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# Quantitative $^1$H NMR. Development and Potential of an Analytical Method: An Update

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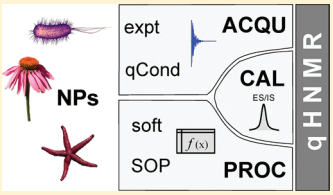
Supporting Information

**ABSTRACT:** Covering the literature from mid-2004 until the end of 2011, this review continues a previous literature overview on quantitative $^1$H NMR (qHNMR) methodology and its applications in the analysis of natural products. Among the foremost advantages of qHNMR is its accurate function with external calibration, the lack of any requirement for identical reference materials, a high precision and accuracy when properly validated, and an ability to quantitate multiple analytes simultaneously. As a result of the inclusion of over 170 new references, this updated review summarizes a wealth of detailed experiential evidence and newly developed methodology that supports qHNMR as a valuable and unbiased analytical tool for natural product and other areas of research.




## INTRODUCTION

In the past seven years since the publication of our earlier review on quantitative $^1$H NMR (qHNMR) for natural product (NP) analysis,[1] the usefulness of NMR spectroscopy as a quantitative tool (qNMR) has received considerable additional attention. This is documented not only by a steady increase in the volume of literature reports that employ qHNMR but also from numerous communications with colleagues who utilize qHNMR or are interested in this methodology. While continuously updating our literature collection on the topic, we have also become progressively aware of a coherence that appears to be characteristic of the field of qNMR. Thus, representing long-standing research and practice, q(H)NMR has produced a wealth of knowledge in industrial settings, e.g., the pharmaceutical, chemical, and food sectors. One plausible interpretation of this phenomenon is that proprietary qNMR methodology provides a competitive advantage for industrial products. This would attest to the superiority of the capabilities of qNMR in terms of work-flow effectiveness, accuracy, precision, and cost–benefit relationships, when compared with other established methods. From a scientific perspective, one unfortunate occurrence is that much of the proprietary knowledge remains undisclosed, such as trade secrets or internal standard operating procedures, and does not usually find its way into the scientific literature. Similar considerations apply for patents involving qHNMR methodology. While there are more than 200 patents on the subject, many of these do not lead to a corresponding peer-reviewed publication. The present contribution covers only peer-reviewed literature.

The current review seeks to update the global picture of qHNMR with respect to recent developments of qNMR methodology, the qNMR work-flow (Figure 1), and advances made in the understanding of metabolomic complexity, which affects NPs at all levels, from crude extracts to bioactive fractions to "pure" compounds (see Residual Complexity of NP Reference Materials section). The organization of the present review generally follows that of the previous work[1] and emphasizes areas of new experimental developments and applications. As high- and ultra-high-field NMR instrumentation (400–1000 MHz) becomes increasingly more available, qHNMR can be considered as a universal method for NP analysis,[2] particularly bioactive NPs,[3] which covers all small molecules (<2000 amu) but also macromolecular systems. As the section on new qHNMR applications shows, there are essentially no restrictions in compound class, as long as the analytes contain protons and provided the dispersion (magnetic field strength) has sufficient resolving power for the complexity resulting from the combination of the $^1$H NMR spectra of the analytes and the composition of the sample (pure compounds, mixtures of compounds, chromatographic fractions, crude extracts).

## BACKGROUND

**The Basics of qHNMR.** A working definition of qNMR, its historic and physicochemical background, the commonly used nomenclature, and an overview of experimental design (data acquisition, postacquisition processing) to establish *quantitative conditions* for qHNMR can be found in our previous review.[1] While the previous report included relevant literature published until mid-2004, the present contribution continues from that point and covers the literature published until the end of 2011.

Following cross references in recent publications, previously unnoticed qHNMR reports were discovered while preparing this review and shall be given credit as very early reports on qNMR applications. This includes work from the period 1963–1976 on the use of an internal standard such as caffeine (**1**) for calibration (see definition of *calibration standards* below, in the

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Reference and Calibration Standards section) in pharmaceutical analysis,[4−7] as well as two reviews on the topic by Rackham,[8,9] which covered the literature until 1975. An early report of qNMR in pharmacognosy research by Hiltunen et al. described the use of this method for atropine (**2**) and scopolamine (**3**) analysis in Solanaceous leaves at a field strength equivalent to 200 MHz for $^1$H and noted that the magnetic field strength employed was insufficient for the quantitation of anthraquinones [e.g., sennoside (**4**)] in *Senna* extracts.[10]

A relatively underexplored field in qNMR is the use of homo- and heteronuclear 2D-NMR experiments. The latter include the inverse-detected sequences such as HSQC. Since these experiments detect $^1$H nuclei, they fall within the scope of this review and are addressed in a newly added section on two-dimensional qHNMR methodologies. Another new section provides an overview of some of the software tools available to practitioners of qHNMR.

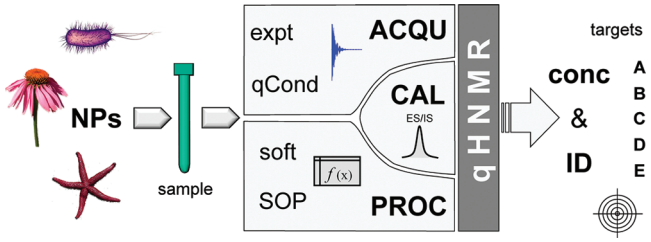


**Figure 1.** Graphical representation of the qHNMR concept and work-flow. Using $^1$H signals for universal detection, qHNMR is amenable to a broad range of pure to crude natural products (NPs). The qHNMR method consists of three main components: (i) ACQU, the acquisition, comprised of a suitable $^1$H NMR experiment (expt) that establishes quantitative conditions (qCond); (ii) CAL, quantitative calibration using a suitable external calibrant and method or an internal calibrant; (iii) PROC, postacquisition processing of the raw NMR data (i.e., FIDs), using a standard operation protocol (SOP) and software tool (soft). Major strengths of the qHNMR work-flow include the ability to simultaneously yield concentration (conc) and identity (ID) information and to perform the analysis for several target analytes at a time and without the need for identical calibration standards.

**Updated Literature Background of qNMR.** In the period since mid-2004, several excellent review articles have appeared that cover various aspects of qHNMR: application in pharmaceutical analysis[11,12] and the quality control of traditional Chinese medicines;[13] metabolomic profiling of plant extracts;[14] an overview of quantitative metabolomics;[15] and an overview of the theoretical foundation of quantitation by NMR.[16] The (semi)quantitative capabilities of NMR in the analysis of carbohydrate mixtures by means of an artificial neural network are addressed in a contribution by Duus et al.[17] In the context of metabolomic analysis, which seeks to characterize multiple individual metabolites both quantitatively and qualitatively, the NMR-based protocol recently published by Kim, Choi, and Verpoorte is a valuable resource for plant metabolomic studies.[18]

An updated statistical picture of the development of the peer-reviewed qNMR literature is given in Figure 2. As the present work focuses on small-molecule NPs, the published literature was analyzed for the NP subset using SciFinder in section titles (see S1, Supporting Information, for details). In recent years, the NP literature has maintained a consistent 10−20% share of all qNMR publications. From a total of ∼13 000 hits on "quantitative NMR", ∼2400 were related to representative NP subsets. Expressing the growth reflected in Figure 2 in numbers, when comparing the seven-year periods of 1998−2004 (covering one-third of the entire qNMR literature from 1954 to 2004) with 2005−2011, the former saw about 81 publications per year, while more recently 133 papers were published annually. Within all of the NP qNMR literature, 16% focus on specific plants or plant parts, as assessed using SciFinder index terms. Of NP-related qNMR publications, 91% were published in English, followed by 3% in Chinese, and 2% in Japanese. The following six NP journals have each published six or more qNMR reports and, thereby, contributed 8% of the NP-related qNMR literature: *Journal of Agricultural and Food Chemistry*, *Journal of Pharmaceutical and Biomedical Analysis*, *Phytochemical Analysis*, *Journal of Natural Products*, *Chemical & Pharmaceutical Bulletin*, and *Phytochemistry*.

In order to gain insights into the nature of the most recent qNMR publications, i.e., whether they report on applications or explore new qNMR methodology, all the publications including the NP subset were analyzed for *research topics*, using the following three representative terms: "acquisition parameters", revealing 25 publications (0.2%) on new qNMR methodology; 152 publications (1.2%) reporting research on "processing parameters"; and 142 (1.1%) found using the term "peak area". On the basis of these data and the authors' general assessment, it is reasonable to conclude that only a very small number (<2%) of publications on "quantitative NMR" have been exploring new qNMR methodology such as experimental parameters, pulse sequences, and experiments, while the vast majority reported on applications of qNMR, with several studies including method validation.

## THE QHNMR EXPERIMENT

**Reference and Calibration Standards.** All qNMR experiments require two forms of quantitative adjustment: one concerns the chemical shifts ($\delta$ in ppm) of the resonances, for which the terms *referencing* and *reference standard* are used in the following sections. The other adjustment relates to the quantitation of the NMR signals, and the terms *calibration* and *calibration standards* (*syn. calibrant*) are used herein for this purpose. While TMS and DSS are the IUPAC-approved NMR standards for *referencing*, in practice chemical shift calibration is often done externally and/or via the residual solvent signal. As a result, the literature is nonhomogeneous with regard to NMR *referencing*, and qHNMR reports should define how both *referencing* and *calibration* were performed.

However, both operations require a well-defined standard material, which is often termed a "reference standard" or "reference compound". While these ambivalent terms were used in the last review,[1] the crucial role of *calibration* and *calibration standards* in qNMR has now become more clearly defined. Thus, we propose to distinguish the different roles and requirements for the two types of standards in qHNMR, i.e., the *reference standard* and the *calibration standard* (*syn. calibrant*). These terms also set them apart from *reference materials*, the term used in this review to designate highly characterized samples ("standards") of a single chemical entity such as a NP.

**Carbon Decoupling and New Experiments in qHNMR.** A routine qHNMR protocol illustrated for paclitaxel, which demonstrates the advantages of $^{13}$C GARP decoupling of the proton NMR spectrum to suppress the $^{13}$C satellites, has been published.[19] When using this protocol on instrumentation equipped with room-temperature probes, in either direct or


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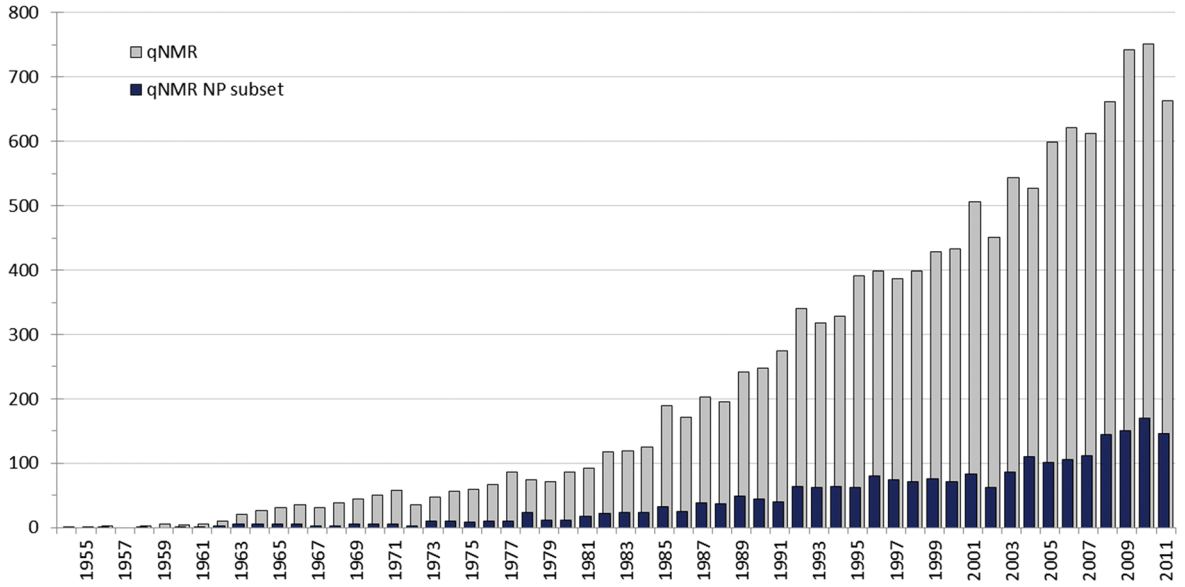



**Figure 2.** Development of the peer-reviewed literature on quantitative NMR (qNMR) since 1954. The survey used ACS's *Chemical Abstracts*, searched via SciFinder for the term "quantitative NMR", resulting in a quantitative measure for all qNMR literature ($N_{tot}$ = 13 047) and the small molecule NPs (NP) subset (for details on subset definition, see S1, Supporting Information).

inverse configuration $^{1}$H mode, sample heating due to decoupling effects during the acquisition (typically 4 s) are usually not a problem. However, on inverse cryoprobes, the lengthy acquisition times can be of concern and damage to the coil may occur. In this case, qHNMR should be run without $^{13}$C GARP decoupling. WURST decoupling, which further reduces sample heating, can be employed when using cryoprobes, but its use in this regard must be verified experimentally to achieve quantitative conditions, as covered in the original review.[1] The inability to use $^{13}$C decoupling may create problems in the quantitation process where spectral overlap is an issue. As long as the signal-to-noise ratio (S/N) of the resulting spectrum is sufficient (>150:1), quantitation can usually be achieved and the presence of the $^{13}$C satellites may be used as an internal reference for the quantitation of low levels of impurities in highly pure reference materials or for low levels of other constituents in a crude extract or fraction.

One interesting new NMR experiment for qHNMR that has recently been reported employs band-selective, adiabatic pulses to selectively excite trace components. Using highly complex samples such as olive oil and honey as test cases, Rastrelli et al. demonstrated how quantitative information can be obtained with high S/N and without interference from strong matrix signals.[20]

**General Aspects of Calibration in qHNMR.** Like any quantitative method, qHNMR requires calibration, which, in principle, can be performed internally or externally. The calibration process traces back ultimately to an accurately weighed "*primary*" *standard*, which ideally is of high purity and has been characterized extensively. The above distinction between *calibration* ("quantitation") and *reference* ("chemical shift") *standards* also falls into place when considering their different requirements in qNMR with an internal standard: the ideal calibrant is a traceable primary standard, but its signals should not overlap with those of the analyte. The second requirement, however, favors compounds with few NMR signals and a certain chemical shift distribution, which makes an appropriate choice difficult or sometimes impossible. In an extension of a comprehensive study of internal calibration standards for qNMR by Rundlöf and co-workers,[21] a compilation of some 40 standard compounds with reported use in qHNMR is provided in the Supporting Information (S2), along with the distribution of their chemical shifts and signal multiplicity. It should be noted that only a few are available as traceable standards [e.g., dimethylsulfone ($DMSO_2$, **5**)], whereas others that have recently been used as external calibration standards [e.g., caffeine (**1**)] may not be listed but are still very suitable.

According to a review by Diehl et al.[22] (and references therein), qNMR methods have been used successfully when chromatographic standard methods have proved to be ineffective. In general, qNMR can be considered a primary ratio method of measurement in which the analyte can be correlated directly to the *calibration standard*. Therefore, in addition to relatively easy sample preparation, the advantages of qNMR over other analytical techniques include (i) the internal *calibration standard* can be a certified reference material and (ii) only one *calibration standard* is necessary for multiple analytes, which can be different from the analyte(s) to avoid signal overlap. (iii) Since the reference compound is different from the analyte, generating a calibration curve becomes unnecessary. (iv) An additional advantage is that for the validation of qNMR conditions the integrals of multiple signals in a spectrum can be used. According to the study of the National Metrology Institutes of Japan and Germany, errors of <1% are commonly achieved in qHNMR when using a certified reference material as internal *calibration standard*.[23,24] The impact of several NMR acquisition parameters on quantification results has been studied and may be summarized as follows: in qHNMR a relaxation delay of at least five times $T_1$ of the slowest relaxing nuclei is essential (when using a 90° flip angle), the acquisition time must be long enough to avoid FID truncation resulting in baseline distortions, the accuracy of integration depends on the S/N achieved, and the receiver gain needs to be set to an optimized value for the spectrometer.[22,25] When validating a quantitative method in which signal averaging is to be done, results should be compared to the

single-pulse experiment using an internal *calibration standard*, which is considered as the most accurate procedure.

**Calibration Methods in qHNMR.** As qNMR is considered a primary analytical method, reference materials do not need to be identical or even chemically related with the analyte (*identical reference standard,* or more distinctively *identical calibrant*). Accordingly, a wide range of pure chemicals have been employed as internal calibration standards for qNMR.[21] Some studies focus on the discovery of a new internal calibrant for qNMR analysis with properties that allow for the most general application. Pinciroli et al. compared an LC-UV-MS method with a qHNMR method using a novel silane standard, 1,4-bis(trimethylsilyl)benzene (BTMSB) in DMSO-$d_6$ solution, to build a generic method for the quantitation of small organic molecules. The present authors have found BTMSB to be an easily weighable solid that is stable for at least one month in DMSO solution and which produces a strong singlet in a signal-free region of the $^1$H NMR spectrum. Quantitative determinations led to ~2% precision and accuracy, which was verified using a set of certified standards and with fully automated sample preparation.[26]

A study by Wells et al. emphasizes the advantages of using one universal reference material (URM) for qNMR. The authors propose $DMSO_2$ (**5**) for this purpose, as it is widely available and of high purity, inexpensive, chemically stable, soluble in a wide range of aqueous and nonaqueous solvents, chemically inert, and nonhygroscopic. In addition, due to its single $^1$H NMR signal, it shows minimum interference with other analytes and is, therefore, proposed as a URM for the certification of secondary reference materials.[27]

The residual solvent signal in $^1$H NMR spectra can be used as the "internal standard signal" for the calibration of the quantitative method with external calibrants.[28] Use of the solvent signal is beneficial when the analyte needs to be recovered after analysis in pure form. A calibration curve relates the solvent signal to another external reference material such as high-purity caffeine (**1**). The first report of the utilization of the residual solvent signal for calibration was by Letot et al.,[29] who established a qHNMR-based quality control (QC) protocol for a combinatorial chemistry library, also a new approach in the field that otherwise is dominated by LC(-MS). Interestingly, they observed an average sample purity of 52%.[29] Later, Pierens et al. compared the qNMR method using the residual solvent signal as internal calibration standard with balance concentrations of 18 NPs.[30] A calibration curve with nine concentrations of **1** showed a linear relationship in the concentration range 0.1−60 mM. Many solvents have comparatively long $T_1$ values. Therefore, when using the solvent signal as internal calibration standard, the acquisition duty cycle needs to be chosen carefully. The authors also found that the amount of residual solvent varied from batch to batch of NMR solvent, but for DMSO-$d_6$ was stable over time and reliable within the same batch. However, the stability of the solvent with respect to deuterium exchange will be dependent on the chemical nature of the dissolved analyte(s) and may require separate stability validation, particularly for strongly acidic or basic analytes. The qNMR concentrations for five crystalline NPs were in good agreement with the balance concentrations, while 18 other NPs differed greatly from their balance concentrations, suggesting again that qNMR is a sharper and more universal method for the purity assessment of NPs.[30]

One key conclusion of the 2005 study by Burton et al.[28] is that the external calibration of an internal (solvent) signal is possible but not necessary: provided precise measurements of the 360-degree pulse length are performed for each sample and single-coil excitation and detection is used, quantitation of the NMR signal is straightforward following the Lorentz principle of reciprocity.[31] Accordingly, a correction factor for variations in probe-Q can be calculated that allows direct comparison of signal intensity across samples, even in different and non-deuterated solvents.[28] Using a series of standards of algal toxins as samples, the authors demonstrated the validity of qHNMR concentration measurements, with excellent validation parameters and errors and without the need for internal calibration standards.[28] This principle was implemented subsequently in the method of "pulse length-based concentration measurement", developed for the determination of protein concentrations by Wider and Dreier.[32] One advantage of calibration methods that utilize the principle of reciprocity to correlate measurements of absolute NMR intensity over methods that utilize electronic reference signals ("ERETIC") in qNMR is that they avoid challenges associated with the practical implementation of the latter. Seeking to combine the best of both approaches, Farrant et al. have developed, very recently, software that integrates the calculation of probe-Q correction factors with the addition of an artificial NMR signal for automated quantitation.[33] A similar method was also implemented by Walker et al. for the validation of metabolite reference materials.[34]

**Sensitivity and qHNMR Calibration of Low-Level Analytes.** Often quoted disadvantages of NMR are its "low" sensitivity and dynamic range, especially when compared to LC methods with selective ion detection. With regard to signal dynamic range, recent developments in the design and manufacturing of NMR instrumentation, specifically faster digitizers for oversampling, can be expected to lead to significant improvements in the detection of trace components and residual complexity in NPs (see section on "Residual Complexity of NP Reference Materials"). From the perspective of the NPs researcher, NMR has recently experienced major gains in sensitivity due to the development of microcryoprobe technology. Martin and co-workers[35,36] (and references therein) are among the leaders in this field and have demonstrated the feasibility of acquiring a 2D-gHSQC spectrum of 540 ng of strychnine (**6**) at 600 MHz utilizing a weekend of spectrometer time. Along the lines of low-level/high-sensitivity qHNMR, Weljie et al. described a method for the detection and quantitation of low-intensity unknown peaks (LIUPs) in spiked urine samples.[37] The method uses database spectra of pure compounds to identify known metabolites in the sample, principal component analysis (PCA) for the identification of the spectral regions responsible for differences between samples, and a subsequent peak fitting method to identify the LIUPs from the spectral regions of interest. The study used 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) as internal calibration standard to relate the analyte spectrum and reference spectrum. It was shown that major influences stem from baseline effects, especially on low-concentration metabolites, as well as from parameters in performing the PCA analysis (binning method, weighting functions). In addition, $^{13}$C satellite signals need to be identified in spectra to ensure legitimacy of low-level metabolites. As a result of the study, a number of metabolites in spiked samples were determined to be correctly and reproducibly quantified in the <10 $\mu$M range.[37]

When isolating NPs with biological activities, initial sample amounts are often limited. Reports by Claridge et al.[38] and Dalisay et al.[39] addressed the accurate qHNMR determination of low-level amounts of impurities and isolates, respectively. Both studies are based on the concept that an internal calibration standard signal of similar integral value as the analyte, such as the natural $^{13}C$ satellites, yields more accurate quantitative results.[2] In the latter study, the $^{13}C$ satellite signals of the solvent ($CDCl_3$) serve as internal calibrants. In addition, the authors used 1.7 mm microprobe technology to reach lower limits of quantitation: when calibrating against cholesterol, a linear correlation was observed over the concentration range 28.5 $\mu$M to 1.42 mM. The study also used a 30° pulse angle for faster relaxation to achieve better S/N in a given time. To ensure sufficient relaxation of the observed nuclei, two relaxation delays were compared, resulting in only a minor difference between the integral ratio of a cholesterol signal and the $^{13}C$ satellite internal calibration signal.[39]

For the quantification of slightly overlapped NMR signals of compounds at the lower concentration limit, a strategy to obtain a higher signal resolution and subsequently accurate quantitation results is the use of peak height instead of integral values.[40] The peak height in a spectrum is not directly proportional to the number of nuclei at that frequency. To obtain accurate results using the peak height method, the peak widths at half-height of both the standard and the analyte signal have to be equal, which can be achieved by applying postacquisition processing. The method can be applied to any qHNMR spectrum, and the results from peak height quantitation and peak area quantitation should be used in conjunction as a way to ensure correct results. Poor phasing, baseline correction, and shimming were shown to influence either qNMR method differently. Therefore, a large variance between the integral and peak height results can give clues to potential errors in data acquisition or processing. This method readily lends itself to automation. Maleic acid, dimethyl fumarate, or **1** was used as internal calibrant.[40]

Another strategy for the quantitation of overlapped or low-concentration signals is peak fitting or deconvolution instead of simple integration of such signal groups. Soininen et al. have described a method for organic impurity determination by qHNMR employing line fitting.[41] The study uses the PERCH software suite for off-line processing, which includes a program for total line shape (TLS) fitting. Upon comparison of various line-fitting methods, one key result is that the ability to enter some constraints into the fitting model, such as assumptions about the multiplicity of the impurity peak or equivalences of certain signal areas, allows impurities to be quantified correctly at levels as low as 0.1 mol %. Results obtained by using the constrained TLS fitting method in determining signal areas remove the problems arising from baseline artifacts in normal integration. This results in higher accuracy, especially for the quantitation of compounds at low concentrations or with overlapped signals. An improvement to the GARP decoupling method for baseline improvement and $^{13}C$ satellite removal also has been described.[41]

**Summary of Calibration Strategies in qHNMR.** Measurement of an internal calibrant added to the analyte and use of a single-pulse experiment are widely considered the most accurate approach to qHNMR and can well be used for calibration purposes. However, an internal standard added to the sample must be carefully selected, and possible chemical interactions with the analyte need to be precluded prior to qHNMR analysis. Alternatively, using the residual solvent or (its) $^{13}C$ satellites as internal calibrant evades the consequences of adding an internal standard. This approach has been shown to give accurate results in combination with external calibration and allows absolute quantitation in qHNMR. For the quantitation of low-concentration samples, multiple transients need to be collected and acquisition parameters must be chosen with particular care to establish quantitative conditions (see ref 1 for an overview). While $^{13}C$ satellites and residual solvent signals can be very useful in high-throughput applications, it should be noted that this form of calibration is specific to the isotopic labeling of the calibrant, e.g., the solvent used.

External calibration is available as a very flexible and highly precise qHNMR concept[28] that has been shown to be suitable for the establishment of traceable NP reference standards. It is based on the Lorentz principle of reciprocity[31] and relies primarily on precise pulse length measurements and the establishment of a correction factor. Moreover, it allows direct qHNMR quantitation across samples and (non)deuterated solvents. Finally, methods such as total line fitting and peak height quantitation are very useful for both calibration and subsequent quantitation. They are advantageous especially in crowded spectra of NPs, do not increase demand on NMR spectrometer time, and require only a more elaborate postacquisition processing work-flow.

**Relationship between qHNMR and Other Quantitation Methods.** A review by Gilard et al. describes several examples of the application of $^1H$ NMR to the quality control of herbal materials and highlights besides the above stated advantages of qNMR ways to deal with its limitations when analyzing complex samples such as plant preparations. For this purpose, when screening herbal medicines for adulteration, the diffusion ordered spectroscopy $^1H$ NMR method (DOSY) has been found to be particularly useful and is regarded as a "virtual chromatography".[42] Numerous studies have reported on the use of $^1H$ NMR spectroscopy for quantitation of constituents in plant preparations,[43−47] and many reports have compared the more customary method with a new qHNMR technique that is less time-consuming and more economical,[47−49] yields the same or better accuracy,[50−58] and supplies more qualitative information about impurity identities as well as impurity counts in a test sample.[59−62] For these reasons, qHNMR purity levels are often found to be lower than those determined by other methods. One example is a study on macrolide antibiotics. A qHNMR procedure was compared with a mass balance method, which represents the gold standard for determining purity of a primary chemical reference material. Both methods yielded similar results for the five kinds of macrolide antibiotics used. 1,4-Dinitrobenzene was selected as internal calibration standard over anthracene. The COSY and NOESY spectra of 1,4-dinitrobenzene and clarithromycin (**7**) indicated no interaction between standard and analyte. The uncertainty of the qNMR method was smaller in four of five cases when compared with the mass balance method. Moreover, the advantages of qNMR include simple sample preparation and a quick and easy analysis. The uncertainty involved with the qNMR method was found to be derived mainly from the sample weight and accurate integration of the monitor signals of analyte and reference. To minimize the integration error, it is best to use similar concentrations for the analyte and standard.[63]

For the determination of oxyethylene group (EO) content of polysorbates used as food additives, qNMR was compared with

a classical titration method. It was demonstrated that the EO contents of commercial polysorbates 20, 60, 65, and 80 (**8**) could be rapidly and simply determined using qNMR with an internal calibrant. Potassium hydrogen phthalate was used as the internal calibrant, and the EO signals were identified through comparison with sorbitan monolaurate and poly(ethylene glycol) distearate prior to quantitation.[64]

LC-MS methods are considered indispensable tools to measure complex analytes at trace levels and for routine analysis. When authentic samples of such complex analytes (and corresponding internal calibration standards) are difficult to obtain, especially in greater amounts where the absolute purity can be established more readily, qNMR experiments are being recognized[64] and employed as the primary analytical method[65−67] or for calibration purposes. A study by Mohn et al. used qNMR for the purity determination of authentic NP reference materials to be used for LC-MS analysis of glucosinolates in cruciferous plants. The purity of nine glucosinolate (e.g., **9**) reference compounds (HPLC purity >99%) was determined by qNMR using 1,3,5-trimethoxybenzene as internal calibrant. The qHNMR data revealed significant differences in purity levels when compared to HPLC values. The purity of the reference materials was found to be in the range 60−90%, showing NMR to be the more universal detector and qNMR a superior method for purity determination of NP reference materials.[68]

Another study by Shao et al. described the use of *p*-toluenesulfonic acid as a reference compound in aqueous solution. Using quantitative NMR, *p*-toluenesulfonic acid was first used to determine the purity of compounds that serve as authentic or internal calibrant in other chemical analyses and was removed from the sample successfully after the analysis.[69]

**Chemical Shift Variations of qHNMR Reference and Calibration Standards.** A qNMR assay is almost always used simultaneously as a *qualitative* tool, evaluating the characteristic coupling patterns and chemical shifts for analyte identification. Noncovalent weak forces that stabilize molecular assemblies through intermolecular hydrogen bonds, aromatic $\pi-\pi$ stacking, and electrostatic interactions lead to "head-to-head" and/or "head-to-tail" dimer formation. In such assemblies, the number of molecules, the orientations in the aggregate, and their mutual interactions and "tightness" of association should vary as a function of concentration, which in turn should manifest in the altered chemical shifts.[70] Working on the quantitation of mebeverine (**10**) HCl from tablets, Blagbrough et al. have studied effects of concentration, pH, and temperature on the chemical shifts of the mebeverine protons.[71] The authors found that concentration differences had the highest influence on chemical shits in $D_2O$, with increasing concentrations resulting in an upfield shift. In $CDCl_3$, DMSO-$d_6$, MeCN-$d_3$, and $CD_3OD$, chemical shifts remained stable. Moreover, changes in pH did not result in major differences, whereas a temperature change from 20 °C to 75 °C resulted in a general downfield shift of the proton resonances. Pulsed-field gradient spin echo (PGSE) experiments were conducted in $D_2O$, $CDCl_3$, and $CD_3OD$, suggesting that the concentration influence on chemical shifts is due to aggregation effects in aqueous media. The amounts determined of mebeverine HCl were in the range of 5−50 mg/mL, as recovered from tablets analyzed. The qHNMR results were close to the label value (100 mg; qNMR mean 99.3 ± 0.92 mg) with a relative standard deviation of 0.84%, which demonstrated the credibility of the qNMR methodology.[71]

Taking these findings into account, any added internal calibrant potentially may influence the weak solute−solute interactions and result in chemical shift changes of the internal calibrant or the analytes. A reliable method for comparing chemical shifts under varying conditions is required for studying the effects of solvent, temperature, and concentration. Especially for an automated identification of known analytes, accurate shift referencing seems crucial. Thus, a study by Hoffmann describing effects on the chemical shift of tetramethylsilane (TMS) gives valuable insights into measuring absolute chemical shifts using a conventional NMR spectrometer.[72] The findings are important because TMS is considered to be the global reference compound for zero-point referencing of the NMR ppm scale. However, the chemical shift of the TMS signal in dilute solutions was found to vary in different solvents and to be dependent on the temperature in the same solvent. The exact chemical shift of TMS was found to depend mostly on solvent anisotropy and polarizability, and it is suggested that the chemical shift be referenced to TMS protons at 0.000 ppm in the same solvent, unless comparison is required with other solvents. IUPAC does not refer to a standard temperature for the chemical shift of the TMS signal. Therefore, temperatures need to be compared carefully before comparing chemical shifts.[72] In addition, it is important to note that temperature precision and stability require careful evaluation when validating qHNMR methods.

**Software for qNMR Analysis.** Software specifically created for analyzing primarily 1D quantitative NMR spectra (1D $^1H$ and $^{13}C$) has appeared. In addition to analysis software provided by the respective NMR instrument manufacturers (Agilent [vnmr], Bruker [Topspin], and JEOL [Delta]), commercial software packages are available from third party software providers (e.g., ACD, AcornNMR [Nuts], Mestrelab [MNova for Windows, iNNMR for OsX], PERCH Solutions [PERCH], NMRTec [NMR Notebook]) and have appeared in addition to open-source software (e.g., Frank Delaglio's NMRpipe [http://spin.niddk.nih.gov/NMRPipe/], One Moon Scientific Inc.'s NMR View J [http://onemoonscientific.com], and Kirk Marat's SpinWorks [ftp://davinci.chem.umanitoba.ca/pub/marat/SpinWorks/]). Most of the software is designed to handle one spectrum at a time, but at least two open-source packages, the statistical software package rNMR[73] and ImatraNMR,[74] are capable of batch integration and analysis of qNMR spectra, while not providing processing capabilities. So far, these software solutions have been applied to the metabolomics problems for which they were developed. They are, however, capable of addressing other problems in qNMR analysis unrelated to metabolomics. Spectral "binning" and parallel signal alignment have been a main approach in making NMR data available to external statistics analysis, using software packages such as Umetrics' SIMCA, Matlab, and Microsoft Excel/XLStat. For example, Bruker's AMIX software and rNMR[73] allow the use of multiple, specifically selected peaks or regions of interest in 1D- and 2D-NMR spectra for further statistical analysis, either within the software (AMIX) or after export (rNMR). Both allow a manual peak alignment, however, only for the entire spectrum. Work by Forshed et al. addresses $^1H$ NMR peak alignment based on multiple points in the spectra by self-written Matlab code, validated by subsequent PCA and PLS-DA analysis.[75,76]

## TWO-DIMENSIONAL QHNMR METHODOLOGY

One-dimensional qHNMR techniques have been demonstrated to work very well for performing purity analysis on reference materials or isolated NPs, fractions, or crude extracts. In general, using $^1$H NMR spectra, with isolated signals associated with a specific component or components in a mixture clearly present and "out in the open", a clean integration of these proton resonances can lead to a quantitative determination of each of the chemical components in the sample. However, in NP research, samples are encountered that are clearly indicative of very complex mixtures of chemical components and may result in significant difficulties in the quantitation of the components in these mixtures. Since the publication of our 2005 review,[1] focusing principally on 1D-qHNMR techniques, there has been considerable interest and activity in studying the application of 2D-NMR techniques for the quantitation of proton NMR spectra of complex mixtures. Complex mixtures exhibit extensive spectral overlap because of the large number of components present in the mixture. This is especially true for crude extracts of plant material and also for the fractions derived from these crude extracts. The use of 2D-NMR techniques for quantitation (2D-qHNMR) is in part designed to bring about a simplification of the chemical information present in a mixture (not a new concept), which may be accomplished by different mechanisms depending on the nature of the 2D-NMR pulse sequence as well as the experiment employed and the chemical information being revealed. A summary of some of the 2D-NMR techniques that are currently being explored for quantitation purposes has appeared in an excellent review published recently by Koskela et al.,[77] and additional papers describing a variety of 2D-NMR methodologies and applications have appeared. Presently, there are a considerable number of 2D experiments that are also actively being exploited for the purposes of quantitation and that have been demonstrated to provide reliable results when compared to the corresponding 1D-qNMR spectral data or quantitation results obtained by chromatographic means, e.g., HPLC.

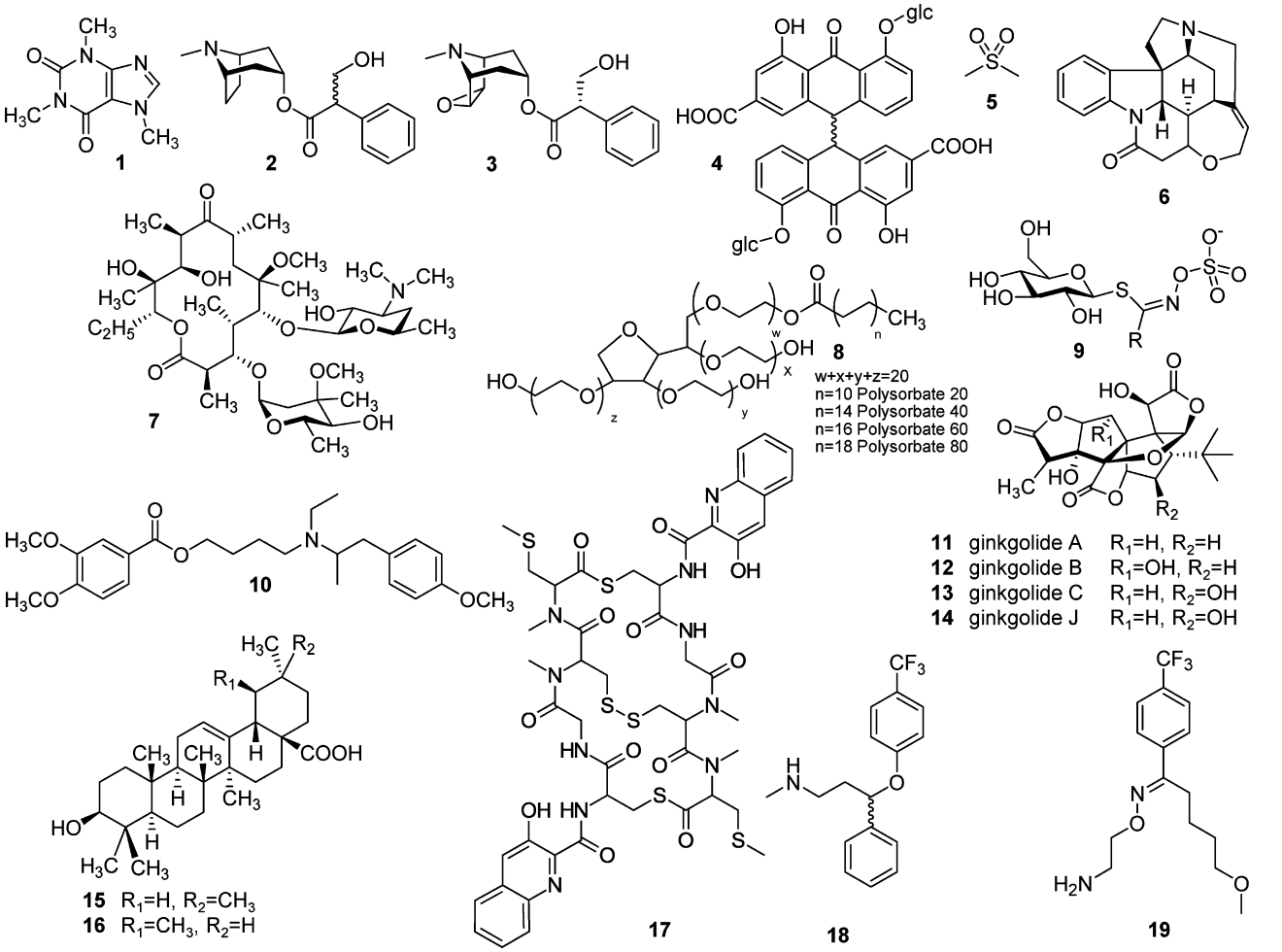
**The 2D *J*-Resolved NMR Experiment.** The 2D *J*-resolved experiment has been used traditionally in a qualitative context to provide a separation of proton chemical shift information from the highly digitally resolved multiplicities of proton spectra for the purposes of extracting *J*-coupling constant information for structural analysis or to assist full-spin analysis of proton NMR spectra. The 2D-spectra of each of the multiplicities appear as slices parallel to the $f_1$ axis (after rotation and symmetrization) and may be plotted and analyzed accordingly. Along the $f_2$ axis, suitable appropriate postacquisition processing of the 2D-NMR data can render a summed projection of the $^1$H data to create a 1D proton NMR spectrum devoid of proton−proton couplings (effectively a 1D "proton-decoupled proton spectrum"). This spectrum will retain the relative quantitative information and can be used therefore for quantitation. While this pulse sequence is conceptually well understood (see ref 78, in particular, Chapter 7), it suffers from the fact that the peaks in the spectrum have a characteristic phase-twist line shape, and therefore the spectra are generally displayed in the absolute value mode. This will affect the accuracy of the quantitative information. Improvements in the basic pulse sequence have been reported,[79−81] which eliminate the phase-twist line shape problem in both dimensions and provide absorption-mode phase-sensitive spectra, thus improving the quality of the projection data while improving both the qualitative and especially the quantitative information by retaining their natural absorption integrated intensities. The advantage of this particular 2D-qHNMR experiment is that each proton in the $f_2$ dimension of the summed projection of the mixture will appear as a singlet. Even though resonance overlap in the projection may still be a problem in some cases, spectral deconvolution techniques may be more robust when applied rigorously to extract quantitative information. A recent review by Ludwig and Viant on the 2D *J*-resolved experiment[82] summarizes the quantitative potential of this experiment to metabolomic analysis. In continuation of the long-standing work on hop constituents by Verpoorte et al., the 2D *J*-resolved experiment has also been used for metabolomic quantitation and differentiation of beer.[83]

**The Homonuclear 2D-COSY Experiment.** The homonuclear 2D-COSY experiment and several of its variations (e.g., TOCSY or 2D-$^1$H-INADEQUATE),[84] all of which are designed to provide qualitative identification of $^1$H,$^1$H spin-coupling networks, have been explored for quantitative 2D-NMR analysis. In heavily overlapped and crowded spectra, the off-diagonal cross-peaks associated with the variety of spin-coupled networks that might be present can provide a way of quantifying the components of very complex mixtures. Since the integrated intensities of the cross-peaks may show variations due to differences in the magnitude of *J*-couplings between the protons giving rise to the cross-peak, the use of calibrations is recommended if accurate quantitative results are to be obtained. Improvements in the pulse sequences directed toward improving quantitative accuracy and speed have been reported. For example, ultrafast 2D-TOCSY and 2D *J*-resolved data have been reported.[85] Both experiments have been shown to result in excellent linearity and reproducibility of the quantitative information. A detailed 1D study describing the qHNMR analysis of the ginkgolides A, B, C, and J (**11**−**14**, respectively), mixtures of ginkgolides, and crude *Ginkgo biloba* extracts has now been completed.[86]

**The Heteronuclear HSQC Experiment.** The standard contemporary qualitative proton-detected $^1$H,$^{13}$C-correlation experiment is the 2D gradient HSQC (2D-HSQC) experiment and its variations. This produces a correlation map of proton chemical shifts vs the chemical shifts of directly bonded carbons via $^1J_{C,H}$. The correlation information is spread out into both the carbon dimension ($f_1$) and the proton dimension ($f_2$). For quantitative purposes, in essence, the $f_2$ dimension, which may be crowded and highly overlapped in a complex mixture, is being separated and resolved by the increased chemical shift dispersion of $f_1$. Cross-peak overlap is in many cases reduced significantly or eliminated, and clean integration of the $^1$H,$^{13}$C cross-peaks is possible. Quantitation of this experiment can be tricky, and independent calibrations may be necessary. This is in part due to (a) the variation/distribution of $^1J_{C,H}$; (b) the efficiency of polarization transfer from protons to carbons and back during the pulse sequence; (c) the nature of the carbon multiplicity (C, CH, $CH_2$, $CH_3$); and (d) differences in observed relaxation times ($T_1$ and $T_2$). All of these parameters contribute to the observed $^1$H,$^{13}$C cross-peak intensity.

Studies concerned with the optimization of quantitative conditions for HSQC have appeared,[87,88] including the development of the Q-HSQC pulse sequence modification to ascertain good quantitative cross-peak areas.[87,89] A comparison, for quantitative purposes, of similar kinds of protons (and carbons) should afford useful quantitation results, but the use of calibrations for the quantitation may still be necessary.

**Chart 1**




The 2D-Q-HSQC pulse sequence has been employed for numerous quantitative applications. For example, 2D-HSQC has been applied to the quantitation of urinary metabolites.[90,91] Both qualitative and quantitative discrimination of oleanolic acid (**15**) and ursolic acid (**16**) in plant extracts has been achieved with the use of 2D-HSQC in combination with 2D-HMBC.[92] The quantitative HSQC results were, in this instance, in excellent agreement with the results of HPLC analysis. Rapid lipid profiling of mycobacteria including *Mycobacterium tuberculosis* has been reported using quantitative 2D-HSQC.[93] A recent study by Markley and co-workers[94] demonstrated that data from a series of HSQC spectra acquired with incremented repetition times can be extrapolated back to zero time to yield a time-zero HSQC spectrum (termed $HSQC_0$). In these spectra, cross-peak intensities are proportional to relative concentrations of the analytes and can afford absolute quantitation by the use of internal calibrants. The same group has also reported an application of the $HSQC_0$ technique to the quantitation of thiocoraline (**17**), present at low levels of 1% (w/w) in an extract from a *Verrucosispora* sp. isolated from the sponge *Chondrilla caribensis* f. *caribensis*.[95]

Quantitative $^{1}H,^{15}N$-HSQC has been applied for the analysis of a labeled protein from *Xenopus laevis* egg extracts.[96] Quantitative $^{1}H,^{13}C$-HSQC has also been used for determining the concentration of metabolites in biological fluids.[97] Quantitative 2D-HSQC has been applied to the analysis of polysaccharide polymers.[98] The latter study outlines a protocol for (i) specifically analyzing this kind of polymer system, (ii) determining the appropriate 2D-HSQC experiment to employ, and (iii) selecting an appropriate internal calibrant to circumvent the $T_2$ problem that is associated with polymers.

**The DOSY Experiment.** The DOSY, or diffusion-ordered spectroscopy, experiment is a 2D experiment in which the individual proton 1D-NMR spectra of each of the components of a complex mixture are separated according to the relative diffusion constant for each component dissolved in the NMR solvent (see ref 78, Chapter 9). This represents a "virtual separation" of the components of the mixture without engaging in a physical separation scheme for the purification of each of the components. Information pertaining to the qualitative identification of each component as well as the quantitative compositional information may be obtained. Studies involving the combined use of 1D-qNMR with 2D-$^{1}$H-DOSY have been reported. For example, qualitative identification and quantitation of fluoxetine (**18**) and fluvoxamine (**19**) in pharmaceutical formulations have been reported using 1D-qFNMR and 2D-$^{1}$H-DOSY.[99] The 2D-DOSY experiment permitted the qualitative characterization of the active ingredients as well as excipients in the formulation. A study involving the application of 1D-qHNMR and 2D-DOSY in the analysis of extracts of *Ligusticum porteri* has also been reported.[100]


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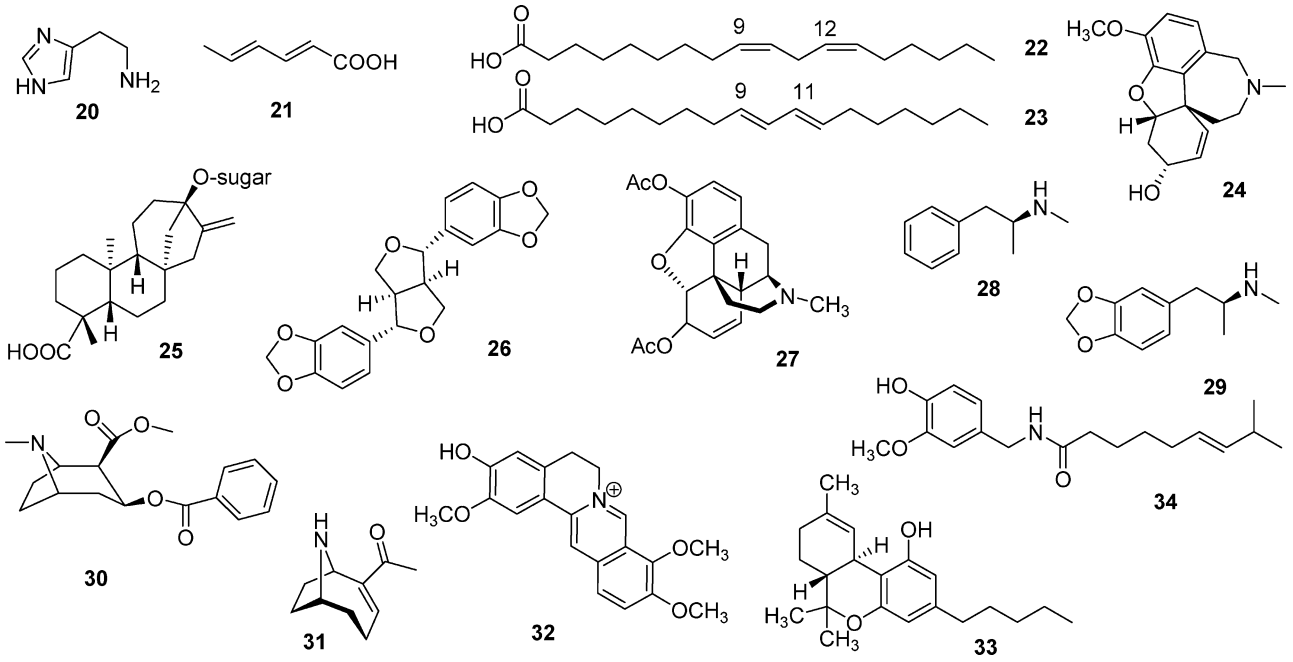
Chart 2




## APPLICATIONS OF QHNMR

The following section provides an overview of new qHNMR applications reported since mid-2004 with particular relevance to NPs. According to these studies, NMR is becoming a capable quantitative tool for metabolic profiling and for the monitoring of metabolic changes from natural sources, especially in the food industry, the analysis of plant constituents, and clinical diagnostics.

**Primary Metabolic Studies of Plants by qHNMR.** Pereira et al. utilized qHNMR in order to establish the metabolic fingerprints of grapes growing on different soil types in Bordeaux, France. NMR analysis of sugars and amino acids in grape skin extracts was more efficient in discriminating grapes from different locations than classical biochemical analyses based on sugar, acidity, and nitrogen measurements.[101] A similar study on Vermentino grapes was carried out by Mulas et al., who investigated the variability in metabolite concentration as a function of the clone and the position of grapes on the bunch or growing area within the vineyard.[102] The fermentation process of Rioja red wine was monitored with qHNMR by Lopez-Rituerto et al.[103] Avenoza et al. used qHNMR to analyze the transformation of the must in wine grapes, specifically the malic and lactic acid levels during the alcoholic and malolactic fermentation process.[104] Schievano et al.[105,106] determined the content of histamine (**20**) in different types of cheeses by qHNMR of acid extracts and were also able to discriminate Asiago D'Allevo cheese samples on the basis of their content of unsaturated fatty acids. As part of the quality control of cod liver oil, qHNMR was employed to determine the content of unsaturated fats, which differ in their triglyceride content and composition.[107]

A global approach to the characterization of changes in metabolic profiles in independent tissues from the same fruits was developed by Mounet et al. for tomato flesh and seeds during fruit development.[108] Quantitation of compounds in both tissues at different time points post-anthesis was obtained through qHNMR, along with liquid chromatography with diode-array detection or gas chromatography with flame ionization detection. The data were analyzed chemometrically, and the compositional changes related to physiological processes occurring in each tissue. Deborde et al. applied qHNMR for the quality assessment of greenhouse-grown tomato fruits through quantitation of 32 major metabolites.[109] This study demonstrated that qHNMR can complement colorimetric analysis and provide a diagnostic tool to assess the changes in organoleptic and nutritional quality of tomatoes.

The changing content of sugars and organic acids on 11 strawberry traits was monitored by Lerceteau-Kohler et al. for up to three years, and qHNMR was found to be a powerful tool to assist efforts to improve fruit quality. The progeny showed a large range variation for most of the traits, with strong positive correlations between the content of fructose and glucose as well as citrate and the sum of organic acids.[110] Del Campo et al. applied qHNMR to the assessment of malic and lactic acid in apple, apricot, kiwi fruit, orange, pear, pineapple, and strawberry juices. The results obtained when applying NMR procedures were compared to those obtained using enzymatic methods, and both were in close agreement.[111] Formic acid content in apple juices of five different cider varieties was assessed using qHNMR by Berregi et al.[112]

Using qHNMR, Wagner et al. determined the ethanol content in several brands of gin and vodka and found significant differences between the analytical results and declared alcohol content.[113] Shripsema has reported the comprehensive qHNMR-based analysis of polar constituents such as the preservatives benzoic acid and sorbic acid (**21**), the organic acids citric acid, butyric acid, and formic acid, and the carbohydrate lactose as well as nonpolar constituents of butter and margarine, such as linoleic acid (**22**) and rumenic acid (**23**), as well as diglycerides.[114]

A new approach to metabolomic qHNMR of biofluids termed "targeted profiling" established by Weljie et al. utilizes mathematically modeled spectra of pure primary metabolites and principal component analysis pattern recognition.[115] Validation against the commonly used approach of spectral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binning determined the method to be stable, scalable, applicable to physiologically relevant low levels (>9 $\mu$M) of the metabolites, and tolerant to solvent suppression schemes including the commonly used WET-CPMG and NOESY presaturation experiments. In a similar approach, Moin et al. used pure reference materials to quantitate 14−17 metabolites in 25 min or less acquisition time, down to low-microgram levels of the compounds.[116]

**Secondary Metabolic Studies of Plants by qHNMR.** The metabolic profiling of the glutamine concentration of ginseng hairy root cell lines with qHNMR by Jung et al.[117] revealed that the inhibition of root growth and lateral root formation depends on glutamine accumulation. Lubbe et al. applied qHNMR for the quantitation of galanthamine (**24**), a benzazepine alkaloid used in Alzheimer's therapy, in the bulbs of some Amaryllidaceaeous plants.[118] Differences in the overall metabolic profiles of bulbs in the two most important cultivation regions were assessed. Very recently, the use of qHNMR spectroscopy for the characterization of *Stevia rebaudiana* extracts was presented by Pieri, Stuppner, and associates.[119] The method allows quantitation of the major steviol glycosides (**25**) in purified extracts and fractions obtained at various stages of the purification process. Moreover, qHNMR proved to be a powerful method to differentiate between *Stevia* glycosides occurring naturally in the plant of origin and artifacts formed in the course of the manufacturing process.[119] Using qHNMR, Tardieu et al. identified and quantified taste compounds in raw onions (*Allium cepa*) and compared mono- and disaccharide release in aqueous solution by raw or fried cubes of onion bulbs.[120,121] Craigie et al. found that qHNMR analysis calibrated with a certified reference material such as **1** is an excellent tool to profile commercial seaweed extracts from *Ascophyllum nodosum* and other brown seaweeds.[122] In their study, the authors also identified seasonal variations in order to standardize the commercially available product.

Employing qHNMR as a key component of a functional genomics platform, *Papaver somniferum* was investigated for the interplay between primary and secondary metabolism with respect to alkaloid biosynthesis in cultured poppy cells treated with a fungal elicitor.[123,124]

**Ex Vivo Metabolic Studies by qHNMR.** Metabolic qHNMR has been applied successfully as a diagnostic tool to analyze metabolomic data in clinical settings. The increase in resolution and sensitivity of NMR instruments has opened up new opportunities for the use of NMR spectroscopy in the quantitative analysis of the most important biological fluids, including urine, blood plasma, cerebrospinal fluid, saliva, and bile.[125] Kline et al. monitored citrate concentrations in human seminal and expressed prostatic fluid from prostatic cancer vs non-cancer-bearing samples. The mean citrate concentration of the cancer patients proved to be reduced by 2.7-fold.[126] Serum citrate content, along with lysine, glycine, suberate, and acetate concentrations, was also assessed by qHNMR in a metabolic profiling effort on serum of high-fed C57BL/6J mice in order to better comprehend the biochemical signature of insulin resistance.[127]

A study carried out by Mochel et al. applied qHNMR analysis on human plasma and developed a method to distinguish Huntington's disease patients at different stages of the condition and presymptomatic carriers from controls. A distinction was attributable to low levels of the branched chain amino acids valine, leucine, and isoleucine.[128] Another study used qHNMR to quantify and identify metabolites present in cultured 3T6 mouse fibroblast cells in their native state and after treatment with an inhibitor of the fibroblast growth factor receptor tyrosine kinase.[129] A recent investigation that utilized qHNMR as the analytical tool showed that blood and red blood cells mediated the transport of hepatotoxic plant pyrrolizidine alkaloids and their reactive metabolites in humans.[130]

Clinical studies of high potential significance were carried out by Ala-Korpela and colleagues on modeled lipoprotein subclasses to assess the eligibility and accuracy of NMR to analyze human plasma in general.[131,132] The subclass models were used to simulate biochemically representative sets of spectra with known subclass concentrations. The spectroscopic analyses revealed 10-fold differences in the quantitation accuracy of different subclasses by $^1$H NMR and questioned the usefulness of qHNMR in serum diagnosis. On the contrary, a study by Oostendorp et al. investigated the potential use of qHNMR to simultaneously identify and quantify the unusual lipids present in the blood of patients with different inborn errors of lipid metabolism.[133] The authors not only found a good correlation between conventional methods and qHNMR analyses for cholesterol and triglyceride concentrations but could also correctly diagnose four inborn errors of lipid metabolism. The study concluded that qHNMR can be applied successfully in clinical diagnoses.

In reproductive medicine, qHNMR is suggested as an option to quickly and noninvasively identify the best embryos in assisted reproduction cycles for transfer.[134] This assessment takes into account a report by Bromer et al., who applied qHNMR for the measurement of the glutamate levels in an embryo culture medium and related these data to the reproductive potential of embryos in women undergoing in vitro fertilization.[135] Gupta et al. proposed qHNMR-based metabolic screening of human seminal plasma as a rapid and noninvasive approach for probing infertility, with sensitivity and specificity similar to the more elaborate traditional methods.[136]

Aimed at monitoring the urinary metabolites of patients with inflammatory bowel disease, Crohn's disease, and ulcerative colitis, a qHNMR method was established to distinguish between these diseases based on the differences of gastrointestinal flora, which in turn influences urinary metabolites.[137] The major metabolite of the cholesterol-lowering sesame oil lignan sesamin (**26**) was quantified by qHNMR in the urine of six volunteers after the intake of sesame oil.[60]

**qHNMR for the Analysis of Drug Metabolism, Toxicology, and Forensic Samples.** Recent applications have applied qHNMR frequently as an analytical tool in drug metabolism and toxicology studies. Several reports have described recent guidance on Safety of Drug Metabolites issued by the U.S. Food and Drug Administration, Center for Drug Evaluation and Research (CDER). The regulatory guidance stresses the importance in drug discovery and development of identifying, characterizing, and quantifying drug metabolites as early as possible and suggests qHNMR combined with chromatography, mass spectrometry, and plasma pooling methods to obtain reliable quantitations of metabolites present in plasma of preclinical species from short-term safety studies.[67,138,139] A study by Hays has reported on the use of qHNMR for purity determination of drug standards and the routine analysis of illicit drugs such as heroin (**27**), methamphetamine (**28**), 4-methylenedioxymethamphetamine (MDMA, **29**), and cocaine (**30**) as well as adulterants.[140] Lesar et al. have shown that qHNMR can meet the challenge of

simultaneously identifying and quantitating γ-hydroxybutyric acid and γ-butyrolactone in spiked alcoholic beverages.[141] Using a capillary internal calibration standard, qHNMR proved to be fast, nondestructive, and sensitive and required no sample preparation that would disrupt the equilibrium between γ-hydroxybutyric and γ-butyrolactone.

By utilizing a qHNMR method to quantify toxins such as anatoxin-A (**31**), Dagnino et al. overcame the very limited availability of toxin reference materials for calibration, while still being able to work in very dilute solutions.[142] Aimed at the simultaneous identification and quantitation of the psychedelic indole *N,N*-dimethyltryptamine in a *Psychotria viridis* preparation, Moura et al. developed a qHNMR method with 2,5-dimethoxybenzaldehyde as internal calibrant that was fast (<30 s), required minimal sample preparation, and exhibited an LLOQ of 12.5 μg/mL and a better than 5% precision, which was interpreted as a limitation of the solvent extraction protocol rather than that of the qHNMR method.[143]

**qHNMR in the Quality Control of Complex Secondary Metabolite Mixtures from Plants.** Quality control of pharmaceutical products is essential for consumer safety and efficacy. Quantitative $^1$H NMR fingerprints have been frequently employed in the analysis of botanical products, including dietary supplements. A review article of Yap et al. compares major analytical techniques including qHNMR in the quantitative analysis of ginseng preparations.[144] A method to detect medicinal yeast and keratin content in pharmaceutical preparations on the basis of qHNMR was developed by Jankevics et al.[145] The quality of *Aloe vera* products was assessed with qHNMR by quantitation of the acetylated polysaccharides, glucose, malic acid, lactic acid, and acetic acid present.[46]

A qHNMR method to quantify the biological active protoberberine alkaloids [e.g., jatrorrhizine (**32**)] of "Huang-bai", the cortex of the dried bark of *Phellodendron chinense* or *P. amurense*, important traditional Chinese medicine products, as well as for Rhizoma Coptidis (*Coptis chinensis*) was developed by Li et al., in order to ensure product quality and authenticity.[54,146]

The composition of the spasmolytic essential oil of the medicinal plant *Brickellia veronicaefolia* was established by NMR spectroscopy together with GC-MS and HPLC studies, and qHNMR was used for quantitation of the major compounds.[147] Components of the essential oil of *Lindera neesiana* fruits were quantified by Comai et al.[148] using qHNMR, with **1** as an internal calibration standard.

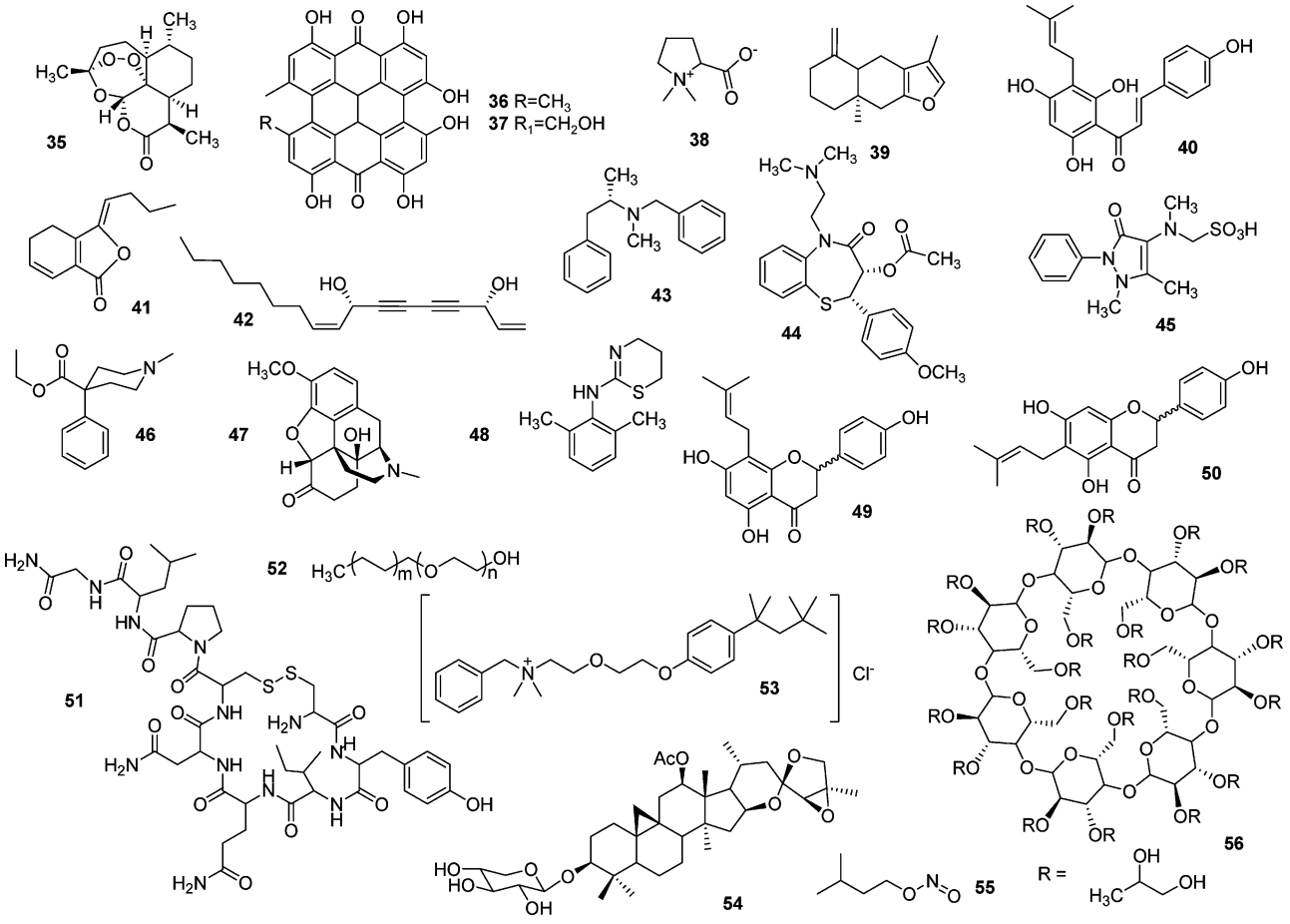
Staneva et al. have used qHNMR for the quantitative analysis of sesquiterpene lactones present in a crude lactone fraction of *Arnica montana*.[149] *Cannabis sativa* water extracts and tinctures were directly analyzed with a combination of diffusion-edited $^1$H NMR (DOSY) and qHNMR spectroscopy in order to quantify $\Delta^9$-THC (**33**), its acid, and other metabolites.[150] Nazari et al. quantified capsaicin (**34**) from a microwave-assisted acetone extract of *Capsicum frutescens*.[151] The antimalarial compound artemisinin (**35**) was quantified in *Artemisia annua* acetone extracts by qHNMR.[50] Using qHNMR approaches, the artemisinin content was also determined in extracts of *Artemisia annua* from Argentina[152] and eight different *Artemisia* samples collected from around the world.[153] A study by Tatsis et al. used the strongly deshielded hydroxy groups of hypericin (**36**) and pseudohypericin (**37**) in the region of 14−15 ppm to identify and quantify both compounds in *Hypericum perforatum* extracts by qHNMR, prior to HPLC separation.[154] The photosynthetic chlorophyll and carotenoid pigments of *Phaseolus vulgaris* have also been evaluated quantitatively by qHNMR.[155] Ritter et al. used qHNMR to quantify the stachydrine (**38**) content in *Leonorus cardiaca* refined and crude extracts.[156] Hasada et al. successfully applied qHNMR to the quantitative detection of atractylon (**39**) in extracts of rhizomes of *Atractylodes ovata*, *A. japonica*, *A. lancea*, and *A. chinensis* without prior purification.[66] Finally, Parys et al. proved that qHNMR along with the use of Folin-Ciocalteu's phenol reagent is among the most reliable and precise methods for the quantitation of polyphenols accumulated in marine brown algae, such as *Ascophyllum nodosum* and *Fucus vesiculosus*.[157]

Two recent reports have explored impurities and degradation of phytochemicals and plant fractions from botanical products by qHNMR (see also the Dynamic Residual Complexity (Dynamic RC) section). Thus, time-resolved studies of the dynamic changes of desmethylxanthohumol (**40**)[158] and *Z*-ligustilide (**41**)[159] were conducted and gave rise to biologically active degradation products. These two reports, as well as the studies mentioned below, demonstrate the potential of qHNMR as a validation tool for the characterization of bioactive NPs.[3] A review on forced degradation studies for pharmaceutical drug candidates and the identification and quantitation of degradation products with qHNMR was published by Alsante et al.[160] A report by Mohn et al. described the purity evaluation of glucosinolates (e.g., **9**) isolated from *Isatis tinctoria* with qHNMR.[68] A publication on *Angelica sinensis* used a combination of 1D- and 2D-NMR methods to determine the absolute amounts of **41** and six of its derivatives, in addition to establishing relative quantitative relationships within a series of phenylpropanoids, falcarindiol (**42**) polyacetylene derivatives, and unsaturated fatty acids present in a bioactive fraction of hydroalcoholic *Angelica* preparation.[161] Another publication described a new concept of qHNMR analysis aimed at the determination of multiple components in herbal extracts. This approach combines the use of comprehensive $^1$H NMR profiles (fingerprints) of marker compounds generated by $^1$H iterative full spin analysis (using the PERCH software tool) with 1D-QHNMR quantitation using extracts of *Ginkgo biloba* leaves as a model.[86]

**qHNMR of Complex Natural Mixtures from Marine and Microorganisms.** To date, relatively few applications of qHNMR for NPs from marine and microorganisms have been reported. Carballo et al. have cultured explants from the sponge *Mycale cecilia* to produce pyrrole-2-carbaldehyde derivatives. These growth inhibitors of the LNCaP human prostate cancer cell line are known to be minor metabolites of the wild forms of the marine sponge. The pyrrole-2-carbaldehyde derivative content of the cultured sponge was detected by qHNMR, and it was demonstrated that marine aquaculture of *M. cecilia* is a viable method for supplying the amount of metabolite needed for advanced bioactivity studies.

Xu et al. described the quantitative analysis by qHNMR of the *Haemophilus influenza* type b polysaccharide, an intermediate of the *H. influenza* (Hib) vaccine PedvaxHIB.[162] The aforementioned report on macrolide antibiotics (see the Calibration Methods in qHNMR section) exemplifies how the purities of five common reference materials of macrolide antibiotics could be measured by qHNMR.[63] The relatively high molecular weight and resulting complexity of the $^1$H NMR spectrum do not preclude qHNMR analysis at 500 MHz, which

Chart 3




demonstrated the applicability of qHNMR concepts for the analysis of NPs with complex spectra.

## QHNMR FOR THE ASSESSMENT OF RESIDUAL COMPLEXITY AND PURITY

**Residual Complexity of NP Reference Materials.** Even after an elaborate analytical separation scheme has been performed, every NP sample, including reference materials, inherits a certain portion of the metabolomic complexity of its natural source. This relationship is perpetuated by the *residual complexity* (RC) of the samples, which in principle affects all isolated ("pure") NPs. RCs can be divided into two groups: static and dynamic. Static RC is found when the kind and level of impurities present in a sample remain constant over time, while dynamic complexity describes the cases where impurity levels change over time and/or the composition of impurities change. For example, this can be due to formation of a chemical equilibrium depending on the solvent or storage conditions in general.[160] In summary, static RC is relatively easy to address and may be reduced by additional purification (if practical). This often is due to chemicals that stem from the isolation procedure such as residual solvents, stationary phases, and cocrystallizing compounds. In contrast, dynamic RC depends on the chemical reactivity of isolates and their storage conditions, following the concept that chemical entities in extracts often stabilize each other, where purification and isolation breaks stable chemical equilibria or stabilizing compounds are separated from chemically labile species that then become prone to chemical modifications ("degradation").

**Static Residual Complexity (Static RC).** Solvent residues are common impurities in NP isolates and are usually not detected in liquid chromatography (LC) analysis. Therefore, qHNMR is a valuable tool to measure their concentration in purified compounds as a prerequisite for obtaining relevant bioassay results. A comprehensive study by Jones et al. led to the documentation of the residual $^{1}H$ and $^{13}C$ chemical shifts of 60 solvents in DMSO-$d_6$, $CDCl_3$, $D_2O$, and $CD_3OD$, which contributed substantially to their identification as impurities in NMR samples.[163] Spectra were recorded at 300 K and referenced to TMS for the organic solvents or trimethylsilylpropionic acid for $D_2O$. The $^{13}C$ NMR spectra were referenced to the solvents (DMSO-$d_6$ 39.5 ppm, $CDCl_3$ 77.0 ppm, $CD_3OD$ 49.0 ppm relative to TMS, respectively) or using the spectrometer default referencing to $D_2O$.

The relevance of an accurate assessment of compound purity for biological evaluation has been recognized previously in the qHNMR context.[1,164] Recently, Jaki et al. have correlated the antituberculosis (TB) activity of various samples of ursolic acid (**16**) with the sample purity as determined by qHNMR spectroscopy.[165] The unexpected finding was that the purity and the anti-TB activity of **16** were inversely correlated, suggesting that the (degree of) impurities contributed to the observed antibiotic activity, either fully or through a synergistic mechanism. The study concluded that the generation of purity activity relationships (PARs) is a powerful extension of the routinely performed quantitative correlation of structure and activity ([Q]SAR). The potential of PARs as a tool in drug discovery and synergy research accentuates the need to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routinely combine biological testing with purity assessment in order to correlate biological activity of NP isolates with their inherent RCs.[165]

Another example for residual complexity of natural product-derived samples was published by Hays,[140] who quantified heroin and its common residual opium alkaloids as well as typical adulterants of 196 samples of illicit materials. The study pointed out that the water-soluble hydrochloride (HCl) forms of all morphine/codeine-type compounds as well as benzphetamine (**43**), diltiazem (**44**), dipyrone (**45**), meperidine (**46**), oxycodone (**47**), and xylazine (**48**) form two different ion pairs with maleic acid, which was used as internal calibrant. For some analytes, this led to minor signals next to major resonances. In contrast, the qHNMR spectra of free bases of these alkaloids showed only one species and, thus, are not affected by this variant of RC that can affect the analysis. These observations serve as a good example that the extent of RC depends on the NMR solvent.

A comprehensive publication by Xu et al. has established an approach for the quantification and identification of small-molecule analytes in complex biological samples with overlapped signals.[166] Their work touches upon the topic of macromolecular components present in biological samples, such as proteins in serum or urine, which further increase the RC of the target analyte and can impact negatively the accuracy of the concentration measurements of small molecules. The approach utilizes a quantification method based on linear least-squares fitting using singular value decomposition (SVD) and a database of reference spectra to identify analytes on the basis of fitted coefficients or concentration values, and at the same time it quantifies them by linear deconvolution. The results obtained indicate that the SVD calculation is efficient, accurate, and reproducible over a wide concentration range; however, peak alignment is important for the success of SVD deconvolution. An increase in line broadening value may help accommodate slight peak misalignment. This method provides a direct and practical approach that might be applicable routinely to complement various existing chemometrics approaches. With regard to RC stemming from the presence of macromolecules, the publication demonstrated that the approach is applicable to the quantitation and identification of small-molecule analytes in complex biological samples utilizing a mixture of small molecule analytes in the presence of macromolecules (e.g., proteins).[166] Another example of the challenges resulting from peak overlap in qNMR applications is the analysis of glyceride oils, which was the subject of a heteronuclear NMR study by Hatzakis et al.[167]

**Dynamic Residual Complexity (Dynamic RC).** A study by Chen et al. has shown the beneficial use of qHNMR for the exploration of the dynamic chemical equilibrium of the potent phytoestrogen 8-prenylnaringenin (**49**), found in hops.[158] The study was initiated because of unexplained variability of estrogenic bioactivity in samples of desmethylxanthohumol (**40**), the chemical precursor of **49**, and the biologically much less active regioisomer 6-prenylnaringenin (**50**). The ratios of the two isomers (**49**, **50**) were measured quantitatively by NMR spectroscopy, and it was shown that they are formed as a function of time through a Michael addition isomerization reaction from **40**. The study also exemplifies that despite its high purity (98.5% by qHNMR), the **40** study material can be predicted to exhibit estrogenicity both in vitro and in vivo as a result of dynamic residual complexity. This underscores the importance of an awareness of potential dynamic complexity in pure NPs, especially those with reactive sites.[158]

As a continuation of a previous study involving qHNMR spectroscopy in process engineering of multicomponent mixtures,[168] Maiwald et al. reported on the use of this method to study reaction equilibria and kinetics in technical mixtures of formaldehyde, water, trioxane, and sulfuric acid, using pressurized sample tubes and an online technique. Their studies showed that reliable quantitative results may be achieved with both procedures, even under difficult experimental conditions such as high temperature and high sulfuric acid concentrations. As most internal calibrant decompose in hot sulfuric acid, a new procedure was applied for the quantitation of the small trioxane signals, in which electronically generated NMR signals were used as highly stable virtual references (VRs). The VR method was shown to be a valuable tool that can be used to circumvent problems with classical internal or external calibration standards.[169]

Especially in complex samples, observing dynamic changes of RC requires a combination of a multicomponent detection procedure such as NMR with a statistical evaluation method, in order to make multiple but small changes visible and measurable.[170] A method developed by Ohno et al. combines $^1$H NMR and principal component analysis to obtain the quality evaluation of biopharmaceuticals, with regard to their quality, consistency, and differences in protein modification patterns.[171] The feasibility of the method was assessed by collecting three $^1$H NMR spectra of oxytocin (**51**) at days 0, 7, and 14. Although the three spectra of **51** seemed similar by simple visual inspection, time-dependent differences among the three spectra were clearly distinguished by a PCA score plot. Peak changes indicating both the decomposition of **51** and the emergence of new decomposition products within the 14-day time frame were also observed by a PCA loading plot. The results demonstrate that PCA in combination with $^1$H NMR is a powerful tool for the evaluation of dynamic RC, especially when differences between spectra cannot be determined visually.[171]

**Purity Assessment and Reference Materials.** Applications of qHNMR for the establishment of commercial and compendial reference materials have started to emerge. Recently, Tan et al. presented a qHNMR method to assess the average polymer length for polyoxyl ether (**52**) reference materials.[172] The superior metrological quality of qHNMR as a relative primary analytical method (see refs 1, 24, 173, 174 and citations therein) has altered the QC of reference materials in pharmaceutical good manufacturing practices (GMP).[175,176] This is also in line with the recent development of qNMR-validated phytochemical standards for botanical analysis (www.qreference.com). A quantitative NMR method for the determination of benzethonium chloride (**53**) in grapefruit seed extracts was validated by Bekiroglu et al.[43] Their method validation addresses specificity, linearity, range, and precision, as well as accuracy, limit of quantitation, and robustness of the qHNMR assay. The structurally complex group of >150 known *Actaea* cycloartane triterpenes such as the major binoxoside of *Actaea racemosa* (syn. *Cimicifuga racemosa*, black cohosh), (12*R*)-12-acetoxy-(24*R*,25*R*)-24,25-epoxy-3-*O*-$\beta$-D-xylopyranosylacta-(16*S*,23*R*-16,23;23,26)-binoxoside (**54**, syn. 23-*epi*-26-deoxyactein),[177] has been associated with numerous biological activities of this popular botanical dietary supplement. In a very recent study, Qiu et al. used NMR spectroscopy for the simultaneous structural dereplication and quantitative analysis

of purified *Actaea* triterpenes and their development into reference materials.[177] The approach utilizes the predictive computational model of classification binary trees for the in silico determination of the aglycone and qHNMR for the quantitation of 10−20 μg amounts of triterpenes in residually complex samples, using 700 MHz 1.7 mm cryo-microprobe instrumentation.

Reference materials for drug metabolism studies represent another area of qHNMR applications. As metabolite standards are very precious samples, the ability of qHNMR to validate such materials is invaluable for the quantitative studies, as has been demonstrated by Walker et al.,[34] who also implemented artificial signals for the concentration measurements (see also above). Reduced cost, efficiency, and suitability for the assessment of early GMP materials of active pharmaceutical ingredients (APIs) for toxicological studies have made qNMR a single-point replacement for early pharmaceutical development work. Webster et al. also noted[178] that, although traditional validation requirements applied in regulated industries to liquid chromatography methods challenge qNMR method validation, qNMR is fully equivalent to LC for early phase potency determinations of APIs, metabolites, and related substances.

Another example of the beneficial application of qNMR is in the quality control of *Angelica sinensis* extracts and fractions. Its main bioactive constituent is *Z*-ligustilide (**41**), which represents an example for dynamic RC and, thus, a challenge as far as the provision of reference materials is concerned. In an earlier publication, qNMR in combination with GC-MS was employed successfully to describe the degradation of **41** qualitatively and quantitatively,[159] including the influence of the solvent on the degradation time course. As **41** appears to be stabilized while being part of a (crude) *A. sinensis* extract, a recent qNMR study explored methods to measure its amount in the bioactive fraction directly, without the need for the identical calibrant.[161] A combination of absolute (external calibration) and relative (modified 100% method) qNMR led to the establishment of quality control parameters with respect to the content of **41** as well as the content of nine minor constituents present in commercial hydroalcoholic extracts.

The *United States Pharmacopeia* (USP34/NF29)[179] lists NMR and quantitative $^1$H NMR as analytical methods for the assessment of pharmaceutical and dietary supplement standards. Two quantitative methodologies are distinguished: (i) the absolute quantitation method, which relies on the use of "internal standards" (equivalent to *calibration standards* used herein), and (ii) the relative quantitation method, which compares integral ratios of signal groups within one molecule. The two monographs on amyl nitrite (**55**) exemplify absolute quantitation methods and use benzyl benzoate as calibrant. Several monographs use the relative quantitation method to determine the content of hydroxypropyl groups in the molecules of hydroxypropyl starches (pea, corn, and potato), to determine the average polymer length of polyoxyl ethers (**52**, polyoxyl 10 oleyl ether, polyoxyl 20 cetostearyl ether), or to determine the molar substitution of hydroxypropyl betadex (**56**), based on integral ratios of protons of the substituents vs protons of the molecular backbone. Finally, the monograph on heparin sodium employs qHNMR as identity test and also includes a semiquantitative purity assay. The *United States Pharmacopeia* is considering an extension of the use of qNMR to future monographs for dietary supplements (Giancaspro, G. I.; Sharaf, M. H. M., private communication), in particular for materials where qNMR protocols have shown advantages such as those containing polysaccharides (e.g., heparin, chitosan, β-glucans, *Aloe vera* [see above]).

## ■ CONCLUDING REMARKS

From the perspective of NPs research, qHNMR has gained substantial popularity as an analytical tool. This is reflected by the predominance of new literature on qHNMR applications. The increasingly abundant use of qNMR as an alternative to LC-based quantitation is also reflected by the citation statistics of the qNMR literature since 2000: the eight most cited publications (SciFinder, >50 citations) relevant to the review topic[1,2,14,15,24,87,116] received 607 citations, and five of these eight including the top three most cited publications (>100 citations) have appeared only since the last review in 2005.[1]

One major driving force is the gain in sensitivity of cryoprobe NMR technology, including the most recent development of microcryoprobes (1.7 mm i.d. and smaller). Their improved mass sensitivity allow for practically feasible qHNMR experiments with limits of detection and quantitation (LODs and LOQs, resp.) from the nanogram to low microgram range and, thus, enable low-level quantitation by qHNMR. While signal dispersion and sample complexity are, and likely will remain, limiting factors for qHNMR analysis, some of the work addressed in this review shows clearly the much enhanced capabilities of 1D-qHNMR for the quantitation of minor metabolites, even in complex mixtures. At the same time, considering the inherent limitation of the engineering of superconducting NMR magnets, dramatic improvements of signal dispersion are unlikely to occur in the near future. While this review focused on qNMR in the $^1$H domain, it should be pointed out that interesting non-$^1$H qNMR methods with relevance to NPs have been published (S4, Supporting Information).

From the perspective of experimental methodology for NP analysis, the authors consider qNMR to still be in a relatively early stage of development. Whereas in qualitative/structural NMR the intrinsic limitation of $^1$H signal dispersion is overcome by 2D/*n*D, selective pulse, and/or heteronuclear experiments, corresponding qHNMR methods are only beginning to emerge. The design of many 2D experiments also provides challenges as far as the establishment of quantitative conditions is concerned. Thus, while their experimental parameters are well understood and documented for 1D-qHNMR,[1,24] 2D approaches require additional and more elaborate steps to establish quantitative correlations between the signals (integral, volume, intensity) and the molarity of the analytes.

From the viewpoint of the NPs researcher, one striking advantage of qHNMR is the ability of this method to function accurately with external calibration, e.g., against a primary standard, only requiring *nonidentical calibrants*. In addition, the use of $^1$H NMR fingerprints obtained through iterative full spin analysis in combination with multisignal quantitation[86] provides a means of optimizing specificity, primarily in 1D-qHNMR. Another remarkable quality of qNMR is that external calibration is straightforward following the principle of reciprocity,[28,31] even in multiuser settings. Moreover, even noncalibrated $^1$H NMR spectra, which can be run routinely under quantitative conditions, can yield meaningful results using the 100% method[2] or the modified 100% method[161] of evaluation. While the lack of knowledge of molecular weights of certain (unknown) components of the sample introduces

potential errors, the advantages of $^1$H NMR spectroscopy as a universal detector often outweigh this limitation.

The insights from numerous qHNMR reports involving method validation, including metrological studies, indicate that qHNMR is generally a highly precise and accurate method, which is to be expected from any predominantly primary analytical method. Interesting unresolved questions in this context are the (potential) errors of the non-qNMR methods used to validate qHNMR studies. They include, but are not limited to, considerations on extraction procedures for crude NPs (accuracy, reproducibility), volumetric operations during NMR sample preparation (precision and accuracy in the $\mu$L range), weighing of NPs (noncrystalline materials, precision, calibration in the sub-mg range), and operations of the NMR postacquisition work-flow (operator influence, hardware stability, repeatability). An ongoing multilaboratory study involving the authors of this review is seeking to address some of these parameters. However, independently from these considerations of potential sources of error, one value of qHNMR can be seen in its ability to quantitate multiple analytes simultaneously, with the same calibration. This offers a unique potential for NPs analysis for two reasons: samples in this field are often (residually) complex, and there is growing evidence that bioactivity results from the (inter)action of multiple chemical entities, which in some cases may even act in synergy. To this end, qHNMR is available as an unbiased analytical tool, which, at the time of writing of this review, is backed up by a wealth of experimental evidence and useful methodology.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional details pertaining to the statistical analysis, potential qNMR reference materials, 2D-qHNMR pulse sequences, and information about non-$^1$H qNMR applications with particular relevance to NP research are available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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