. D. Searle and ultimately served as supervisor of the NMR facility. At University of Illinois at Chicago, he is a Research Professor and member of the UIC/NIH Botanical Center since 2004. His research interests focus on the applications of NMR to natural products research, specifically structure elucidation and purity/composition by qNMR methodologies. He has (co)authored ~100 papers and been co-inventor on four patents.

Tanja Gödecke is a licensed pharmacist with a Ph.D. degree in Natural Products Chemistry from the Freie Universität (FU) Berlin, Germany. She joined University of Illinois at Chicago in 2006 as a postdoctoral researcher and has since been pursuing her interest in studying medicinal plants and botanical dietary supplements. Her experience lies with the development of analytical methods for the standardization of herbal preparations, especially with respect to identifying and quantifying active ingredients in mixtures. Currently a practicing pharmacist, she is interested in the public health impact of botanical dietary supplements and holds an appointment as Adjunct Assistant Research Professor at University of Illinois at Chicago.

Birgit U. Jaki is a licensed pharmacist (University of Heidelberg, Germany) and obtained a doctoral degree (Dr.sc.nat.) from the Swiss Federal Institute of Technology (ETH), Zurich, Switzerland, in Pharmaceutical Biology, followed by postdoctoral experience at ETH/ZA (Central Institute for Drug Research) and the Institute for Tuberculosis Research at the University of Illinois at Chicago (UIC). Currently, she is a Research Associate Professor at UIC and principal investigator in a method development project for antituberculosis drug discovery. Her research interests are focused on natural products and anti-TB drug discovery.

J. Brent Friesen received his Ph.D. in Natural Products Chemistry from the University of Minnesota, focusing on the biosynthesis of pyridine alkaloids in tobacco. He has spent 10 years in Africa teaching organic chemistry at the Faculté des Sciences Exactes et Appliquées de Farcha in N'Djamena, Chad, and studying native plants used in Chadian traditional medicines. Currently a Professor of Chemistry at Dominican University, River Forest (IL), he holds an appointment as Adjunct Research Professor at University of Illinois at Chicago. His research encompasses the use of innovative NMR applications in undergraduate laboratories and research as well as the chromatography of bioactive natural products. He has participated in the international countercurrent separation community since 2003 and published articles both independently and in collaboration with the Pauli group at University of Illinois at Chicago.

James B. McAlpine obtained a Ph.D. from the University of New England, Armidale, New South Wales, Australia. Postdoctoral work followed at Northwestern University Medical School, studying the biosynthesis and mode of action of macrolide antibiotics. In 1972, he joined Abbott Laboratories and worked on macrolides, aminoglycosides, and quinolones before heading up their natural product discovery project in 1981−1996, which discovered tiacumicin B, the API of Difficid. He joined Phytera Inc. as V.P. Chemistry in 1996 discovering drugs from manipulated plant cell cultures, and in 2002 he joined Ecopia BioSciences as V.P. Chemistry and Discovery using genomics to discover novel secondary metabolites. He authored 100+ papers, is inventor on 50 U.S. patents, and joined University of Illinois at Chicago as Adjunct Research Professor in 2011.

José G. Napolitano received his Ph.D. in 2010 from Universidad de La Laguna (Tenerife, Spain), where he worked on the characterization of new marine natural products under the tutelage of Drs. Manuel Norte, José Javier Fernández, and Antonio Hernández Daranas. After receiving his Ph.D., he moved to University of Illinois at Chicago, where he carried out postdoctoral research with Dr. Guido Pauli on the application of NMR techniques for phytochemical analysis. He recently joined the NMR group at AbbVie Inc. His research interests include computer-assisted NMR analysis and the development of computational and spectroscopic approaches to solve complex stereochemical problems in organic chemistry.

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■ ABBREVIATIONS USED

qNMR, quantitative nuclear magnetic resonance; qHNMR, quantitative ^{1}H nuclear magnetic resonance; HiFSA, ^{1}H iterative full spin analysis; HPLC, high performance liquid chromatography; *S/N*, signal-to-noise; IC, internal calibrant; EC, external calibrant; ECIC, combined external and internal calibration; LOD, limit of detection; LOQ, limit of quantitation

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