Reviews

Quantitative ^{1}H NMR: Development and Potential of a Method for Natural Products Analysis[§]

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Based on a brief revision of what constitutes state-of-the-art "quantitative experimental conditions" for ^{1}H quantitative NMR (qHNMR), this comprehensive review contains almost 200 references and covers the literature since 1982 with emphasis on natural products. It provides an overview of the background and applications of qHNMR in natural products research, new methods such as decoupling and hyphenation, and analytical potential and limitations, and compiles information on reference materials used for and studied by qHNMR. The dual status of natural products, being single chemical entities and valuable biologically active agents that need to be purified from complex matrixes, results in an increased analytical demand when testing their deviation from the singleton composition ideal. The outcome and versatility of reported applications lead to the conclusion that qHNMR is currently the principal analytical method to meet this demand. Considering both 1D and 2D ^{1}H NMR experiments, qHNMR has proved to be highly suitable for the simultaneous selective recognition and quantitative determination of metabolites in complex biological matrixes. This is manifested by the prior publication of over 80 reports on applications involving the quantitation of single natural products in plant extracts, dietary materials, and materials representing different metabolic stages of (micro)organisms. In summary, qHNMR has great potential as an analytical tool in both the discovery of new bioactive natural products and the field of metabolome analysis.

Introduction

A number of recently published excellent reviews have documented the importance of natural products in drug discovery. The reviews have shed light on the subject from various perspectives such as structural and biological diversity,[1−3] lead compounds for novel drugs,[4−6] screening strategies,[7−9] metabolic engineering[10] and (combinatorial) synthesis,[11,12] diversity of natural sources,[3,13] and also NMR.[14] Viewed from the biological and pharmacological perspectives, natural products offer two fascinating fields of research inquiry. In the first, they are well-defined single *chemical* entities (SCEs), most of which are "small" and "organic" molecules with a carbon-based skeleton. In the second arena, they are *biologically* active (re)agents, which are capable of acting as endogenous or exogenous ligands or modifiers, and are investigated as pharmacological and toxicological agents or as scaffolds of potential new drugs.

The necessity for isolation from complex matrixes is a key difference between an SCE obtained by any means of synthesis, including synthetic and semisynthetic natural products, and materials from natural sources. While the synthetic process is much under human control, their purely natural counterparts are created by nature in a combinatorial assembly line and, therefore, typically need to be separated from a very complex combinatorial cocktail. Nevertheless, whenever materials—natural or synthetic—cross the chemistry−biology interface, chemical structure is *the* predominant piece of information communicated between both worlds, typically by labeling with the name of the (major) compound. This places a 2-fold demand on any related interdisciplinary research: (a) The *chemical structure* has to be definitive, or at least as definitive as technology allows; and, most important from the perspective of this review, (b) the *singleton character* of the SCE has to be demonstrated and/or validated. A key challenge resulting from the requirement to isolate natural products from complex matrixes is to generate evidence for how far any given natural product sample deviates from the ideal *singleton character* of a SCE.

Because of the complex origin, this represents a significant challenge, which is rarely addressed in the current scientific literature. One intention of this review, besides providing a thorough literature review of the subject, is to demonstrate that nuclear magnetic resonance (NMR) in general is capable of making substantial contributions with regard to both demands. Accordingly, one main specific objective is to show that quantitative NMR (qNMR) bears enormous potential by providing simultaneous access to both the qualitative (*chemical structure*) and the quantitative information (*singleton character* of the SCE, purity). In fact, qNMR reveals its extraordinary potential particularly well when applied to natural products.

Background

Before going into the details of the qNMR experiment, and in order to develop a full appreciation of how the experiment is conducted and what its potentials are, it is

[§] Dedicated to Dr. Nikolaus H. Fischer on his retirement, with best wishes for the achievement of another level of academic freedom.

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appropriate to provide a working definition of qNMR and touch upon the following fundamental concepts first: the physicochemistry, the characteristics of the underlying qualitative ^{1}H NMR experiment, and the historical and literature background.

Working Definition of qNMR. Generally, NMR becomes quantitative NMR (qNMR) whenever it is applied as a quantitative analytical tool. In principle, qNMR is amenable to all NMR-sensitive nuclei and unrestricted in dimensions. Different acronyms have been used historically for "quantitative NMR", such as "qNMR", "QHNMR", "quantNMR", and constructs including the observed nucleus such as "qHNMR". To stay in line with the nomenclature of other NMR derivative techniques such as gradient selection (e.g., gCOSY), and to avoid confusion with heteronuclear NMR (e.g., accepted CNMR for ^{13}C NMR vs QHNMR), the authors feel most comfortable in using "qNMR" as a general abbreviation and "qHNMR" as the proton-specific abbreviation.

Physicochemical Background of qNMR. An important, if not the most important, aspect of qNMR is that the underlying physicochemical mechanism is completely different from other common quantitative analytical techniques. Therefore, qNMR offers a unique and critical view of the analyte, whether it is of biological, nutritional, pharmacological, or toxicological importance. In particular, qNMR is distinctly different from chromatographic procedures, which for the majority of these materials represents the current method of choice for quantitation.

Following the diagnostic dictum that "separation means knowledge", chromatography is dependent on detection when used as a quantitative tool. Furthermore, the chemical (not integration) dynamic range of a single chromatographical step is rather limited and determined by the choice of mobile and stationary phases. This does not even consider irreversible adsorption, which is commonly known to affect silica gel-based chromatography. From a similar point of view, NMR is intrinsically limited by two factors: the achievable signal dispersion and the dynamic range of the observed nuclei. While the former is a function of magnetic field strength and experimental design, the latter depends on the presence of diverse diagnostic nuclei and can mean both a paucity or an essential lack (e.g., caffeine only has 3 × CH_3 ^{1}H NMR singlet signals with no coupling information; the exchangeable imidazolyl protons are not considered viable for qHNMR) and an overflow of information (e.g., triterpene aglycones exhibit highly complex skeletal ^{1}H NMR resonances that require extensive 2D NMR for interpretation).[15] Regardless of complexity, qNMR by nature offers a unique physicochemical view of the target analyte.

Background to Qualitative ^{1}H NMR. Six decades after its discovery,[16,17] NMR can be considered a leading nondestructive analytical tool for—but not limited to—the analytical chemist in structural analysis of biomolecules. The NMR toolbox comprises a set of qualitative methods to measure chemical shift (δ), spin−spin coupling and dipolar coupling (J, leading to connectivity), through-space interactions (NOE, also establishing connectivity), and relaxation (T_1/T_2). Initiated by Jeener's design[18] to combine chemical shift and coupling dispersion, the advent of correlated spectroscopy (COSY)[19] marked the point of entry into n-dimensional (nD) NMR space, in which the aforementioned physicochemical properties are implemented into one experiment. Two-dimensional (2D) experiments, such as COSY, HMBC, and HMQC/HSQC, have become essential tools in modern structure elucidation, and reviews as well as related key publications on NMR as a structural tool for natural products are available.[20−25] 2D NMR experiments are typically represented by topographical plots, in which signal intensity is the third dimension, and is primarily evaluated as a determinant of sufficient signal/noise (S/N) ratio. A discussion of the semiquantitative and quantitative evaluation of NOESY and ROESY-type 2D experiments for contour mapping of biomolecules shall be deferred for the purpose of this review. Regardless of the underlying principle, the power of n-dimensional NMR lies in the increase of signal dispersion into multidimensional space, combined with spin−spin coupling (J), through-space (NOE), or through the lattice (T_1) linkage of structural fragments.

In principle, the same S/N considerations apply to the quantitative capability of 1D NMR, which has been acknowledged since the early days of NMR. Today, it is routine practice in Fourier transform NMR (FT-NMR) to accumulate FIDs (time domain free induction decays) until the acquired spectra exhibit a S/N that is sufficient for the target analyte. Next, the spectra are interpreted in terms of the resolution of signal splittings. Because the target is typically the major component, other components in the sample remain invisible by being buried in the noise. Accordingly, proton spectra are considered rapid experiments, and in contrast to ^{13}C NMR, it is hardly perceived that there is an exponential relationship between the achieved S/N and the experiment time. As a result, sample components at abundance of ca. 10% or possibly more are easily overlooked in routine ^{1}H NMR screening. It can be shown, however, that properly selected ^{1}H NMR acquisition times and delays and other parameters (hereafter referred to as "quantitative conditions" in the following discussion), which allow the accurate quantitation of the ^{13}C satellite signals in ^{1}H spectra, are already sufficient to successfully characterize sample composition at the 1% level.[15,26] Therefore, instrument time is well spent with work in the ^{1}H domain, when considering both the amount of information extracted and the overall sensitivity of the observed ^{1}H nuclei. Accordingly, there are a number of benefits to using qHNMR as a routine analytical tool such as sensitivity, universality, precision, reduction, and/or replacement of other quantitation tools,[26] and—it should not be forgotten—the nondestructive nature of the method. Altogether, these benefits should reinvigorate the power of 1D ^{1}H NMR in the natural products laboratory.

Historical Background of qNMR. Quantitative NMR (qNMR) is almost as old as NMR itself. Early reports regarding the achievable precision of quantitation are inconsistent, and some of them even tended to deny NMR a role as a precision method by estimating the error to be in the 10% range. Interestingly, and with notable exceptions,[27,28] textbook literature often does not emphasize the quantitative aspects of NMR and, thus, does not motivate educators and researchers to consider qNMR as an analytical tool. This stands in contrast to the authors' recent personal discussions with experienced NMR spectroscopists, as well as to the tenor of the publications cited in this review, according to which the quantitative power of ^{1}H NMR and its broad applications are greatly underestimated. Moreover, recent developments in the field have provided evidence that NMR can be developed as a precise quantitative tool and, in time, can even be a primary analytical method.[29]

As can be seen from Figure 1, there is a steadily increasing interest in qNMR over the past 40 years, as measured by the number of publications in the field

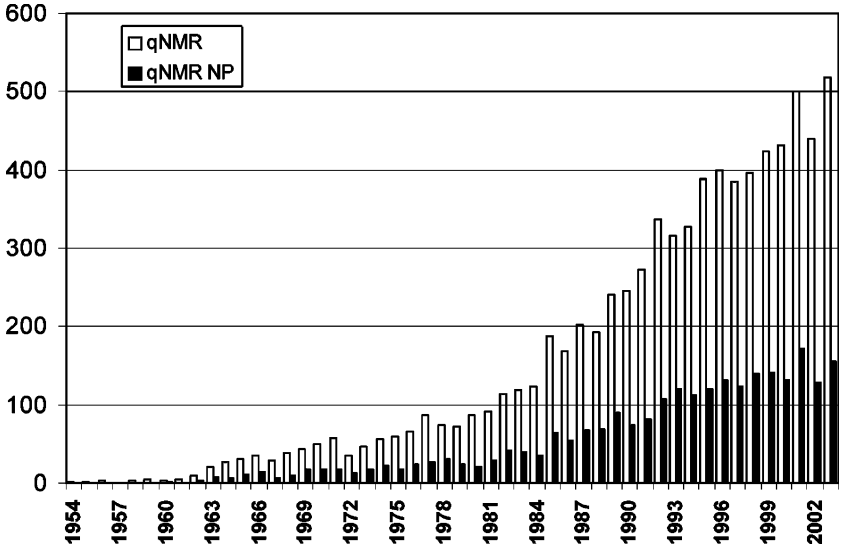


Figure 1. The development of quantitative NMR (qNMR) between 1953 and 2003 measured by its publications record as indexed in the ACS's *Chemical Abstracts* (N_{tot} = 7981). The subset of publications relating to natural products is represented by solid bars (qNMR-NP, N = 2610) and was selected based on CAS sections assignments including natural products chemical classes, biochemistry, and food chemistry sections, while excluding publications marked unassigned (ca. 1900 entries).

(*Chemical Abstracts*). However, taking into account the overall rapid increase of publications in science, and especially when considering the statistics for natural products related qNMR (solid bars in Figure 1), there seems to be almost no gain in interest in the past 15 years, a period that has been exceptionally productive in terms of NMR hardware development. It must be noted, however, that the metabolomic studies mentioned below, which often involve (semi-) quantitative NMR analysis, are not included in this statistical picture, because the necessary qNMR keywords cannot be searched successfully since they are not included in the database entries of the corresponding publications. The importance of the qNMR methodology in this recently emerging area of research, however, indicates the rising impact of qNMR methodology on natural products research in general.

Literature Background of qNMR. Because qNMR has been living in the shadow of the multifaceted and multidimensional qualitative NMR used in structure analysis, neither has it been used as widely and routinely nor is a recent and comprehensive overview of the literature available. However, Szantay[30] and Evilia[31] have reviewed systematically the general experimental factors known to interfere with quantitative determinations in NMR. Their articles cover relaxation, digitization, and instrumental parameters and provide valuable sources of information independent from the observed nuclei. Certainly noteworthy, while exclusively dealing with analyses of drugs and pharmaceuticals, is the extensive qHNMR work by Turczan and co-workers at the FDA,[32−51] which to our best knowledge has not been summarized in a review format. Their experience shows that typical errors fall in the 0.5−2% range, and their reports serve as a valuable resource when it comes to the selection of qNMR reference standards (see below). The essential lack of reports describing the application of qHNMR in natural product research is confirmed in a 1989 $^{1}H/^{13}C$ NMR review by Pieters and Vlietinck,[52] who concluded that, despite the great potential of qNMR, suitability has to be established for each individual case. The excellent review series focused on ^{1}H NMR by Rackham[53] that begun in 1975, unfortunately, has been discontinued, leaving almost all of high-field qHNMR uncovered. The present review seeks to fill this gap and to provide a comprehensive survey of the qHNMR literature by discussing recent and forthcoming technological innovations, while concentrating on the applications of qHNMR to complex samples (mixtures) such as materials that are obtained from natural sources. Because the second most studied organic NMR nucleus (^{13}C) is considerably less sensitive (1.6% of ^{1}H sensitivity for an equal number of nuclei, augmented by a sensitivity loss due to the 1.1% natural abundance of ^{13}C) and affords quantitative information significantly more difficult to obtain, in particular for small natural product samples, this review will focus on the ^{1}H variant of qNMR (qHNMR). Unless NMR technology achieves another quantum leap in sensitivity, it is reasonable to hypothesize that proton NMR will remain the most suitable nucleus for quantitative studies, especially for natural products, and is preferred over the much more dispersive, but inherently less sensitive heteronuclei. One exception is ^{19}F, with 88% of ^{1}H sensitivity (100% ^{19}F natural abundance), which due to its negligible background interference is evolving into a preferred quantitative tool in drug metabolism studies.[54−56] The application of ^{19}F NMR for studying natural products is very limited at this time,[57,58] and while not presently enjoying practical widespread utility, it could become an important qualitative and quantitative screening tool in the search for new naturally occurring organofluorine compounds. Another relatively sensitive nucleus, which has been used for extensive qualitative and quantitative applications, is ^{31}P NMR (7% of ^{1}H sensitivity, 100% ^{31}P natural abundance), but this is also beyond the scope of this review.

The present review (see organizational Figure 2) covers the scientific literature from 1982 to July 2004. A thorough manual screening has been performed of ca. 8000 primary hits obtained from the *Chemical Abstracts* database through the use of SciFinder, when searching the concept "quantitative NMR" (see Figure 1). It has been the experience of the authors in their own research involving qNMR, and from extensive communications with colleagues, that qNMR is much more frequently applied in the industrial sector and in the regulatory environment than is reflected in the published scientific literature. Therefore, the proceedings of two conferences have been included in the literature survey: first, beginning with 1988, the abstracts of the Experimental NMR Conference (ENC),[59] which represents a foremost platform for information exchange on NMR

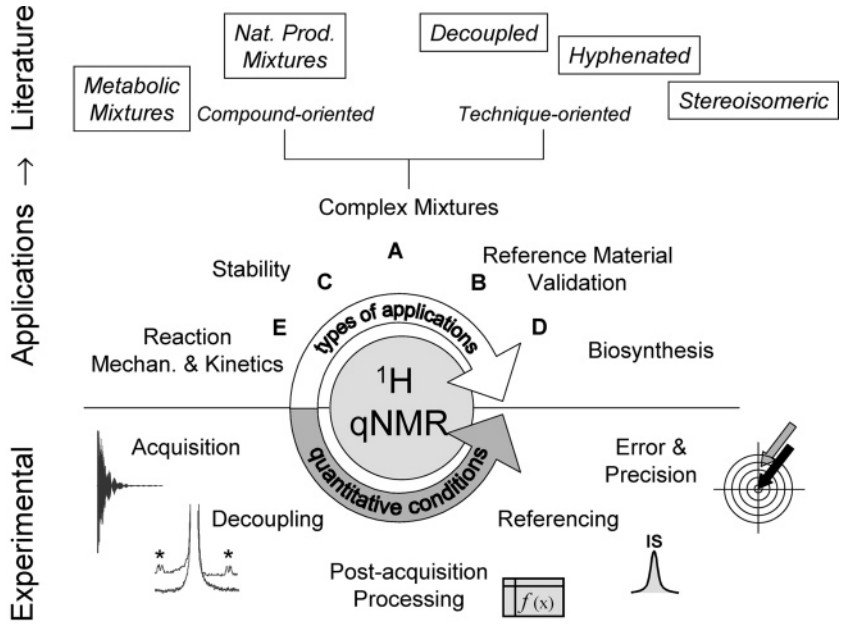


Figure 2. Overview of the topics of the present review. Based on a general overview of experimental parameters (bottom) that lead to "quantitative conditions", reported applications of the main areas of qHNMR analysis (middle) are compiled with particular focus on analytical procedures for natural products (top).

topics, and second the recent "Small Molecules Are Still Hot" (SMASH) NMR conferences,[60] albeit providing few of the documented qNMR applications. The review is organized in two major sections. The first section deals with a discussion of the experimental aspects of the qNMR experiment, and the second section is devoted to a compilation of relevant applications of the qNMR technique for natural product analysis.

The qHNMR Experiment

The first major section of this review provides an overview of the technicalities of the qHNMR experiment with regard to experimental design, data acquisition, ^{13}C decoupling, postacquisition processing, reference compounds, the aspects of a quantitative assay, and the level of quantitative assessment.

Experimental Design and Parameter Selection. From its inception, NMR, and specifically ^1H NMR, has been used extensively for the structure elucidation of organic molecules. One important aspect of this is the general fact that the integrated intensity (or the area under the NMR signal) is proportional to the number of nuclei giving rise to that NMR signal. Thus, integration permits determination of the ratio of the number of protons contained within a molecule. The observed chemical shift (δ) positions and spin−spin coupling pattern (J) for each proton absorption provide information as to what kinds of protons are found in the molecule and subsequently how the protons are arranged (structural information). This is a fundamental tenet for the structural application of proton NMR spectroscopy.[61−63]

Since the integrated intensities of proton resonances are proportional to the number of protons within a molecule (intramolecular context), it is reasonable to assume that the same approach could be used for the analysis of simple or complex mixtures (intermolecular)[27] or, as another focus here, for the determination of impurities in isolates of natural extracts.[15] To make the integrated intensity of a 1D NMR signal accurately and reproducibly proportional to the number of observed nuclei, a number of data acquisition parameters need to be carefully optimized. Because qHNMR places certain demands on the parameters that are crucial, attention must be paid to the details of appropriate experimental conditions for quantitation ("quantitative conditions"; refer to Figure 2 for an overview, and to Table 1 for a "cookbook approach" to qHNMR). Unless the NMR signal is represented by a single Lorentzian line, which it rarely is, signal intensity alone usually cannot be used for quantitation. Instead, integration of the signals must be performed. Accordingly, achievement of proportionality and the integration method represent two key factors for quantitation. Their limitations and/or consequences[30] and their prospective solutions will receive major attention in this review. It shall be pointed out early on, however, that both factors represent a 2-fold approach that is needed to make NMR a precise analytical tool: (i) "quantitative experimental conditions", including appropriate parameter selection such as relaxation delay, digitization, and pulse sequence design, and (ii) selection of appropriate postacquisition processing parameters for optimized spectral integration.

Data Acquisition. All contemporary NMR spectrometers employ pulsed NMR techniques for the acquisition of NMR data. A radio frequency pulse excites the nuclei in the sample, which produces a time domain response to the pulse, i.e., the free induction decay (FID). The time domain response is then subjected to a mathematical Fourier transformation (FT), which converts the time domain response into a frequency domain spectrum. It is this "familiar" frequency domain spectrum that is analyzed and used for spectral interpretation in terms of structure and quantitation. For quantitative purposes, a simple 1D proton NMR spectrum is typically obtained using this technique. The signals are then integrated and the ratio of components in a mixture is determined. There are numerous factors in this simple pulsed NMR approach that must be carefully optimized, to achieve what are referred to collectively as "quantitative conditions" for good quantitation.

The basic pulse scheme for obtaining 1D proton NMR spectra (Figure S1) contains three basic parameters that are under control of the user: *d1* (relaxation delay), the pulse with a pulse width (*pw*) in μs (corresponding to a

Table 1. Cookbook Approach to qHNMR Providing General Guidelines for the Choice of NMR Acquisition and Processing Parameters

parameter (abbreviation in text)	suggested value	comments
acquisition		
acquisition time (*aq*)	2−4 s	varies with sample/spectral window; choose short value for inverse-gated decoupled qHNMR
relaxation delay (*d1*)	3−10 s	should reflect 5 times the longest T_1 in the sample; shorter delays reduce precision, but may be acceptable (see text); longer delays are used to reduce the duty cycle of the decoupler in decoupled qHNMR
pulse width (*pw*)	15−45°	ideally use Ernst angle calculated for each sample (see text; *d1* and longest value of T_1 in the sample have to be known)
time domain	64 k	should be zero-filled, but not linear predicted
spectral width (*sw*)	sample spectral window ± 3 ppm on each end	depends on type of electronic filtering used (analogue/digital)
transmitter offset (*o1*)	center of the spectral width	automatically set by spectrometer
receiver gain	closely below the highest possible setting	automatically set by spectrometer
number of scans/transients	128−1024	dependent on sample molar concentration, desired S/N, and level of quantitation to be achieved
processing		
exponential multiplication	lb = 0.1−0.3	other window functions may be considered (Gaussian, TRAF)
phasing	manual	still the best way to do it
baseline correction	polynomial *n*th order	manually optimized

pulse angle α), and the acquisition time (*aq*) of the time domain data. The latter must adhere to the Nyquist theorem[27,28] and is related to the extent of digitization of the final spectrum after FT; that is, the longer the *aq*, the more data points are used for defining the spectrum. The length of *aq* is generally selected on the basis of the spectral width (*sw*) and the required digital resolution for that *sw*. For most 1D proton NMR spectra, *aq* is selected so that the spectral resolution (aq^{-1}) is around 0.25 Hz or better. While *aq* is set prior to measurement of the NMR spectrum, improvement of the digital resolution can be further enhanced by postacquisition data processing techniques such as zero-filling or linear prediction.

The relaxation delay (*d1*) represents the time, in seconds, for the equilibrium magnetization, or the equilibrium state, to be established, or re-established, between pulses when accumulating co-added FIDs (Figure S2A). Immediately after application of a radio frequency pulse (*pw*, in this case, a 90_x° pulse), all of the magnetization that was along the *z*-axis is now converted into transverse (or *x*,*y*-) magnetization (Figure S2B). If a 90° pulse is used, then maximum sensitivity will, in theory, be obtained; that is, all of the *z*-magnetization is converted into the detected *x*,*y*-magnetization. However, a long *d1* must be used in order to allow the equilibrium magnetization state to be re-established before the next pulse cycle can begin. A long *d1* will have the effect of considerably lengthening the time necessary to complete the experiment, since, in most cases, signal averaging is required to achieve sufficient signal-to-noise (S/N) in the final spectrum. The length of *d1* is governed by the relaxation properties of the nuclei in the mixture being excited and is characterized by individual relaxation times of the various proton resonances associated with the sample. If a 90° pulse is used, a *d1* delay of $5T_1$(max), where T_1(max) is the value of the proton with the longest relaxation time in the sample (not necessarily the target analyte!), must typically be employed.

Relaxation times fall into two types: longitudinal relaxation (T_1) and transverse relaxation (T_2). If nuclei are subjected to a 180° pulse (inversion pulse, Figure S3), the magnetization that started out on the +*z*-axis (Figure S2A) is now found on the −*z*-axis. If the recovery of the magnetization is then sampled at regular intervals by application of a 90° pulse to create transverse *x*,*y*-magnetization during the *z*-magnetization recovery process, relaxation times (T_1) can be obtained for each resonance in the NMR spectrum of a mixture by analysis of the relaxation recovery data. This is the essence of the inversion−recovery T_1 experiment (Figure S4). Once the range of actual T_1 values, typically 0.3−5 s, for the protons in the molecule, or in the mixture being studied, are accurately known, adjustment of *d1* and *pw* may be established (typically < 90° pulse, e.g., Ernst angle,[64] see below and Table 1) and optimized for quantitative conditions associated with the sample. If the longest relaxation time, T_1(max), in the sample is known, and the *d1* delay is set to $5T_1$(max), *pw* may next be optimized. The optimum *pw* (*syn.* flip angle or tip angle) is known as the Ernst angle.[64] The goal is to perform the qHNMR experiment in a reasonable time without distorting the quantitative information by saturation or incomplete relaxation of the signals in the sample. The Ernst angle α_E allows optimizing the steady state of the observed *x*,*y*-magnetization when accumulating co-added FIDs. It is an average optimum value for the pulse flip angle that can be calculated from the relaxation delay *d1* and the longitudinal relaxation T_1 as follows:

$$\cos \alpha_E = e^{-d_1/T_1}$$

The transverse relaxation component (T_2) or spin−spin relaxation is inversely proportional to the line-width-at-half-height ($w_{1/2}$): $T_2 = (\pi w_{1/2})^{-1}$ or $w_{1/2} = (\pi T_2)^{-1}$. Lines that are broadened because of some chemical exchange or dynamic exchange processes will experience a faster rate of decay of their *x*,*y*-magnetization than lines that are much sharper with narrower $w_{1/2}$. For purposes of quantitation, it is important to factor in the proton resonances that might be useful for the quantitation of a mixture. Errors in the ratio of the components of a mixture can be experienced if this is not born in mind when attempting to quantify nuclei with a short T_2. Another set of problems can arise from the interference with other signals (signal overlap) present in the NMR spectrum of such samples or from interference between molecules (e.g., with solvent or relaxation agents) in the NMR sample. Sources of interference are (i) the

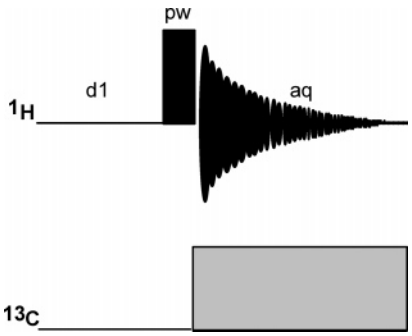


Figure 3. Inverse-gated decoupling scheme for eliminating ^{13}C satellites from ^{1}H NMR spectra.

presence of spinning sidebands, which are associated with the rotation of the NMR tube in the probe, (ii) differences in relaxation times between the analyte(s) and the internal standard, and (iii) the presence of ^{13}C satellites.

The first problem can be overcome by simply not spinning the sample. Contemporary NMR spectrometers operating with superconducting magnets have very good B_0 (static) magnetic field homogeneity. In many cases, the B_0 field homogeneity is so good that the spinning and the nonspinning performance (line shape and S/N) of the NMR spectrometer are almost indistinguishable. This kind of performance can be achieved on instruments that are well cryoshimmed and are equipped with higher order room-temperature shim hardware. In the early historical development of NMR spectrometer design, which included the use of electromagnets to produce the magnetic field necessary for the NMR experiment, it was a requirement to spin the sample (15−20 Hz) to obtain a high-resolution NMR spectrum. The need to spin the sample tube was associated with (a) the relatively poor B_0 field homogeneity associated with the electromagnet system and (b) magnet field instability. Generally, the magnitude of the spinning sidebands, while minimized through proper shimming, can potentially be present at the same intensity or greater than that of impurity signals in a given sample. To overcome problem (ii), *d1* has to be chosen such that sufficient relaxation is allowed for the nuclei with the longest T_1, or by adding a relaxation reagent to the sample.

$^{1}H\{^{13}C\}$ Decoupling. The third problem, relating to the presence of ^{13}C satellites in the spectrum, can be overcome by performing heteronuclear decoupling at the radio frequency of the ^{13}C nuclei to collapse the ^{13}C satellites.[65,66] The ^{13}C isotope is present to the extent of 1.1% in the sample. Because it is a spin 1/2 nucleus, it will couple to the proton resonances, producing satellites at 0.55% integrated intensity that flank the principal proton resonance bound to ^{12}C (spin = 0). The separation between the centers of the satellites is the one-bond ($^{1}J_{H,C}$, typically 100−200 Hz) coupling constant. These satellite resonances can overlap with other signals of the primary analyte or with signals arising from impurities present in the sample. By irradiating at the center of the carbon frequency range (ca. 100−110 ppm) during the acquisition (*aq*) period of a proton NMR experiment (Figure 3), the result will be a ^{1}H NMR spectrum with the ^{13}C satellites collapsed. It then becomes a simple task of integrating the proton spectrum for assessing the quantitative composition of the sample. This represents an excellent general protocol for obtaining qHNMR spectra of samples that are mixtures of, for example, natural products.

Several caveats to this technique need to be mentioned. First, a composite pulse-decoupling scheme is used for the experiment to reduce, in part, sample heating. The use of continuous decoupling, because of the power levels necessary to fully decouple ^{13}C, is not viable since it can potentially cause damage to the probe. In addition, sample heating can be extensive and may run the risk of decomposing the sample. The Waltz-16 composite pulse-decoupling scheme[65,66] can and has been used, but a better scheme using GARP decoupling (globally optimized alternating-phase rectangular pulses)[69] may be employed. GARP provides more efficient decoupling over the wider spectral window associated with the chemical shift range (ca. 220 ppm) of the ^{13}C spectral region and should be used especially on higher field instruments with proton observation frequencies of 400 MHz or greater. Decoupling should be performed only during the acquisition (*aq*) of the FID (inverse gated decoupling), again to reduce the problem of sample heating. To reduce concerns associated with the possible buildup of NOE during an inverse-gated decoupled qHNMR experiment, one option is to shorten the acquisition time and regain the sacrificed spectral and digital resolution by zero-filling. Selection of the *d1* delay should also be judicious in this regard. If *d1* is too short, this will increase the percentage of time that the decoupler remains on (decoupler duty cycle) and lead to an increase in sample heating. So, while a suitable repetition rate (*d1* + *aq*) may have been optimized to yield quantitative conditions, additional care should be taken with respect to the selection of the length of the *d1* delay to minimize sample-heating effects.

Postacquisition Processing. Once the NMR data have been acquired, it is necessary to process the data using appropriate postacquisition processing techniques. Prior to Fourier transformation (FT) of the FID, appropriate zero-filling should be done.[27] The FID has two components: a real and an imaginary component (0° and 90° phase shifted), corresponding to absorptive and dispersive components of the FID. Since interpretation and quantitative analysis will make use of only the absorptive component (dispersive component is used for phasing only), FT will essentially throw away half of the data points collected. By zero-filling, the data points are added back as zeros prior to FT, and the original spectral resolution of the FID will be regained. This will contribute to maintaining both spectral and digital data point resolution of the spectrum. The "rule of thumb" for zero-filling is to zero-fill by at least a factor of 2, i.e., double the number of data points being Fourier transformed, to regain or maintain spectral resolution. Zero-filling beyond a factor of 2 is permitted, but will only have the effect of improving the overall digital (data point) resolution in the final spectrum.[27]

Application of windowing or weighting functions to the NMR data is also performed prior to FT, generally to enhance resolution at the cost of reduced signal-to-noise (S/N), but can also be performed to enhance S/N[70] in the final frequency domain spectrum. The typical S/N enhancement weighting function for ^{1}H NMR spectra is exponential multiplication (EM), which improves the S/N, but leads to a degree of line broadening. For optimum S/N enhancement, an EM function is applied that is matched to the decay rate of the FID. Typically, for proton NMR spectra, line-broadening factors (*lb*, *syn.* EM factor) in the range 0.15−0.40 Hz are applied. The zero-filling process noted earlier also helps to regain some of the additional loss of resolution that is incurred in the exponential weighting process. There are other weighting functions such as the TRAF function[70−72] that optimize sensitivity enhancement, while fully retaining quantitative information. These functions should be used with some caution, however, and

Table 2. Reported Reference Compounds for qHNMR Validation[a]

standard	references	comments
1,3,5-benzenetricarboxylic acid	86, 87	aromatic singlet, acidity
1,3,5-trimethoxybenzene	85	aromatic and OMe singlets
1,3,5-trioxane	89	singlet in −OR shift range; complexation?
1,4-bis(TMS)-benzene	77	used in automation qHNMR, >99.9% purity by vacuum sublimation; moisture?
1,4-dinitrobenzene	32−51	aromatic singlet, acidity/complexation?
1,4-dioxane	83	volatility, singlet in −OR shift range; complexation?
anthracene	88	interaction (π stacking)?
benzyl benzoate	32−51	ester hydrolysis?
biphenyl	32−51	interaction (π stacking)?
dimethyl isophthalate	89	complex aromatic spin pattern and OMe
dimethylformamide	97	two *N*-methyl and one aldehyde singlet, high temperature dependent (N-Me); reactivity?
dimethylsulfone	89, 90	99.95% pure by DSC; moisture? reactivity?
formic acid	92	acidity
hexamethylcyclotrisiloxane	32−51	Me singlets; stability?
maleic acid	32−51, 78−82	olefinic singlet; acidity?
methenamine	32−51	stability?
phloroglucinol	84	known instability (redox)
sodium acetate	91	aliphatic Me singlet, aqueous only
tert-butanol	32−51	aliphatic Me singlet, dehydration?
tetramethylpyrazine	89	aromatic methyl singlets; asymmetric protonation? interaction (π stacking)?
TSP-d_4	92, 93	aqueous only; known protein interaction
etacrynic acid	15, 94−96	simultaneous validation of quantitative conditions and calibration
2,5-dimethylfuran	98	

[a] The choice of reference compound should be exercised with great care, keeping in mind that either reaction, complexation, acid/base, or any other type of chemical/physical transformation can occurr. This table merely represents a compilation of all reported reference compounds and can give only brief guidance about potential problems.

validation carried out to ensure that the quantitative information is accurate. Care should be exercised when applying windowing functions, since too severe a window function (i.e., resolution enhancement) can distort the line shape and compromise quantitative accuracy.

Careful phasing of the spectrum ensures that the integrals representing the various signals in the transformed spectrum will have minimal distortion and will, therefore, contribute to quantitative reproducibility. Interestingly, all of the qHNMR literature that has evaluated phasing methods favors manual phasing over automatic phase correction routines. In addition, baseline correction routines should also be applied prior to the integration process since they will further contribute to both the accuracy of integrals and their reproducibility. Further improvement in the accuracy of NMR integrations and, thus, the accuracy of NMR-based quantitation using various computer algorithms has been reported.[73−76] A spreadsheet-based software tool that aids the subsequent evaluation of the quantitative data using the "rule of three" has been reported.[77]

Reference Compounds for Validation. As noted above, the validation of qHNMR experimental conditions may be a necessary exercise to ensure that proper "quantitative conditions" have been established leading to accurate and reproducible quantitative results. Some compounds or mixtures of compounds have been used for this purpose. Traditionally, "synthetic" mixtures, i.e., mixtures in which the ratio of the components within the mixture is known with certainty, have been created, and the NMR experimental conditions optimized using the mixture. This will provide confidence that the NMR conditions will afford accurate and reproducible quantitative results. It will, however, provide only reproducible ratios of the specific components present in the "synthetic" mixture. In addition to the need to validate the qHNMR experimental conditions, reference compounds are required for internal and external quantitative calibration. Therefore, a brief and annotated overview of all the reference compounds that have been employed as part of the applications cited in this review is provided in Table 2.

An ideal internal standard for assay purposes would be one that is readily available in a highly pure form, inexpensive, stable and chemically inert, nonvolatile and nonhygroscopic, and soluble in all (or most) of the NMR solvents that are used routinely. If used as a weighed internal standard in an assay determination, its ^{1}H NMR spectrum should contain a minimum of interfering signals with the signal associated with the spectrum of the target analyte. This makes the finding of one universal qHNMR reference substance to serve all purposes (i.e., the validation of quantitative conditions and a weighable internal standard) an impractical task. Depending on the particular application, various compounds have been suggested to serve this purpose. There is a tendency in the literature to propose internal reference standards with simple ^{1}H NMR spectra, preferably singlets. The most widely used is maleic acid, which was the reference compound of choice in a number of quantitative qHNMR studies involving catecholamines,[78] muscle relaxants,[79] and antidepressants,[80] as well as in the semiquantitative analyses of herbal drug preparations such as kava-kava[81] and in mixtures of methamphetamine and ephedrine in substances of abuse.[82] The publications by Turczan and co-workers[32−51] are a valuable source of qHNMR reference standards and include *tert*-butyl alcohol, 1,4-dinitrobenzene, hexamethylcyclotrisiloxane, methenamine, biphenyl, benzyl benzoate, and again maleic acid. One report proposes the ^{1}H singlet signal of 1,4-dioxane for calibration,[83] which raises a caveat with regard to its volatility (bp 101 °C). The simple singlet spectrum of phloroglucinol has been chosen as reference for the qHNMR analysis of ginkgolides from *Ginkgo biloba*,[84] while subsequent studies found solution stability problems associated with this compound and replaced it with 1,3,5-trimethoxybenzene.[85] A related compound with a similar spectrum, 1,3,5-benzenetricarboxylic acid, was

used in the qHNMR analysis of apple phenols.[86,87] Although providing a total of three ^{1}H signals, only the singlet of anthracene was used in a qHNMR study of cannabinoids, which showed no spectral overlap with the selected reference compound.[88] Wells et al. evaluated the following inexpensive high-purity compounds as internal standards: dimethylsulfone (DMSO2, 99% declared, 99.95% by differential scanning calorimetry), 1,3,5-trioxane (99+% declared, 99.2% qHNMR against DMSO2), tetramethylpyrazine (99% declared, 99.0% qHNMR against DMSO2), and dimethyl isophthalate (99% declared, 99.8% qHNMR against DMSO2).[89] The same authors subsequently used DMSO2 as a reference for the purity assessment of technical grade agrochemicals.[90] In a combined ^{1}H and ^{31}P qNMR study, they employed trimethyl phosphate and sodium acetate as reference standards for quantitation.[91] In a recent study by de Graaf and Behar on blood plasma metabolites, out of the two reference compounds that were added to the samples, i.e., sodium 3-trimethylsilyl[2,2,3,3-D_4]propionate (TSP-d_4) and formic acid, only the latter was found to be useful for quantitation, because interactions between TSP and serum albumins led to partially invisible signals as a result of dynamic interaction.[92] In urine samples, a titer solution of TSP-d_4 was employed in the form of a coaxially inserted capillary tube for a field-frequency lock and a chemical and quantitation reference,[93] underlining the omnipotence of this established NMR reference standard compound.

Etacrynic acid (EA) represents one of the few suggested reference compounds that allow both the validation of qHNMR experimental conditions and quantitative calibration.[15] EA is a titer-stable substance with a highly dispersive set of proton resonances, it is available in high-purity, and it is soluble in a wide range of solvents. It has been successfully employed to internally calibrate solvent signals for subsequent secondary/external calibration. Applications have been reported for various natural product reference materials,[15] including arbutin,[94] oligomeric proanthocyanidins,[95] and glucoiberin.[96] Like any reference compound used for quantitation and to simultaneously validate experimental conditions, the applicability of EA as an internal calibrating agent will vary with sample and depend on the absence of overlap with signals of the target analyte, which in turn depends on spectrometer frequency and the solvent used. Analogous to the EA calibration of the residual ^{1}H signals of deuterated NMR solvents, dimethylformamide (DMF) has been used as an indirect calibrating agent for the study of the pungent components in supercritical fluid extracts of chili pepper, black pepper, and ginger.[97] However, since DMF does not offer validation of the qHNMR experimental conditions, a possible alternative is to use 2,5-dimethylfuran (DMFu). This low-boiling liquid provides two singlet ^{1}H peaks at 5.80 and 2.20 ppm and has been proposed as internal standard for "traceless" quantitation.[98] As part of an effort to establish an automated qHNMR setup, which involves robotic solution preparation and an autosampler for spectral collection, Pinciroli et al. found 1,4-bis(trimethylsilyl)benzene to be the most suitable internal reference compound.[26] Prior to use, the compound is purified to >99.9% purity by vacuum sublimation. For the certification of qHNMR conditions, the present authors have used five certified reference standards: doxorubicin HCl, niacinamide, penicillin G Na, acetanilide, and caffeine.

Assay Aspects. For simple compositional analysis, integration of the spectrum or selected spectral regions is performed, followed by adjustment of the integrated intensities to reflect the number of protons giving rise to the integrated signals. The individual integrated intensities are summed and are then expressed as a percent of the summed integrations (normalization), which represents the molar composition of the mixture (mole %). For most quantitative purposes this is usually sufficient. However, if an absolute determination of purity of the principal component of a complex mixture is required, it is necessary to develop a weight-percent-quantitative assay. This procedure would involve (i) obtaining a weight (mg) of a sample of the crude mixture, (ii) adding a precise quantity of a known internal standard, (iii) obtaining the solution qHNMR spectrum on the resultant crude mixture plus the internal standard, (iv) calculating the actual weight of the desired component of the crude mixture, and (v) expressing it as a percent of the weight of the total sample of the crude mixture.

Level of Quantitative Assessment. Whenever the nature of NMR quantitation experiments described here pertains to the detection of impurities in natural product isolates, the essential goal is usually to assess the purity of the major constituent of any given mixture. To do this, assumptions about the nature of the observed proton signals present in the impurities need to be made in order to quantitate against the same kind of proton signals present in the major constituent and to estimate the impurity level. In routine practice, proton NMR can perform this task reasonably well down to a level of about 1−2%. While this is a comfortable lower level of detection, levels much lower than this can be achieved and have been reported (see below). Regarding the investigation of complex natural product mixtures and the metabolome analyses addressed below, the aforementioned assumptions run a much higher risk (up to almost certainty!) of being flawed. Because the samples are much more complex, single components would have to be identified and/or dereplicated, e.g., by 2D NMR. Alternatively, assumptions need to be made on the basis of the combined coupling and chemical shift information contained in a given signal. But even if the component cannot be quantitated in an *absolute* fashion (i.e., reliable molecular weight and structure-based determination of mole %) because of the uncertainty about identity, qHNMR is still capable of *relative* quantitation of the component relative to a reference or to another analyte that is similar in structure and/or molecular weight. Just like any chromatographic method, however, the risk remains of uncalculated signal overlap. In qHNMR the discerning factor, at this point, becomes chemical shift dispersion (δ, in ppm) rather than retention time in chromatography. The chemometric applications cited herein demonstrate the promising potential arising from this distinctive physicochemical parameter of qHNMR, which arises from the persistence of a highly significant link between measured signal and chemical structure. This is documented in a recent discussion pertaining to the high accuracy of quantitation by NMR.[99,100]

The structural significance of qHNMR is favorably paired with its high accuracy. Briefly, the accuracy and precision of reported qHNMR applications typically fall in the 0.5−3% range. According to Pinciroli et al., the errors appear to be slightly higher (2−3%) in an automated setting compared to manual setup. However, use of the same NMR setup, but with manual sample preparation, reduces the error to about 1%.[26] Very recently, the "uncertainty budget" (term coined by the National Institute of Standards and Technology, NIST) for single qHNMR purity determinations was reported to be 0.66% for a 95% confidence

interval.[101] Because of the uncertainty in the recognition and identification of potential minor components, qHNMR of natural products is by default less accurate due to unpredictable sample complexity (metabo(l/n)omics!). In some cases this may even apply for those natural products that are thought to be pure "standards".

There are ongoing efforts to establish qHNMR as a primary analytical method, i.e., a method that measures the value of an unknown without the necessity to have on hand a chemical standard of the same quantity.[102] Due to limitations of the quality of integration, NMR is currently considered as a *relative* primary analytical method by the Comité Consultatif pour la Quantitè de Materié (CCQM, Sevres, France), which so far considers only coulometry, titrimetry, gravimetry, and the colligative methods as being primary. For this purpose, a worldwide interlaboratory test series has been organized by Malz and Jahncke, and four phases have been completed through 2000.[29,103] Several qHNMR factors were tested ranging from acquisition parameters to operator influence. Although it is hard to derive a single statement about accuracy from these globally performed interlaboratory studies, it is reasonable to say that accuracy for multicomponent mixtures was generally found to be within 2%, as long as certain experimental conditions were met, and often was between 1.0 and 1.5%.[29,99] A remarkable result of the interlaboratory test CCQM-P20a is that ^{13}C decoupled qHNMR can be as accurate and almost as precise as differential scanning calorimetry (DSC).[29] At present, to our knowledge, there seems to be only one comprehensive report that is readily available to the academic community (Ph.D. dissertation of F. Malz)[29] and one follow-up publication.[104] For the purpose of this review, it can be posited that qHNMR fulfills the criteria of a relative primary analytical method and, therefore, within its limits of detection, is superior to any chromatographic (hyphenated!) method for quantitation. Fortunately, only very recently one participating national institution has published a practical guide for accurate solution state qHNMR,[99] which summarizes the key parameters of "quantitative conditions" on qHNMR. An important conclusion of this study is the insight into the correlation between the relaxation delay (*d1*) and the achievable precision. While *d1* needs to be greater than 5 times T_1(max) for the peaks of interest in order to achieve 0.1% precision, it can be significantly shorter than the $5T_1$-(max) rule if less precision is acceptable,[99] which confirms our previous conclusions.[15] With the exception of high-purity reference materials, this will be the case for most natural products, especially when looking at complex mixtures, and, therefore, greatly reduces the demand for instrument time of any routine qHNMR method.

Obtaining a standard proton NMR spectrum of a mixture, which contains the ^{13}C satellites from all prominent constituents, however, can assist in the determination of impurity levels. Because natural abundance ^{13}C is present to the extent of 1.1% abundance, the two carbon satellites each appear at the 0.55% level (neglecting heteronuclear higher order effects). Comparison of the integration of satellites with respect to the integration of impurity signals, subject to the assumptions above, can provide a confidence factor for the level of impurities that are present and further define the approximate limits of detection of a given sample.[15] The sensitivity as determined by the signal-to-noise (S/N) of a given sample will contribute significantly to the accuracy and reproducibility of the determination. Another factor that must be considered relates to integral limits (i.e., where the integral trace starts and stops), which can serve as a contributing factor to variability in a given determination. Appropriate definitions or suggestions for the standardization of integral limits are currently lacking in the literature. However, it is important to document them in any given qNMR study, e.g., as a straight interval width in Hz. It shall be noted that any choice will largely depend on the use of magnetic field strength, window functions, and overall resolution. Therefore, each analytical problem will have to be evaluated separately.

Applications of qHNMR

The second major section of this review provides an overview of reported natural product-relevant qHNMR applications and covers a wide range of experimental targets (Figure 2): (A) quantitation in mixtures, complex matrixes, and formulations, (B) validation of reference materials including purity analysis, (C) biosynthetic studies, (D) stability studies, and (E) reaction mechanisms and kinetic studies. While D and E are important areas of qHNMR application in the pharmaceutical industry and in synthetic chemistry, such types of studies involving natural products are essentially lacking in the literature. Topic C continues to be a domain of ^{13}C NMR due to the fact that the carbon backbone is the typical study target, making ^{13}C labeling/incorporation the most straightforward technique to use. At the present stage of method development, A and B are the prime areas of qHNMR of natural products, covering the analysis of (complex) natural mixtures, (bio)active principles, isolated natural products for wide use in chemical and biological assays, and the emerging field of metabolomic studies. Accordingly, the applications cited in the following will primarily fall into categories A and B, while some examples cover areas C and D.

Metabolic qHNMR. NMR is becoming an increasingly important quantitative tool for natural products analysis, as this field of research is moving into the metabolome era. This includes "metabolomics" and "metabonomics", in the sense that both seek to analyze the total mass ("-ome") of primary and/or secondary metabolites (often called "small molecules") of an organism or cell. Key concepts in metabolome analysis involve the qualitative and quantitative evaluation of "fingerprints" and "biological signatures" recorded by NMR, making qHNMR an essential component of the chemometrics toolbox.[105,106]

The highly complex resonances of hundreds of overlapping metabolites result in indiscernible multiplets and represent the major problem in the ^{1}H NMR analysis of biological samples and fluids such as urine.[107] To increase spectral dispersion, 2D NMR experiments can be utilized to generate suitable quantitative variables for multivariate statistical analysis including Linear Discriminant Analysis (LDA), Hierarchical Clustering Trees (HCT), and Principal Component Analysis (PCA). For this purpose, for example, Dumas et al. have utilized 2D gHMBC and gCOSY as multivariate parameter generators and as alternatives to Watergate 1D ^{1}H NMR spectra to investigate endocrine disruptions caused by anabolic steroids in cattle.[77,107]

^{1}H NMR is a useful tool for metabolic profiling and monitoring metabolic changes in natural sources. Schoen et al.[108] proposed a quantitative metabolic profiling assay based on NMR of perchloric acid extracts of *Hymenolopsis diminuta* (cysticercoids) parasitized *Tenebrio molitor* beetles. Infected beetles possessed less glycerophosphocholine but more glycogen and a higher percentage label in glucose and trehalose than their respective controls. The beetles were fed with D-(1-^{13}C-glucose), and the incorporation of label

from this substrate was examined by in vivo ^{13}C NMR. In addition, ^{1}H NMR spectra were obtained for perchloric acid extracts of the same beetles and of the cysticercoids. (2,2,3,3-$^{2}H_4$)-3-Trimethylsilylpropionate (TSP) was added as an intensity and chemical shift standard to the aqueous samples. ^{13}C decoupled ^{1}H spin−echo NMR spectra, with and without ^{13}C population inversion, were obtained on a 500 MHz instrument. Inverting the ^{13}C population puts the attached protons 180° out of phase with respect to protons attached to ^{12}C and other nuclei. Consequently, resonances from protons attached to ^{13}C cancel when the spectra are added. When the spectra are subtracted, all other resonances cancel, leaving only resonances of protons attached to ^{13}C. Chemical shifts were expressed relative to TSP at 0 ppm. Quantities of metabolites present and the percent ^{13}C enrichment were calculated from integration values of the metabolite peaks relative to that of TSP.[108]

The metabolism of the tomato saponin α-tomatine (**1**) to the aglycon tomatidine, and further to 7α-hydroxytomatidine and the minor corresponding Δ^5-dehydro product 7α-hydroxydehydrotomatidenol, by *Gibberella pulicaris*, was published by Weltring et al.[99] For structure elucidation, the major metabolites were isolated, and the purity and the content of the minor dehydro metabolic side product (20%) were evaluated by qHNMR, measured at 600 MHz. High-resolution qHNMR has turned out to be a promising screening technique, which could answer some of the concerns caused by genetically modified organisms. Le Gall et al.[109] described the usefulness of ^{1}H NMR in metabolite profiling of tomato to detect potential unintended effects following a genetic modification. The study showed that chemometric NMR at 400 MHz combined with statistics could successfully trace even small differences in metabolite levels between plants. Thus, potential unintended effects in the genetic modification of crops can be detected. Two maize transcription factors were simultaneously overexpressed in tomato with the aim of producing lines with increased amounts of flavonols. The metabolite composition of these genetically modified tomatoes was compared with that of nonmodified controls. Le Gall et al. observed metabolic changes in both types at different stages of maturity.[109] ^{1}H NMR spectra showed that the levels of glutamic acid, fructose, and some nucleosides and nucleotides gradually increase from the immature to the ripe stage, whereas some amino acids were present in higher amounts in unripe tomatoes. Significantly increased concentration levels were observed for six main flavonoid glycosides as well as at least 15 other secondary plant metabolites.[109]

In a metabolomic study on the prediction of Alzheimer's disease, Partial Least Square Analysis (PLA) of ^{13}C decoupled ^{1}H NMR spectra of the cerebrospinal fluids successfully allowed classification of myo-inositol, glucose, and other sugars. The quantitation was based on a statistical approach to total-line-shape fitting and compared assigned and unassigned models.[110]

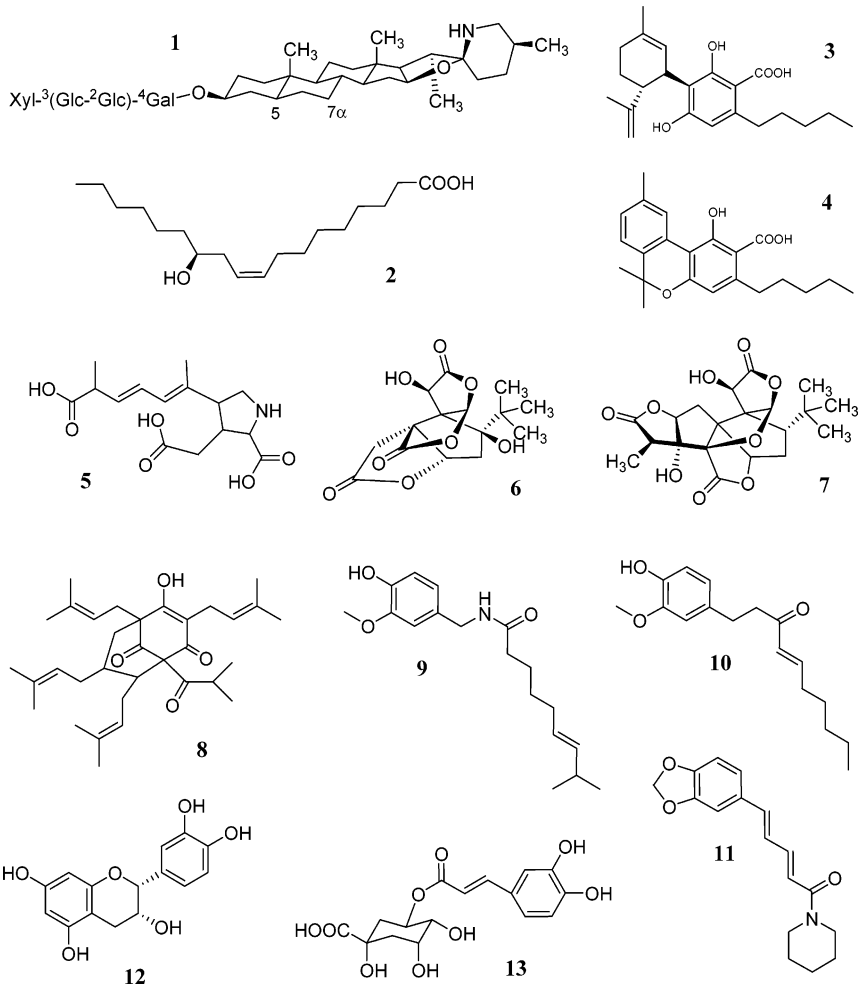
qHNMR of Complex Natural Mixtures from Plants. Quantitative ^{1}H NMR fingerprints have also been employed in the analysis of natural food products such as wine, fruit juices, and olive oil,[111] as well as beer[112−114] and port wine.[115] Forveille et al. used HMBC-GAS, HMQC, and HMQC-HOHAHA 2D maps to differentiate grapevine cultivars in an ANOVA analysis of the vine polyphenols.[116] The composition and geographical origin of olive oils have been analyzed by 1D qHNMR.[117,118] A series of recent publications by Kosir and Kidric et al. deals with the chemometric analysis of wines.[119−121] While large (solvent) signal suppression is reported to be an important general prerequisite, a signal intensity-based qHNMR analysis could even be achieved for minor constituents such as anthocyanin glycosides and amino acids in a semiquantitative fashion after chromatographic enrichment.[119,120] More recently, the qHNMR determination of aging indicators in balsamic vinegars has been demonstrated to supplement their quality assessment.[122] In this context it is noteworthy that the qHNMR observation of deuterium in site-specific natural isotope fractionation (SNIF-NMR) became the first officially adopted stable isotope method in wine analysis in the European Union in 1990,[123,124] allowing the discrimination between natural and enriched wines and differentiation of geographical origins.[125] For the differentiation of beer types and labels, mono- and oligosaccharides[112,114] as well as aromatic profiles were obtained with triple suppression of the water and the ethanol methyl and methylene resonances and were chemometrically evaluated by qHNMR.[112,113] Only very recently, two reports on isotopic qHNMR appeared, dealing with the quantitation of natural abundance deuterium (D, ^{2}H) itself. Quantitation of the heavy isotope allowed the assignment of the mechanisms of ricinoleic acid (**2**) biosynthesis in *Ricinus communis* and *Claviceps purpurea*[126] and revealed marked differences in the isotopic fingerprint from both the desaturase and elongase steps in oleic and linoleic acid biosynthesis.[127]

The ability of qHNMR to rapidly quantitate plant secondary metabolites without the need for prepurification or to obtain reference compounds was underlined by Verpoorte's group, who investigated cannabinoids from *Cannabis sativa*.[88] The team specifically quantitated four cannabinoids using a 300 MHz magnet and concluded that their method allowed the analysis of the 66 known natural cannabinoids that are commercially unavailable as reference substances. In a subsequent metabolomic analysis at 400 MHz, the quantitation of Δ^9-tetrahydrocannabinolic acid (THCA, **3**) and cannabidiolic acid (CBDA, **4**) was based on signal intensities rather than integrals. Principal component analysis (PCA) led to the chemometric discrimination of *C. sativa* cultivars, again without any need for prepurification of the plant extracts.[128]

QHNMR has also proven to be useful for quantitation of single compounds in complex mixtures such as crude extracts without requiring fractionation or isolation procedures. A qHNMR method for the determination of the molar substitution of acetylated and hydroxypropylated starches was developed by de Graaf et al.[129] Two qHNMR techniques were applied. The first used acetic acid and *tert*-butyl alcohol as internal standards for hydroxypropylated starch and acetylated starch, respectively. Alternatively, the anomeric proton signal of starch at 5.4 ppm, which depends linearly on the amount of anhydro-glucose units present in the sample, was used as an internal standard. Interestingly, instrumentation was comparatively low-field; all experiments were carried out on a 200 MHz spectrometer.

In the course of a recent study to find nonlethal bird repellents, Hile et al.[130] compared various preparations of garlic oil-impregnated granules. Employing qHNMR at 500 MHz proved to be useful for the rapid quantitation of major and minor components. For a complex mixture of naturally derived products, and especially for an essential oil, distilled garlic oil has a surprisingly simple ^{1}H NMR spectrum. The percent molar composition can be estimated on the basis of the 3.1−3.7 ppm region of the simple thioallylic proton resonances.Erickson et al.[131] have devised

Chart 1



a method for the quantitative analysis of cyanophycin in cyanobacterial extracts based on qHNMR spectroscopy, by integration of the signal attributed to the proton attached to the δ-carbon of arginine. The concentration of cyanophycins, representing nonprotein nitrogen storage polymers, was proportional to the nitrogen content of the cell and, therefore, an indicator of the nitrogen status of the bacteria. Burton et al.[82,132] discussed the application of qHNMR in research on phycotoxins such as domoic acid (**5**). Monitoring of these toxins is challenging, because isolation of the compounds from marine matrixes such as shellfish or plankton is tedious, with total yields typically being in the low milligram to sub-milligram range. According to their report, qHNMR has the potential for accurate determination of molar phycotoxin concentrations in crude extracts of marine organisms.

Van de Velde et al.[133] reported the usefulness of qHNMR as a tool for the analysis of samples of carrageenan, representing a family of linear, sulfated galactans contained in extracts of red seaweed species. Quantitation of different carrageenan types was based on the resonances of the α-anomeric protons in the region from 5.1 to 5.7 ppm. Field strengths of 400−600 MHz were necessary to produce sufficient dispersion for obtaining reliable results. In a similar approach, Tojo and Prado were able to analyze nondestructively by qHNMR batches of intact carrageenans and to distinguish and quantitate κ-, ι-, and λ-carrageenans.[134] To determine the 5-*N*-acetyl neuraminic acid (Neu5Ac) level in lipopolysaccharides (LPSs), Bauer et al. investigated *O*-deacylated LPs (LPS-OH) from different pathogenic *Haemophilus influenzae* and *H. ducreyi* strains using qHNMR.[135] Due to the Neu5Ac detection limit of ca. 2%, their method was of limited use, however, due to the relatively homogeneous mixtures of silylated glycoforms in the LPS-OH samples.

Among medicinal plants, so far ginkgo has been the most attractive target for qHNMR analysis. The first reported qHNMR method to determine the terpene trilactones in *Ginkgo biloba* dates back to 1993.[136] Using a preseparation method, van Beek et al. were able to quantitate five trilactones based on their H-12 olefinic proton signals using a 200 MHz spectrometer.[136] The authors estimated that within a 30 min total experiment time they were able to quantitate ca. 0.1 mg of each lactone and concluded that the method was as sensitive as HPLC with refractometric detection, but with the advantage of not requiring pure trilactone reference substances. Later, the method was modified by Lichtblau et al. and employed to optimize the extraction yield and purity of terpene trilactone preparations used as herbal dietary supplements.[137] A rapid method for the quantitative analysis of bilobalide (**6**) and ginkgolides [e.g., ginkgolide A (**7**)] from harvested *Ginkgo biloba* leaves, but also in commercial *Ginkgo* herbal products, without any chromatographic separation by qHNMR was reported by Choi et al.[84] The authors laid the groundwork for further study by performing an evaluation of the optimum qHNMR solvent that yielded the best signal resolution. As a result of this, experiments were performed in a mixture of acetone-d_6 and benzene-d_6 (50:50) analyzing the singlet signals of H-12 of each of the lactones, which were well separated in the range 6.0−7.0 ppm. The quantity of the compounds was calculated by the relative

ratio of the intensity of each compound to a known amount of the internal standard, phloroglucinol (25 μg/mL). The NMR spectra were measured on a 600 MHz instrument. Very recently, Li et al. extended qHNMR methodology to the simultaneous analysis of ginkgolides and flavonoids and have addressed problems due to limited extract solubility and the degradation of the internal standard phloroglucinol.[85] As a result, the authors proposed a modified procedure with an altered solvent mixture (a 65:35 mixture of methanol-d_4 and benzene-d_6) and a different internal reference compound (1,3,5-trimethoxybenzene).

A combination of fingerprint and semiquantitative analyses was used by Bilia et al. to analyze the complex matrixes of herbal drug preparations such as from *Piper methysticum* (kava-kava),[81] *Hypericum perforatum* (St. John's wort),[138] and *Arnica montana* (arnica).[139] Although quantitation was only a minor aspect of these studies, the suitability of qHNMR to address questions of extract stability as exemplified for the unstable *Hypericum* phloroglucinol derivatives such as hyperforin (**8**) was clearly pointed out.[140]

qHNMR of Complex Dietary Mixtures. Catchpole et al.[97] reported the extraction of the hot food plants ginger, black pepper, and chili pepper powder using near-critical carbon dioxide, propane, and dimethyl ether. The pungency of the extracts was determined by a qHNMR technique using an aliquot of each extract, dissolved in $CDCl_3$ with 0.05% dimethylformamide as an internal standard and TMS as a chemical shift reference. NMR spectra were measured at 500 MHz. For spectral analysis, a macro was written that located and integrated the indication peaks for capsaicin (**9**, 2H doublet at 4.351 ppm), 6-gingerol (**10**, 3H singlet at 3.871 ppm), and piperine (**11**, 1H doublet at 6.452 ppm). These integrals were compared to the integrated value of the dimethylformamide singlet at 8.02 ppm and enabled the quantitation of the pungent principles.

The quantitative determination of (−)-epicatechin (**12**) and chlorogenic acid (**13**) in cider apple juices by qHNMR was presented by Berregi et al.[86,87] Compunds **12** and **13** were determined by quantitating signals at 7.05 and 7.20 ppm, respectively. The addition of 1,3,5-benzenetricarboxylic acid to the juice as an internal standard allowed the determination of the concentration levels of both compounds. The analyses were performed on a 500 MHz instrument and statistically evaluated for precision, repeatability, and reproducibility, to demonstrate qHNMR applicability in an industrial setting.

Knothe et al.[141] reported a procedure for the determination of the fatty acid profile of triacylglycerol mixtures in vegetable oils and the methyl esters of oleic, linoleic, and linolenic acids by ^{1}H NMR spectroscopy with a 400 MHz instrument. The signal of the terminal methyl group of linolenic acid is shifted downfield from the corresponding signal in the other fatty acids investigated, permitting their separate integration. The integration values of the signals of the allylic and bis-allylic protons allowed the quantitation of oleic and linoleic acid. There are numerous qHNMR applications in the analysis of milk and dietary products, which have been summarized in a review by Belloque and Ramos.[142] However, due to spectral complexity, ^{1}H NMR applications so far are limited to the determination of the proportion of liquid to solid fat and micellar disaggregation, observed through relaxation-based mathematical and analysis enhancement of signal intensity of Ca-depleted micellar casein, respectively.[142]

qHNMR of Complex Alkaloid Mixtures. A qHNMR method for strychnine (**14**) and brucine (**15**) in *Strychnos nux-vomica* seeds and stems was developed by Frederich et al.[143] and accomplished the quantitation of the alkaloids in the crude plant extract. In a study of pyrrolizidine alkaloids, the composition of six Brazilian *Senecio* species was investigated and the alkaloids were quantified by both ^{1}H and inverse-gated decoupling ^{13}C NMR spectroscopy by Krebs et al.[144] Seasonal and local differences were found in the pyrrolizidine alkaloid composition, as determined by qHNMR at 300 MHz. Naqvi et al.[83] reported the analysis of the tropane alkaloids, atropine (**16**) and scopolamine (**17**), in Solanaceous plants by a simple, accurate, and specific ^{1}H NMR spectrometric method, which is based on a comparison of the integrated peak areas of the N-CH_3 protons of both compounds with the sharp singlet of the internal standard 1,4-dioxane at 3.5 ppm. Another precise and specific ^{1}H NMR method for alkaloids was developed to assay papaverine hydrochloride as a bulk drug as well as in injection dosage forms at 270 MHz.[145] The assay depends on the integration of the 12 protons of the four methoxy groups relative to that of the three methyl protons of the internal standard, acetanilide.

To conclude the metabolic and complex natural products application section of this review, two concepts with future potential for qHNMR applications shall be mentioned. First is the use of NMR as a hyphenated technique with chromatography, either on-line or off-line, as a means of overcoming the overwhelming complexity of metabolomic samples. An important factor in this context is that dereplication of target analytes can be achieved, and quality NMR reference data are available for this purpose, as could be recently demonstrated for liquid food products.[146] Second, with the availability of high-resolution solid-state NMR (HR-MAS), qHNMR also has potential for those natural product samples that represent an unusual challenge because of their physicochemical characteristics (insolubility, solids, semisolids). As was shown by Gil et al., HR-MAS qHNMR allowed the semiquantitative fingerprinting of the overall biochemistry (e.g., sugars, organic acids, amino acids) of mango pulps at different ripening stages.

Decoupled qHNMR. Published reports on ^{13}C decoupled qHNMR measurements are sparse, although the reduction in ^{1}H spectral complexity at the 1% signal level would be an obvious application. The aforementioned study by Schoen et al.[108] of perchloric acid extracts of infected *Tenebrio molitor* beetles used ^{13}C decoupled ^{1}H spin−echo sequences, with and without ^{13}C population inversion. From their qHNMR work, Meusinger et al.[147,148] described the simultaneous qualitative and quantitative determination of single components present in petrochemical samples at levels between 1 and 17%. All analytes were quantitated from the ^{13}C decoupled ^{1}H NMR spectra to eliminate ^{13}C satellite signals. The chosen experimental condition for decoupling comprised the GARP[69] pulse sequence in the inverse-gated mode and long (200 μs) "soft" decoupler pulses at high attenuation levels (20 dB).

Hyphenated qHNMR. Hyphenation of chromatography with NMR (e.g., LC-NMR) has been successfully added to the toolbox of the analytical chemist in the recent past,[149−153] but is still not widely available and still limited in terms of sensitivity compared with regular nonflow probes. On the other hand, evidence is growing that NMR alone offers sufficient dispersion to yield meaningful and sensitive results. It is reasonable to assume that the combination of separation (chromatography) and NMR will offer the most comprehensive quantitative analytical picture ("fingerprint") of a given material. This, however, does not neces-

sarily imply that hyphenated NMR techniques have to be used all the time. Instead, qHNMR can benefit from the much-increased sensitivity of modern probe design (cryoprobe, microprobe, nano/capillary probe technology)[14,24,154−158] when used in "off-line" combination with separation technology.

On-line HPLC-qHNMR at 600 MHz was applied for the first time to the analysis of environmental samples after solid phase extraction by Godejohann et al.[159] The results were compared to those obtained by HPLC-photodiode array analysis, demonstrating that hyphenated qHNMR was capable of identifying more compounds as a result of coelution of major and minor components in the HPLC chromatogram. This example emphasizes the potential of NMR to resolve coeluting compounds, which represents a common problem in the analysis of natural products. In other words, (q)NMR can add another dimension of separation to chromatography.

Fischer et al.[160] proposed the hyphenation of supercritical fluid chromatography (SFC) with qHNMR. Due to the lack of disturbing proton signals, supercritical CO_2 is an excellent solvent for ^{1}H NMR spectroscopy. As a result of decreased viscosity, ^{1}H T_1 relaxation times of analytes in supercritical fluids are 3−10 times longer than in liquid solvents. Therefore, synthetic immobilized free radicals were used to shorten the spin−lattice relaxation times and to obtain NMR spectra that could be integrated. The method was substantiated with examples quantitating phthalate ester mixtures on a 400 MHz spectrometer. Maiwald et al.[161] recently reported a study of flow NMR spectroscopy in reaction and process monitoring. On-line NMR, using 400 MHz instruments, allowed investigation of reaction processes almost in real time and under process conditions in a wide range of temperatures and pressures. For many engineering and physicochemical applications, such a "noninvasive" analytical technique is desirable since process conditions are not disturbed.

Stereoisomeric qHNMR. ^{1}H NMR spectroscopy can also be applied for the quantitation of stereoisomerism, such as the epimeric compositions of a sample. Chiu et al.[162] reported a chemotaxonomic study of the sterol composition of bryophytes. The epimeric compositions of 24-methylcholesterol (**18**), 24-ethylcholesterol, and 24-ethyl-5,22-cholestadienol were analyzed by comparing the ^{1}H NMR spectra at 220 MHz with those of authentic standard mixtures. The data indicated that the 24-methylcholesterol contained in all the tested bryophyte species was actually a mixture of the 24α-epimer campesterol and the 24β-epimer 22-dihydrobrassicasterol. The 24α-epimers accounted for 20−80% of the methylcholesterol fraction. Epimeric mixtures were also found in the 24-ethylcholesterol fraction. Although sitosterol, the 24α-epimer, was the only epimeric form of 24-ethylcholesterol in some species, 10−40% of the 24β-epimer, clionasterol, was found in some bryophyte species. The 24α-epimer stigmasterol was the only epimeric form that existed in all of the studied bryophytes. In conclusion, qHNMR analysis supported the phylogenetic placing of the bryophytes between the thallophytes and the tracheophytes.[162]

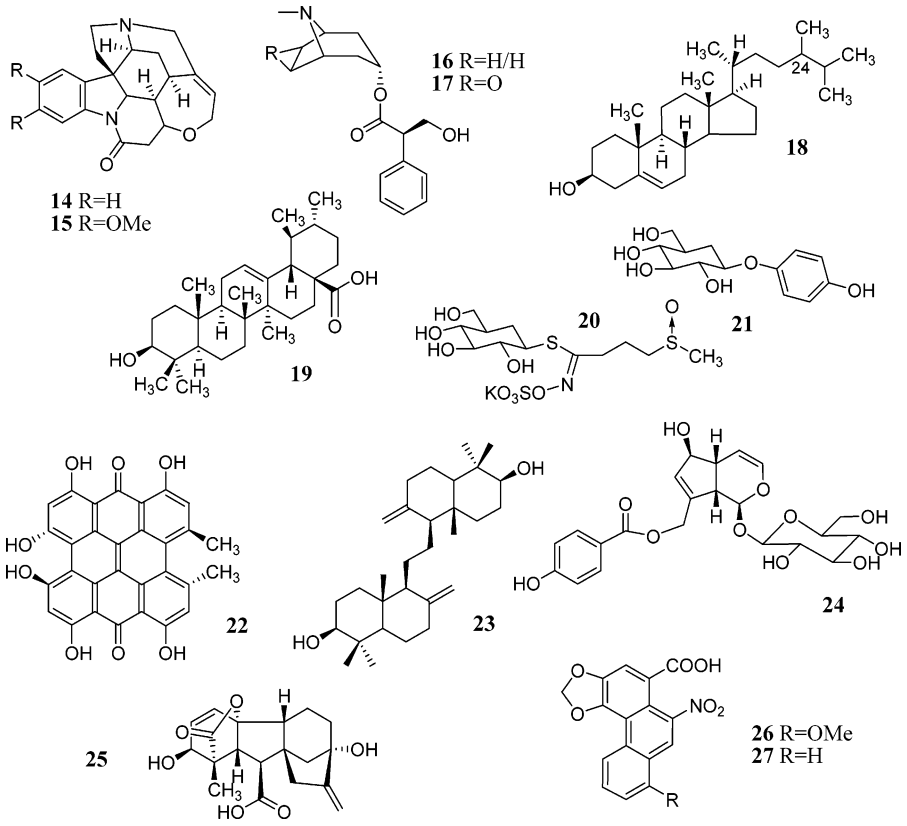
^{1}H NMR spectroscopy has further been applied for assessing the enantiomeric composition of chiral natural or synthetic drugs in preparations. Thunhorst et al.[163] described the chiral analysis by means of ^{1}H NMR of phenylethylamines with the shift reagent heptakis(2,3-diacetyl)-β-cyclodextrin. Several reports on this subject were also published by Hanna and colleagues at the FDA between 1989 and 2001, describing the determination of the enantiomeric purity/composition with the aid of chiral lanthanide shift reagents (LSR), containing lanthanum (La) or europium (Eu) as Lewis acids, of tramadol hydrochloride,[164] indacrinone,[165] tranylcypromine sulfate,[166] threomethylphenidate,[167] chlorpheniramine maleate,[168] timolol maleate,[169] ibuprofen,[170] prilocaine,[171] propranolol,[172] and bupivacaine.[173]

Regarding cis/trans stereoisomerism, Deubner et al.[80] reported a method for the qHNMR spectrometric determination of the *E*/*Z*-isomer ratio of the antidepressant drug fluvoxamine and were able to determine *E*/*Z*-isomeric impurities down to the 0.2% level in a 15 mg sample with a 400 MHz instrument requiring less than 30 min acquisition time. Interestingly, although the application of lanthanide shift reagents is well established in natural product chemistry,[169,174,175] we are aware of only one recent publication applying a chiral variant of LSRs for chiral qHNMR analysis of natural products.[176]

Reference Materials and Regulatory qHNMR. The worldwide increase in regulations governing the various aspects of the pharmaceutical and environmental sectors, which require increased efforts toward validation of analytical and pharmacological reference standards, has recently made qHNMR one of the essential tools for the analysis of these reference materials. The use of qHNMR proves to be both simple and highly applicable for the determination of quantitative purity of isolates, impurity or metabolic profiling, and the quantitation of single entities in complex mixtures. In comparison with traditional methods, such as chromatography, qHNMR is a universal, nondestructive technique and can be much more precise and reliable, as shown in a recent study investigating different samples of ursolic acid (**19**), which lacks a chromophore or other physicochemical properties required for chromatographic detection.[177] Another concern that particularly applies to natural products, and for which qHNMR can offer analytical solutions, is the limited availability of highly pure certified reference materials. Other complicating factors are that very often natural products are hygroscopic and present in unknown salt forms. One such example is phycotoxins. As demonstrated by Walter et al., they can be easily monitored over a wide range of concentrations using qHNMR.[82,132]

A study published by Maniara et al.[178] evaluated the method, performance, and validation for ^{1}H and ^{13}P NMR quantitative analysis, including the investigation of experimental precision, accuracy, specificity, linearity, limits of detection and quantitation, and ruggedness of the method. The authors concluded that qHNMR rivals chromatography in sensitivity, speed, precision, and accuracy. The level of the major chemical ingredient could be determined with accuracy and precision significantly better than 1%, and impurities could be quantified at the 0.1% level or below. Very recently Soininen et al. demonstrated that ^{13}C GARP decoupled qHNMR, combined with a total-line-shape fitting routine, significantly improved the quantitation of signals emanating from minor components. The method allowed determination of salicylic acid and ethanol impurities in vanillin with a limit of quantitation of 0.05 mol % and a limit of detection of 0.016 mol %.[179] Improved quantitative reference validation can be achieved by a qHNMR method as proposed recently,[15] by focusing on the quantitative evaluation of fingerprints of both the analyte and its impurities. An example of a comprehensive qHNMR-based reference compound analysis is a recent report on the evaluation of glucoiberin (**20**) reference material from *Iberis amara*.[96] Two methods of the qHNMR

Chart 2



concept were applied for quantitation: the relative reference method representing a "semi"-absolute quantitation, and the 100% integral method, which is comparable to quantitation by relative peak areas in chromatography. Both methods permitted the determination of the compound content (% purity) and the exact amount, while simultaneously providing information about the probable nature of the impurities. Using the relative reference method, other natural product reference materials investigated by qHNMR were arbutin (**21**),[94] oligomeric proanthocyanidins,[95] hypericin (**22**), and hyperforin (**8**) from St. John's wort,[180] α-onocerin (**23**),[181] and agnuside (**24**) studied together with the quinic acid derivatives chlorogenic acid (**13**) and tetragalloyl quinic acid.[15] The spectra were measured at field strengths of 300−400 MHz. The use of higher field strengths becomes necessary only when analyzing compounds with extensive overlapping spectral patterns. One example, the triterpenoid aglycon ursolic acid (**19**), had to be analyzed at 500 MHz. The qHNMR impurity profiling provided evidence for the presence of several nontriterpenoid impurities, present in different commercial batches of this widely used natural reference compound.[182]

Quantitative impurity profiling of technical grade agrochemicals was performed by Wells, Al-Deen, and coworkers.[89,90] Although only indirectly related to natural products, Wells' method should be mentioned, because it permitted the full assignment of the NMR spectra of 2,4-dichlorophenoxyacetic acid, sodium-2,2-dichloropropionate, gibberellic acid (**25**), metsulfuron, clopyralid, thiram, iprodione, dicamba, and methabenzthiazuron as well as their related organic impurities in technical grade samples. The normalized percentage impurity method, as well as an assay employing the internal standard method using dimethyl sulfone, revealed that the qNMR techniques at 600 MHz constitute the more precise and accurate methods of analysis than commonly employed chromatographic methods. Thus, the purity of the herbicide glyphosate and the insecticide profenofos was determined by ^{1}H and ^{31}P qNMR.[89,90] Sodium acetate and sodium phosphate of known purity were selected as internal standards. An impurity profile and the quantitation of the impurities of both compounds could also be established.

Hanna[183] reported a qHNMR method for the simultaneous determination and characterization of the nephrotoxic components aristolochic acids I (AA-I, **26**) and II (AA-II, **27**) originating from plants of the genus *Aristolochia* utilizing a 400 MHz NMR spectrometer. The quantity of the toxins was calculated on the basis of the integrals for the signals of H-7 and H-8 of the phenanthrene ring of AA-1 and AA-II at 7.38 and 8.31 ppm, respectively, and the vinyl protons of the internal standard maleic acid at 6.06 ppm. The accuracy of the method was established through the analysis of "synthetic mixtures" containing the weighed internal standard maleic acid and containing purified AA-I or combined AA-I and AA-II sodium salts.

Finally, application of qHNMR can play an important role in the analysis of pharmaceutical preparations of natural and synthetic drugs. Hanna et al. reported two qHNMR methods pertaining to the evaluation of drug (API) content and stability testing in pharmaceutical preparations of natural products, e.g., quinidine, dihydroquinidine[184] in tablets, and ephedrine, pseudoephedrine, and norephedrine[185] as mixtures in bulk material. Guerrini et al.[186] reported a qHNMR method to distinguish, characterize, and quantitate the sulfatation patterns (sulfate esters) of pig and bovine heparin preparations and contaminants from industrial processes. In a recent report, Talebpour et al.[78] proposed a method utilizing qHNMR to confirm the identity and quantity of the catecholamines levodopa, carbidopa, and methyldopa in human serum and in pharmaceutical preparations. The method is based on 500 MHz proton NMR. For each of the catecholamines characteristic chemical shift positions exhibiting minimum overlapping were selected: levodopa 6.74−6.91, 3.12−3.29, 4.32−4.35

ppm; carbidopa 6.68−6.89, 2.96−3.18, 1.57 ppm; methyldopa 6.70−6.91, 3.02−3.30, 1.68 ppm. Experiments were performed to validate the qHNMR method, and the linearity and reproducibility of the proposed method were verified.

A method to be used at the laboratories of the Drug Enforcement Administration (DEA) was developed by Meyers et al.[82] to analyze controlled substances, in this case the methamphetamine and ephedrine contents, in evidentiary samples. These samples ranged from pure drugs to mixtures containing a variety of drug and nondrug additives. The use of ^{1}H and ^{13}C NMR provides easy identification, e.g., of cocaine and its derivatives, while qHNMR allows for the quantitation and profiling of the drug mixtures. The use of qHNMR was also successfully applied to several synthetic drugs in pharmaceutical preparations. Relevant examples are bethanechol,[187] furosemide,[188] chlorpheniramine maleate,[168] dicyclomine hydrochloride,[189] propantheline,[190] diphenhydramine hydrochloride,[191] iopamidol and iothalamalate meglumine,[192] azathioprine,[99] and methocarbamol.[193]

Concluding Remarks. From the surveyed literature and current conference communications summarized here, it has become evident that qHNMR possesses the required accuracy and precision to readily become a routine quantitative tool in many analytical laboratories and, further, has great potential for widespread applications in natural products laboratories. In view of recent reports on successful automated qHNMR installations in industrial laboratory environments employing robotic sample preparation,[77] and considering the available body of existing experimental methods, routine qHNMR evaluation of samples is no longer a technological challenge. One of the distinctive characteristics of qHNMR lies in its ability to characterize natural products, just as any organic material, routinely and efficiently by providing simultaneous qualitative and quantitative information. For the reasons described in this review, and by analogy to suggestions made for synthetic molceules,[26] the authors feel that the enormous latent potential of qHNMR will be realized once it is implemented into the routine course of natural products research methodology. The only crucial prerequisite relates to the intelligent application of appropriate data acquisition parameters and likewise appropriate postacquisition processing treatment ("quantitative conditions") when dealing with qHNMR. As far as materials from natural sources are concerned, qHNMR is easy, efficient, and beneficial to implement, from the level of crude products/extracts to final purified natural compounds and reference materials.

As a rule of thumb, qHNMR provides an opportunity for the routine determination of constituents at the 1% level with 1% error and is well capable of analyzing possibly down to the 0.1% level and below with errors as low as 0.1%, depending on the nature of the sample. Because qHNMR methodology is based on a unique physicochemical mechanism, as compared to quantitation by chromatographic techniques, qHNMR offers a more critical view when compared to traditional methods of chemical composition and purity analysis, which tend to overestimate purity.[15,26,176,182] Most importantly, since the *singleton character ideal* discussed in the Introduction is particularly vulnerable in the case of natural products, as opposed to synthetic entities, the opportunities offered by qHNMR should not be missed. Considering that substantial resources are routinely devoted to the NMR analysis of natural products, and taking into consideration the body of evidence compiled in this review, it is reasonable to propose qHNMR as a routine analytical method for natural product analysis. Besides, this comes at almost no extra cost since only the following ingredients are required: accurate weighing/dosing of the sample, adoption of qHNMR conditions (pulse sequences and parameters, *vide infra*) for routine ^{1}H NMR spectra, and proper postacquisition data processing. Software tools for the latter are provided by the manufacturer of the NMR equipment, as well as being widely available for personal computers (see ref 194 for a review).

In summary, qHNMR has enormous potential in the identification, characterization, and discovery of bioactive natural products and in the area of metabolome analysis, which together will mutually drive the future development of natural products research.[195]

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Supporting Information Available: Pulse profiles and magnetization figures of NMR experiments relevant to the qNMR methodology. This material is available free of charge via the Internet at http://pubs.acs.org. Additional information on this subject can be found at http://qnmr.org.

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