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qNMR in natural products: practical approaches. What nobody tells you before starting your qNMR study!

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NMR (Nuclear Magnetic Resonance) spectroscopy is a well-established technique for elucidating the structure and composition of Natural Products (NP). However, while NMR spectra offer qualitative insights, there is often a growing demand within the NP research community for quantitative analysis using NMR techniques. Unfortunately, this demand is sometimes met with misconceptions and lacks the methodological rigor typically associated with analytical procedures, particularly concerning validation and mandatory adjustments of quantitative NMR (qNMR) parameters. Therefore, this minireview highlights the practical approaches concerning preparation and validation for qNMR analyses of plant extracts. Specifically, we provide a concise protocol detailing experimental settings crucial for ensuring the accuracy and reliability of quantitative measurements. It is noteworthy that such guidance is often absent in existing literature, and our minireview wants to bridge this gap by offering clear methodological recommendations.

KEYWORDS

^{1}H qNMR, qHNMR, quantitative nuclear magnetic resonance, plant extracts, validation, quantification, secondary metabolites

1 Introduction

Quantitative Nuclear Magnetic Resonance (qNMR) is a primary method of measurement for quantifying substances (King, 2000a; King, 2000b; Malz and Jancke, 2005) and has been increasingly utilized over the years. Concerning the application of qNMR in organic compounds quantification, ^{1}H, ^{31}P, ^{19}F, and ^{13}C nuclei are widely used, and the definition of experimental NMR parameters is beyond the lab routine acquisition of NMR spectra. In the Natural Products (NP) field, qNMR applications usually cover both target metabolomics (Ramos et al., 2019; Rebiai et al., 2022) and determination of the content of bioactive compounds (Yu et al., 2020; Zhao et al., 2022), with quantities of substances based on the mass of dried plant extract (mg/g of dried extract) or based on the mass of the analyzed plant material (mg/g of plant).

Considering a recent search on ScienceDirect online database using different combinations of the keywords “qNMR”, “plant”, “natural products”, “quantitative NMR”, and “qHNMR”, the results revealed that the majority of papers reports some of the NMR parameters important for quantitative analysis, such as acquisition time and relaxation delay. These two parameters are extremely important for a quantitative analysis

using NMR spectroscopy. It is mandatory to use sufficiently high acquisition time and relaxation delay to guarantee the complete longitudinal relaxation (T1) of the nuclei which are responsible for the NMR signals selected for the quantification. However, most of the papers found in the literature do not report either T1 estimation, usually performed by the inversion-recovery experiment, or validation data to increase the reliability of the results. Thereby, the data bank search demonstrates the gap between the expectation of applying an NMR quantification method and obtaining it adequately, indicating the importance of a joint approach to approximate the quantification expectations of NP researchers with the language of the NMR spectroscopists. This joint approach can facilitate the development of projects involving qNMR and NP, allowing to achieve more reliable results, showing the required analytical rigor, to make better discussions and to publish paper with excellent quality, such as publication of Pauli et al., 2005; Pauli et al., 2012; Thapa et al., 2022.

Therefore, this minireview aims to bring some practical details to perform the ^{1}H qNMR method in NP samples, including sampling plants, extraction preparation, qNMR parameter adjustments, and validation. In addition, this minireview brings a brief protocol for helping NP researchers to organize their experimental designs for application of qNMR in their NP works. Subsequently, we shall elucidate critical aspects frequently overlooked in scientific papers yet of paramount significance for starting a qNMR study.

2 Practical approaches for qNMR in natural products

2.1. Sampling plants for qNMR investigation

Sampling approach for qNMR investigation in Natural Products typically follows the same criteria as sampling for metabolomic studies, among them: 1) sample acquisition, involving the collection of plants in their natural state followed by immediate cryogenic freezing in liquid nitrogen (Salem et al., 2020), or the acquisition of plant material, particularly in the case of studies involving commercial samples, such as herbal medicines; 2) statistical representativeness, considering the balance between the number of analytical and biological replicates; 3) analytical rigor in the weighing of material for extract preparation, as well as in processes such as grinding/pulverization of the plant material, among others. In all these cases, the decision on whether plants are processed *in natura* or after drying depends on the specific objectives of the study. This multifaceted decision-making process ensures that the sampling strategy aligns with the research proposal and optimally addresses the analytical and biological aspects inherent in qNMR investigations in NP.

In this minireview, we aim to raise a specific inquiry regarding sample representativeness, as it is common to observe analyses conducted on pooled individuals in qNMR studies applied to NP area (Chauthe et al., 2012; Tan et al., 2020). Depending on the research objectives, sampling that aggregates diverse individuals may indeed be a good strategy, resulting in a reduced number of analytical replicates, because biological diversity is reduced into a pooled sample. Conversely, these practices are not observed in studies with other types of samples out of NP area, such as in metabolomics including plasma/serum or urine analyses, where samples from various individuals can never be combined to present results for the population studied. Regarding this sampling difference, it seems provocative to consider that the use of pooled samples in the NP area reduces the sampling Universe too much and, whenever possible, it would be interesting to avoid it.

In this context, it appears more logical that in NP studies, quantification of the analyte in each biological replicate is performed. This approach facilitates the demonstration of sample variability among the studied groups and, ultimately, allows for the presentation of statistical significance for the identified differences/similarities. Thus, an effective sampling should encompass diverse individuals (biological replicates) and some analytical replicates for each individual (different extracts/spectra for the same specimen). This comprehensive approach ensures a robust evaluation of both the biological and analytical variability within the study, enabling a more detailed interpretation of the results obtained.

2.2 Plant extraction: "tips and tricks"

Obtaining plant extracts is crucial to access the plant material composition for quantification of the components of interest. It is important to decide which solvent or solvent system is suitable for each specific case, as NP researchers well know (Deborde et al., 2019; Abubakar and Haque, 2020; Verpoorte et al., 2022; Bitwell et al., 2023). The importance of solvent choice is because signal position might change depending on the solvent. Additionally, some solvents could improve extraction and dissolve the extracted chemicals better.

For quantitative purposes, we must consider two different scenarios to present the results: 1) based on the mass of dried extract, or 2) based on the mass of the analyzed plant. In both cases, it may be more effective to use a small mass of the plant (between 50 and 200 mg) for testing. This amount is suitable for obtaining an extract that is completely soluble in 600–700 µL of deuterated solvent for qNMR analyses. However, the potential effect of heterogeneity in the distribution of metabolites within the plant when such a small sample is analyzed might lead to biased results. Therefore, it is crucial to start with a homogeneous plant sample. This can be achieved by pooling the studied plant tissue and ensuring the material is ground, finely divided, or even sieved when possible (Deborde et al., 2019; Ramos et al., 2019). In large extracts (made from a greater quantity of plants), only a small portion can be satisfactorily solubilized, which is far from the optimal strategy. In addition, to work with a large plant extract with the intention of resuspending a small portion of the extract can be tricky. The solid material formed during the freeze-drying processes of a large liquid extract can be non-uniform due to differential deposition of the material, besides the texture of the extract may hinder the effective weighing of the material. In this case, it is extremely important to completely homogenize the dried extract before resuspending it.

If the goal is to express the quantification of the substance based on the mass of the analyzed plant (e.g., mg of the analyte/g of plant) and not based on the mass of dried extract, exhaustive extraction is required (Martins et al., 2016; Owczarek et al., 2019). In practice,

successive extractions should be performed and combined before drying (which can be done in a fume hood, using gaseous N_2, or by freeze-drying, depending on the case). The use of a rotary evaporator can be also an alternative, especially when a large amount of extract is prepared.

A tip for obtaining exhaustive extraction is to perform a pilot with a minimum of seven consecutive extractions without combining the material. ^{1}H NMR spectra of each resuspended fraction should be measured to determine the last sequential fraction that no longer shows the analyte signals. In practice, to determine the number of sequential extracts needed to complete extraction of the analyte from the plant material, it would be interesting to consider the first fraction without it. Based on the pilot experiment, it is recommended to pool all the sequential fractions before evaporating the solvent when the final experiment is accomplished. The combined extract will contain the total amount of the analyte (or of the mixture of analytes, when applicable) in the portion of plant analyzed (qNMR results, in this case, can be expressed as mg of each analyte per g of plant).

Another inevitable point, especially in exhaustive extractions, although also important in a single extraction, is the need to check if the final combined extract can be dissolved in the recommended deuterated solvent volume (~600 µL). The presence of insoluble material can compromise the presented result of analyte mass per plant mass. Additionally, it is recommended that the extract must be dissolved in a slightly larger volume (~700 µL) of deuterated solvent, followed by centrifugation to ensure the deposition of any suspended particulate material. After that, the resuspended solution must be transferred with a slightly smaller volume (~600 µL) to the NMR tube. However, in quantitative calculations, never forget to consider the total volume of the resuspended solution when expressing in mass (g) rather than molar concentration (mmol/L)!

In the pilot experiment stage, it is important to remember to test the stability of the analyte in solution during the time required for the analyses. It is usual to prepare a batch of samples and it is important to remember that the NMR acquisitions are made one by one. A ^{1}H NMR experiment acquired a few hours after sample preparation can reveal whether the conditions are adequate (solvent choice for resuspension, for example,), as well as the permissible waiting time between extract resuspension and the beginning of the acquisitions.

2.3 qNMR parameter adjustment

Even though ^{1}H and ^{13}C nuclei are usually common in NP samples, the choice of the quantifiable nucleus depends on the NMR sensitivity and the amount of the interested substance in the plant extract. For this reason, ^{1}H qNMR is more common and more applicable in NP studies (Gödecke et al., 2013). Herein, all discussions will be based on ^{1}H qNMR analyses. Another important matter is to know undoubtedly the complete NMR spectral assignment of the analyte to select a free and non-overlapped quantifiable ^{1}H NMR signal (see selectivity and specificity, item 2.4).

Thereafter, it is essential to define which qNMR method to use (Schönberger et al., 2023): internal calibration (a standard substance together the sample, inside the NMR tube, with the application of the qNMR equation) (Westwood et al., 2019; Choi et al., 2021; Solovyev et al., 2021; Wang et al., 2021) or external calibration (calibration curve or electronic methods). Considering that the NP samples have a highly overlapped ^{1}H NMR spectrum, it is sometimes challenging to find an internal standard with quantifiable ^{1}H NMR signal fitted in a free spectral region. On the other hand, external calibration by a calibration curve with different analyte concentrations has been less used in recent papers (Choi et al., 2021; Nishizaki et al., 2021). Thus, electronic methods are important alternatives, such as pulse length–based concentration determination (PULCON) (Monakhova et al., 2014; Monakhova et al., 2015). PULCON is also referred as ERETIC2 by Bruker company. In fact, ERETIC2 is an electronic calibration that functions as an external standard method applying PULCON (Paniagua-Vega et al., 2019; Parra-Naranjo et al., 2022). Therefore, the PULCON method is usually desirable for qNMR determinations in complex matrices, such as NP samples. For more details about PULCON (or ERETIC2, in a Bruker jargon/language), please see Burton et al., 2005; Wider and Dreier, 2006; Cullen et al., 2013; Garrido and Carvalho, 2015; Giraudeau et al., 2014; Wang et al., 2021.

The longitudinal relaxation (T1) for the interested signal is an extremely important NMR parameter in a quantitative study. It is indispensable to determine the 7*T1 (seven times the longitudinal relaxation time) for the quantifiable ^{1}H NMR signal, since quantitative NMR parameters depend on 99.93% of recovery of magnetization (at least) between the scans (Wang et al., 2023). Ideally, the T1 estimation experiment using inversion-recovery pulse sequence should be performed using the real samples in different molar concentrations (e.g., concentrated and diluted samples prepared from the plant extract resuspended in the chosen deuterated solvent), because the T1 time the different protons in one compound can differ; this holds also true for the T1 of the same proton measured in different solvents and different concentrations. Obviously, *acquisition time* + *relaxation delay* ≥*7*T1* is a *sine-qua-non* condition for all qNMR studies. However, how to decide the distribution of 7*T1 in acquisition time and relaxation delay? One point of view is to guarantee the complete FID decay to obtain a good spectral resolution. On the other hand, FID extremely long includes noise in the processed spectra, reducing the signal-to-noise (S/N) ratio. Therefore, the best cost-benefit can be found about 2–4s for ^{1}H nucleus (Malz and Jancke, 2005; Bharti and Roy, 2012), the remaining time to complete 7*T1 depends on the relaxation delay, in most of the observed studies (Wang et al., 2023).

Concerning the choice of the pulse sequence as a fundamental part in qNMR method development, it is important to avoid pulse sequence for solvent signal suppression whenever is possible. Common solvent suppression techniques, such as presaturation, are not advisable in quantitative studies because of their lack of frequency selectivity, which can cause a partial suppression of the interested signal (Maiwald et al., 2008). However, there are more selective suppression methods available in two main categories of experiments: those which selectively eliminate the water signal, and those which selectively excite or refocus the signals of interest (Giraudeau et al., 2015).

TABLE 1 Some suggested ^{1}H qNMR parameters based on important reviews on the literature (Malz and Jancke, 2005; Pauli et al., 2005; Bharti and Roy, 2012; Westwood et al., 2019) for spectral acquisition and processing in organic compounds.

NMR parameter	Suggested values, based on literature[a,b,c,d]	Additional comments
Acquisition		
flip angle	90[a,b,d]	90° degree pulse angle leads to the maximum signal intensity with respect to the measurement time spent (Schönberger et al., 2023)
acquisition time	2–4s[b,c] >2.5s[d]	This value will depend on: 1) T1 for quantifiable signals; 2) number of points acquired and spectral width (*Acquisition time = number of points acquired/2*spectral width*); 3) flip angle (in case the flip angle is below 90°, see Ernst angle[a,c]). Remember: longer acquisition time must be avoided to prevent compromising the S/N ratio
relaxation delay	*7*T1 - acquisition time = relaxation delay*	Considering *acquisition time + relaxation delay* ≥*7*T1* in quantitative condition, relaxation delay must be sufficient to complete relaxation It is important to note that *acquisition time + relaxation delay* ≥*7*T1* is a condition for a 90° flip angle. If a pulse angle lesser than 90° is used, a proportional *acquisition time + relaxation delay* is required to achieve complete relaxation
pulse width	dependent on the pulse calibration	It is essential to calibrate the 90° flip angle (*pulsecal*) before starting the quantitative NMR parameters adjustment
number of points acquired	64k[a,c]	Good values of acquisition points are also extremely important for resolution of the ^{1}H qNMR experiments
spectral width	30 ppm	Larger spectral width facilitates phase and baseline corrections
transmitter offset	center of the spectral width[c]	In the case of the analyte signal having an extreme chemical shift, it would be better to set O1 close to the signal to be used for quantification
receiver gain	closely below the highest possible setting[a,c]	Firstly, check the receiver gain value automatically set by the spectrometer (*auto receiver gain* setting). Test concentrated samples to avoid overloading the receiver gain. We recommend keeping a constant receiver gain value for all samples
number of scans or transients	enough to reach a good S/N	The ns must be sufficient to reach S/N > 150 (ideally) It is important to manage together the total acquisition time, the amount of plant material, and extract solubility. Please see 3.3b section
signal-to-noise ratio (S/N)	>250[a] >150[b] >1000[d]	S/N > 150, ideally. However, sometimes it can be difficult to reach a good experiment time (number of scans/transients) in NP samples that usually have compounds in a very low concentration
Processing		
data size of real spectrum	64k	Use zero-filling (data size of real spectrum ≥ number of points acquired)
integration	20 times the peak width in both directions[a] 64 times the full-width at half-height[b] >60 times the full-width at half-height or ^{13}C satellite ±30 Hz[d]	The integral definition is one of the most important steps in a qNMR study. Put all spectra together (overlapped) to define the better integral range to avoid close signals. If ^{1}H-^{13}C satellites were considered for integral definition, use them for standard and analyte (but in complex spectra, it is probably difficult to include satellites)
exponential multiplication (line broadening values)	LB = 0.3[a,b,c] LB = 0.05[d]	Larger line broadening values improve the S/N, despite compromising the signal resolution and the definition of the integral area
phasing	automatic (e.g., *apk0.NOE*) followed by manual phasing	Can be interesting to start with automatic (e.g., *apk0.NOE*; Bruderer et al., 2021) followed by manual phasing. Do not forget to compare all spectra together (overlapped) to check similar phase processing
baseline correction	automatic (polynomial *n*th order)[c]	It is important to set the spectral baseline at zero in the scale of intensity to slightly avoid either negative or positive baselines. We also suggest to use *baseopt* (Bruderer et al., 2021) to standardize baseline corrections avoiding non-randomized errors

[a]Bharti and Roy, 2012.
[b]Malz and Jancke, 2005.
[c]Pauli et al., 2005.
[d]Westwood et al., 2019.

To ensure accurate measurements in ^{1}H qNMR, it is essential to perform a careful selection of integrals of both the analyte and the standard signals. This choice aims to ensure that the chosen integrals are free from interference (Table 1) (Bharti and Roy, 2012; Westwood et al., 2019). Common interferences include ^{13}C satellite signals from adjacent integrals, low-concentration impurities, and tautomer signals. Additionally, ^{13}C satellites can decrease quantification accuracy in complex matrices due to the risk of overlapping signals. An effective approach to identify and prevent this interference is decoupling of ^{13}C satellites, representing one of

the simplest strategies in this context (Bahadoor et al., 2021). ^{1}H-^{13}C-decoupled NMR pulse sequence (for example, zgig pulse sequence (Bruker language)) usually is an excellent option in complex matrices to avoid the ^{1}H-^{13}C satellites of other signals being overlapped with those interested (Pauli et al., 2012). Besides, the integral of the signal is centered in one single signal (without satellites) when ^{13}C-decoupled pulse sequence is employed in ^{1}H qNMR. In time, it is important to note that qNMR measurements are performed without NMR tube rotation, to avoid spinning side bands due to rotation, which could impair the measurement of the signal integral.

Regarding the flip angle definition, the most commonly used flip angles in qNMR are generally in the range between 30° and 90°. However, a 90° degree pulse provides maximum signal strength, improved signal-to-noise (S/N) ratio and reduced artifacts. The use of 90° degree pulse in quantitative NMR leads to the maximum signal intensity with respect to the measurement time spent (Schönberger et al., 2023). For example, in experiments using 30° degree pulses, to double the S/N ratio and achieve the same S/N ratio as with the 90° degree pulse, it would be necessary to perform four times more scans (transients). This makes experiments using 30° degree pulses more time-consuming than those using 90-degree pulses when the S/N ratio is a limiting factor. Additionally, according to the EUROLAB Technical Report 1/2023 (Schönberger et al., 2023), in qNMR, especially when using external calibration, the 90° degree pulse angle is usually preferred since this pulse angle provides the smallest deviation in signal intensity when the angle is not set entirely correctly.

Several other important NMR acquisition parameters adjustments are not individually discussed here, considering that they are similar to all qNMR studies. A useful summary including practical comments can be found in Table 1. Concerning the spectral processing, baseline correction is a particularly sensible step even more intricate in case of complex spectra such as for NP samples. Sometimes, an automatic phase correction (e.g., *apk0. noe*) followed by manual correction are suitable for obtaining good phased spectra. In all cases, a deep learning-based phase and baseline correction of 1D ^{1}H NMR spectra is recommendable (Bruderer et al., 2021) and a visual inspection of the results obtained (all spectra together, overlapped) is essential.

2.4 qNMR validation

qNMR results should be validated to guarantee that the quantitative data is reliable. In analytical studies, some essential figures of merit are shown in a quantitative study. Herein, we will discuss the following figures of merit for validation of qNMR method: specificity/selectivity, linearity, trueness/recovery, precision, limits of detection and quantification, and robustness. Useful information about definitions can be found in these references: VIM4 CD, 2021; Schönberger et al., 2023.

Specificity and selectivity: qNMR is intrinsically a specific and selective method since the interested signal for quantification appears in a free and non-overlapped spectral region. Thus, because NP samples often have a large number of overlapping ^{1}H NMR signals, the acquisition of 2D NMR experiments is essential to prove that there are no small signals overlapped to those interested ones. 2D NMR experiments, especially HSQC, TOCSY, and Jres, using a large number of scans are excellent options to prove the selectivity/specificity of an qNMR method.

Linearity: When quantitative NMR parameters are achieved (acquisition time + relaxation delay ≥7*T1), the NMR signal area is directly proportional to the sample concentration. Thus, qNMR is essentially a linear method (Schoenberger, 2019). Nevertheless, considering that linearity is a usual figure of merit shown in current analytical papers, it can be performed in qNMR for NP samples using a concentration range in which the analyte concentration is centered, similar to an calibration curve. Therefore, the linearity assessment can be performed using a standard solution of the analyte at different concentrations (at least five). In this case, a pilot experiment with real samples is essential to determine the concentration range of the standard substance. Interesting papers with the expression of linearity of qNMR method can be found in the literature (Li et al., 2020; Tan et al., 2020; Zhang et al., 2021). The linearity is expressed by the correlation coefficient (R^2), which a good result shows $R^2 \geq 0.99$.

Trueness and Recovery: The closeness of agreement between the average of measured values obtained by replicate measurements and a reference value is extremely important in a qNMR method (as well as any analytical method). Obviously, some deviation from exact value is observed, according to Benedito and co-workers (2018), an uncertainty of up to 5% is acceptable. In fact, the trueness measurement could be measured using some samples from the linearity test, because the concentration from the weighed quantity of the substance is known for these samples. It is important to ensure the trueness is determined using different concentrations (e.g., three different linearity test samples, in triplicate at least). The trueness measurement results normally can be shown using Eq. 1.

$$Trueness\ (\%) = \frac{analyte\ calculated}{analyte\ by\ weight} x100 \quad (1)$$

In the case of the recovery test, the addition of a reference (the standard analyte) can reveal the range of recovery. In fact, there is a different acceptable range of recovery (Tan et al., 2020), and it is important to know the calculated amount of the analyte before and after reference addition, and the true amount of reference addition, as the following equation:

$$Recovery\ (\%) = \frac{A_x - A_0}{A_S} x100 \quad (2)$$

Where A_x is the calculated amount of analyte after reference addition, A_0 is the calculated amount of original analyte before reference addition, A_s is the true amount of analyte after reference addition.

In the recovery test for plant extraction, the use of exhaustive extraction is the best way to prove the complete recovery of the substance. However, we can also see in the literature the addition of the interested substance (a purchased standard) in the NP samples, performing the extraction and measuring the recovery in these cases (Tan et al., 2020).

Precision: The precision is usually divided in intraday precision (repeatability) and interday precision (intermediate precision). The

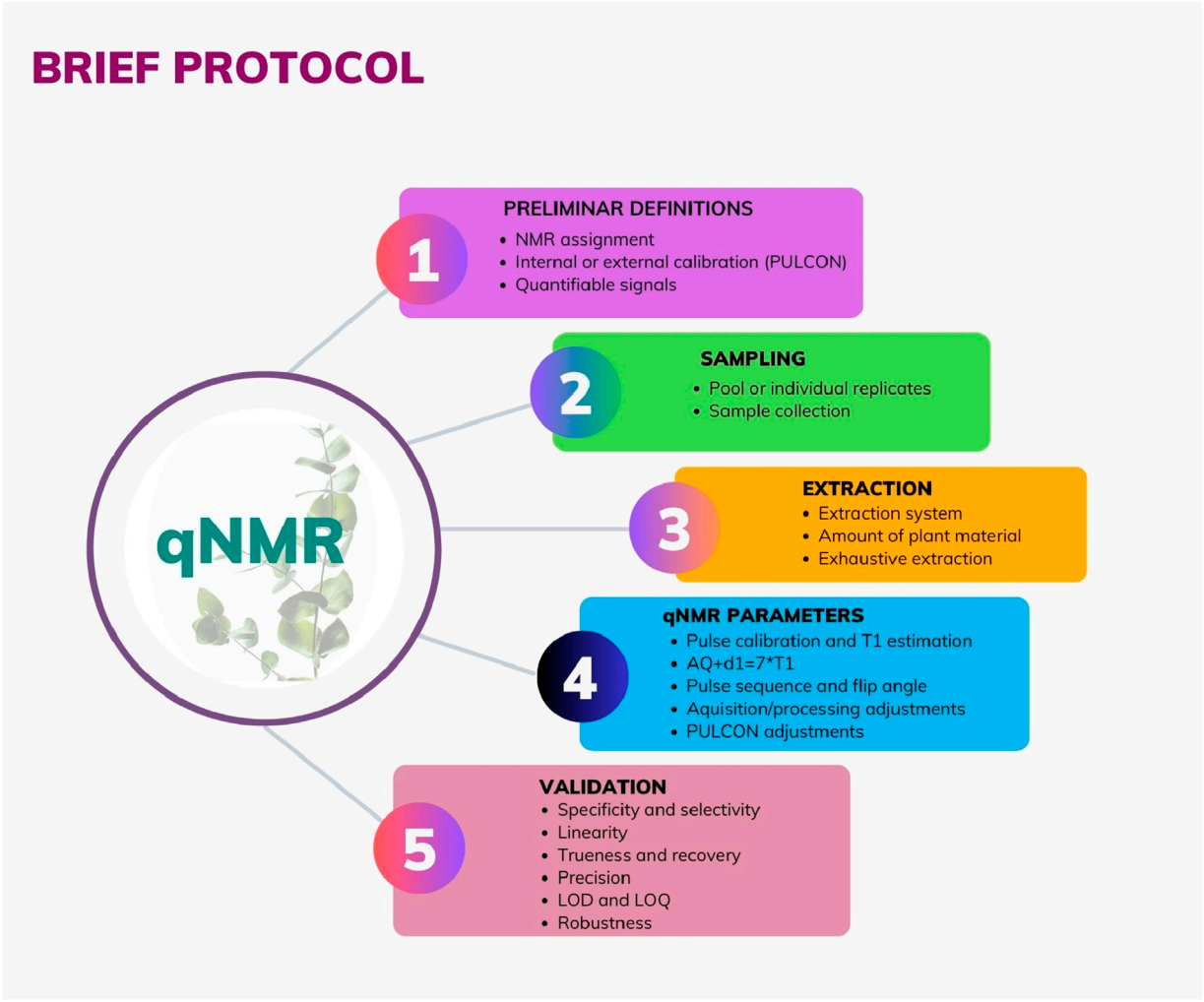

FIGURE 1
Graphical brief protocol in a qNMR study for Natural Products samples: sampling, extraction, qNMR parameter, and validation.

reproducibility also can be performed to evaluate the influences of aleatory factors (such as instrument, operator, etc), since it is the measure of dispersion of results when different laboratories are performing the measurements. Herein, we will show practical approaches for intra- and interday precision.

The samples for evaluation of intra- and interday precision could be either one plant extract sample (Cao and Hahn, 2023) or one standard sample (Windarsih et al., 2018; Thongphichai et al., 2022) (e.g., samples from linearity test). It is important to carry out ^{1}H qNMR experiments along the day (repeatability/intraday precision) and on non-sequential alternate days (reproducibility/interday precision). One suggestion is to perform five to seven intraday ^{1}H qNMR acquisitions of the same sample. This procedure will also be important to test the stability of the sample (How much time the prepared samples can wait for NMR analysis, for example, in an autosampler?). For reproducibility, plant extract samples could be measured by running the experiments once a day for five non-consecutive days (Owczarek et al., 2019), e.g., 1st, 2nd, 4th, 7th, and 10th days. Precision results could be expressed as relative standard deviations (RSD). RSD up to 3.6% has been reported on plant extracts (Giraudeau, 2017).

Limit of detection (LOD) and limit of quantification (LOQ): LOD and LOQ are usually found by performing replicates of blank samples (without the substance of interest). Three to ten replicates could be performed. Thus, only the solvent is added in the NMR tube and the optimized ^{1}H qNMR experiments are carried out. LOD and LOQ values are found using the Eqs 3, 4, respectively (Owczarek et al., 2019):

$$LOD = X_B + 3\sigma_B \quad (3)$$

$$LOQ = X_B + 10\sigma_B \quad (4)$$

Where X_B and σ_B are the mean and standard deviation of blank measurements. X_B and σ_B were determined using free regions of the spectrum, similar in size to the integral regions of the appropriate analyte.

The working range of an NMR method usually starts from the LOQ (lower limit) to the analyte solubility (upper limit) for an analyte in a given matrix (Schönberger et al., 2023).

Robustness: The robustness of the method is assessed by changing some NMR parameters and checking the differences found in comparison with the optimized parameters. Among NMR parameters included in robustness, one possibility is to evaluate two levels of number of scans, relaxation delay, acquisition time, and spectral width (Owczarek et al., 2019; Tan et al., 2020), although other acquisition and processing NMR parameters could be also evaluated (Benedito et al., 2018). Contrast values should be calculated and a confidence interval (95%) must be determined. qNMR is characterized by a high degree of robustness.

3 qNMR in natural products: a brief protocol

A qNMR work in the NP area usually involves important steps aiming for a successful performance. Here, you can see a brief protocol which is summarized in Figure 1 and described below.

3.1 Preliminary definitions

a) Accomplish the complete ^{1}H NMR assignment of the analyte.
b) Select a free and non-overlapped quantifiable ^{1}H NMR signal of the substance of interest and define the integral regions. Check signal purity by 2D NMR experiments. Sometimes, the overlapped signals might be influenced by the solvent or solvent mixture in a way that the signals do not overlap anymore (Beyer et al., 2010).
c) Select the qNMR method: internal standard or external standard (calibration curve or PULCON). The PULCON method is an experimental qNMR technique used for the quantification of analytes through an electronic reference signal applied during acquisition. ERETIC2 is a Bruker's nomenclature for the PULCON method. Herein, we focus on PULCON (Burton et al., 2005; Garrido and Carvalho, 2015; Choi et al., 2022).
d) Select a suitable calibration standard (Malz, 2008; Westwood et al., 2019).

3.2 Sampling

a) Decide between a pool or individual (biological) replicates.
b) Collect samples for all extracts: quantitative analysis, as well as larger extracts (for 2D NMR analysis) and additional extracts for all upcoming tests (pilot experiments).

3.3 Extraction

a) Define the extraction system (How the sample is going to be pulverized? Which solvent is going to be used? The solvent system is going to be a mixture? Is freeze-drying an option?).
b) Test the amount of plant material required for an adequate NMR sample. Balance three important points:
i) the amount of plant material.
ii) the mass of extract obtained and the solubilization in the quantity of deuterated solvent required for analysis.
iii) NMR parameters (e.g., number of scans/transients) to obtain a signal/noise ratio (S/N) suitable for the qNMR study.
c) Decide the type of extraction depending on the expression of the quantities of substances based on the mass of dried plant extract (mg/g of dried extract–one single extraction process) or based on the mass of the analyzed plant material (mg/g of plant–exhaustive extraction).
d) In case of exhaustive extraction: set the number of consecutive extractions.
e) Sample preparation for NMR analysis.
i) Define which deuterated solvent is suitable for extract resuspension (the solvent must solubilize 100% of the analyte and must not overlap its NMR signal).
ii) Define the volume of the solvent (ensure that the entire amount of the analyte is soluble in the resuspension in the same way as it was observed in the exhaustive extraction test. Use at least 700 µL of deuterated solvent to accomplish this.
iii) Centrifuge the resuspended extract to guarantee a limpid solution. After centrifugation, put 600 µL into the NMR tube.

3.4 NMR parameter adjustments

Important: See Table 1 and Supplementary Table S1 (Supplementary Material) for checking all NMR parameters and the list of abbreviations and equivalence for different instruments.

a) Calibrate the pulse width (use *pulsecal*).
b) Perform the T1 estimation for the nuclei responsible for the interested NMR signals (selected signal of the analyte, the internal/external standard, etc) in different conditions, like using different sample concentrations into the range of the study. Choose the greater value found for T1 and use it in the equation $AQ + D1 \geq 7*T1$ to balance the acquisition time and relaxation delay values simultaneously.
c) Pulse sequence

Consider.

i) Flip angles of 30° or 90° are possible (See Table 1).
ii) ^{13}C decoupling could be of interest to avoid ^{1}H-^{13}C satellites close to analyte signal.
iii) Avoid, whenever possible, solvent suppression pulse sequences (Bharti and Roy, 2012).
d) Define acquisition time value.

Take care of the dependency of acquisition time with number of points acquired and/2*spectral width (Acquisition time = number of points acquired/2*spectral width).

It also depends on the equation AQ + D1≥ 7*T1 (see Table 1 and Supplementary Material).

e) Define relaxation delay value.

Balance acquisition time and relaxation delay to complete relaxation (≥7*T1, for a 90° flip angle).

f) Define a suitable number of scans/transients.

Observe signal/noise and balance the acquisition time for the shortest possible time, references usually cite S/N > 150.

g) Define the number of points acquired and data size of real spectrum values.

For ^{1}H, the number of points acquired is at least 64k to keep good resolution. It is important to use zero filling (number of points acquired = data size of real spectrum, at least).

h) Use a large spectral window.

Large spectral window helps phase and baseline corrections.

i) Define receiver gain value.

Closely below the highest possible setting. It is important to maintain a constant receiver gain value for all samples.

j) Center the O1 frequency (center of the spectral width) close to both quantifiable signal (or signals) and the standard compound (in the case of using an internal standard).
k) Processing parameters (Fourier transform + exponential function (LB = 0.3) + automatic/manual phase correction + automatic baseline correction + chemical shift calibration).
l) In the case of the PULCON method, acquire a ^{1}H qNMR spectrum for the standard substance designated for electronic calibration (employing a solution of known concentration). Use the optimized NMR parameters.

3.5 Validation

a. Specificity and selectivity

Select a non-overlapped signal; check 2D NMR experiments.

b. Linearity

Use standard solutions of the analyte at different concentrations (at least five concentrations being equidistant). Decide the concentration range based on the pilot with NP real samples.

c. Trueness and recovery
i) For the trueness measurement, use the same samples from linearity (Eq. 1).
ii) For the recovery test, add a known quantity of the analyte in a standard solution with known concentration (Eq. 2).
iii) For the recovery test in plant extraction, the exhaustive extraction is the best way to prove the complete recovery of the substance.

d. Precision
i) For repeatability/intraday precision, acquire five to seven measurements at the same day.
ii) For intermediate precision (interday precision), run experiments once a day for five non-consecutive days.

e. Limits of detection (LOD) and quantification (LOQ)

Prepare blank samples. Use Eqs 3, 4. However, LOD/LOQ are not necessary when the main component is determined. They are required for determination of minor components.

f. Robustness

Define the number of samples and which parameters you would like to vary: pulse sequence, number of scans/transients, receiver gain, number of points acquired, acquisition time, etc.

4 Final remarks

The quantification of natural product samples using a qNMR method highlights the significance of NMR for this area. This approach extends beyond NMR's traditional role in structural elucidation and, therefore, requires a set of experimental adjustments responsible for the feasibility of the proposal, providing reliability to the results. Consequently, the application of qNMR to NP samples requires a previously planned experimental design and can only proceed following a thorough validation stage.

Actually, regardless of the nature of the sample, a qNMR work is always an Analytical Chemistry work! Thus, the same rigor as analytical chemists should be maintained, unless researchers are in fact interested in a broader, more qualitative overview, where relative proportion measurements derived from integral comparisons may suffice for the investigation. In these cases, the terminology "qNMR" or "quantitative NMR" should be avoided, and terms like "relative proportion" could prove more apt.

Therefore, this minireview hopes to encourage NP researchers to explore the applications of qNMR in their work in order to strengthen results, increase the impact of publications and, why not, facilitate collaboration with NMR spectroscopists.

Author contributions

LR: Conceptualization, Investigation, Visualization, Writing–original draft, Writing–review and editing. PO: Conceptualization, Investigation, Supervision, Writing–original draft, Writing–review and editing. UH: Visualization, Writing–review and editing. GA: Conceptualization, Funding acquisition, Investigation, Supervision, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fntpr.2024.1416195/full#supplementary-material

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