

Quantitative ^1H NMR spectroscopy

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This review illustrates the need to use nuclear magnetic resonance (NMR) spectroscopy for the quantitative analysis of small molecules in their crude forms and in mixtures.

We provide the basic concepts of quantitative NMR (qNMR), a brief description of important acquisition and processing parameters responsible for obtaining high-quality, reproducible NMR spectra in order to maximize accuracy, and the latest referencing techniques used for quantitative analysis.

We also describe methods that are used for quantitative analysis including calibration-curve and standard-addition methods. Further, we briefly address validation of qNMR spectroscopy and its major applications in various scientific disciplines.

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Keywords: Calibration-curve method; Electronic referencing; Metabolomics; NMR spectroscopy; Nuclear magnetic resonance (NMR); Pharmaceutical analysis; Quantitative NMR (qNMR); Referencing technique; Standard-addition method; Validation

Abbreviations: ARTSI, Amplitude corrected referencing through signal injection; DNP, Dynamic nuclear polarization; ERETIC, Electronic reference to access in-vivo concentration; FID, Free induction decay; QUANTAS, Quantification by artificial signal; qNMR, Quantitative nuclear magnetic resonance; RG, Receiver gain; S/N, Signal to noise ratio; T_1 , Longitudinal relaxation time; TD, Time domain

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is a well-known analytical technique for structure elucidation of small and macro molecules. The ^1H (proton) NMR spectrum with chemical shift and coupling constants also gives information about the quantitative relationship between intra-molecular and inter-molecular resonances (Fig. 1).

Proton NMR spectroscopy as an analytical tool for quantitative analysis was first reported in 1963 by Jungnickel and Forbes [1] for determining the intra-molecular proton ratios in 26 pure organic substances. Simultaneously, Hollis [2] analyzed the fraction of aspirin, phenacetine and caffeine in their respective mixtures. In the past three decades, there has been growing interest in quantitative NMR (qNMR) [3]. In this context, the contribution of Turczan et al. to qNMR was significant. They published several papers in the period 1965–90 on the absolute quantitation of active drugs and their purity in various formulations (e.g., tablet, gelatin, ointment and inhalants) [4].

One of the major advantages of qNMR is its primary analytical characteristic, because of which it can be applied in the quantitative estimation of purity of compounds without using any specific

reference standard. The applications of qNMR to other nuclei (e.g., ^{31}P and ^{13}C) are limited due to low sensitivity or low natural abundance. However, the recent progress in the development of high-field magnets and CryoProbe technologies, which have dramatically reduced the limit of detection (LOD), provided an opportunity to analyze samples in low concentrations in metabolomics, pharmaceuticals and natural products.

^1H qNMR has grown significantly in metabolomics in the past two decades [5], so it is one of the most suitable techniques for quantitative measurement of multi-components in a complex mixture (e.g., cell extracts, tissue extracts, body fluids, natural-product isolates and drug formulations) (Fig. 2). NMR-based metabolomics provides absolute and relative quantification of several metabolites in biological samples without separation of individual components in normal or modulated metabolism, so qNMR spectroscopy has been widely applied in environmental toxicity, drug toxicity, disease diagnosis, cancer metabolism, pathophysiology of disease, stress, nutrition, drug metabolism, plant metabolism, bacterial metabolism, and cell-virus interactions.

The quantitative inaccuracy of qNMR has been reported to be less than 2.0%, which is an acceptable limit for precise,

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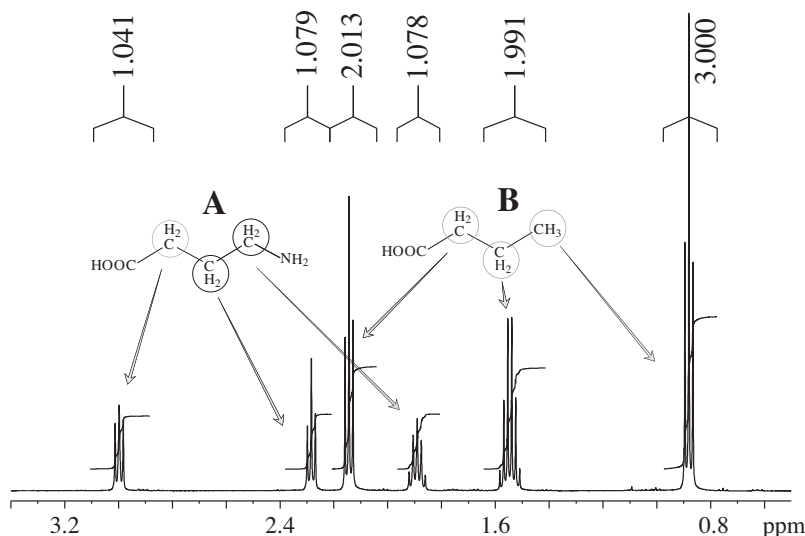


Figure 1. Proton NMR spectrum of a mixture of (A) γ -amino butyric acid and (B) n-butyric acid showing relative integral values of intra-molecular resonances that depend upon the number of nuclei per resonance and their relative concentration.

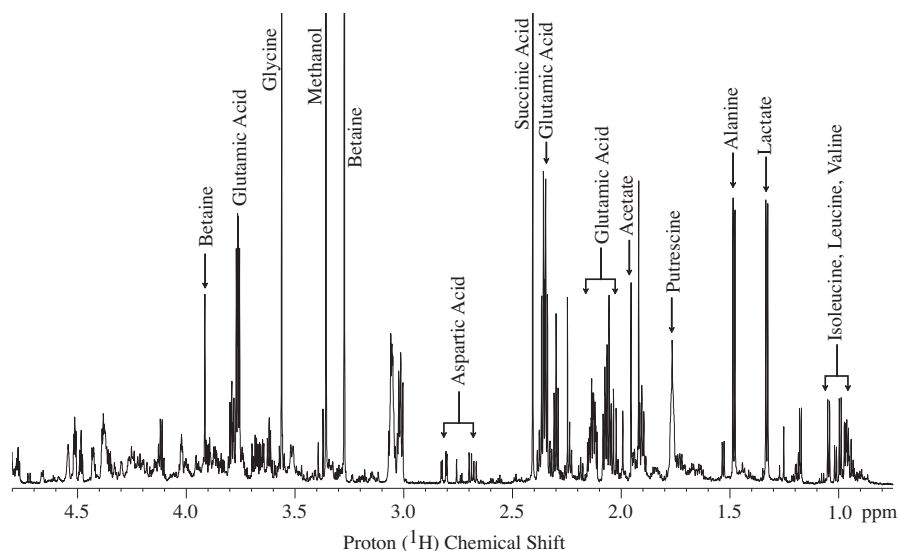


Figure 2. Expansion of one-dimensional single pulse ^1H NMR spectrum (0.50–4.80 ppm) of aqueous extract of bacterial cells. It represents a number of metabolites detected using a single pulse NMR experiment. The intensities of resonances depend on their respective concentrations in the extract.

accurate quantification [6]. Validation processes (e.g., precision, accuracy, linearity, reproducibility, robustness, selectivity, and specificity) had proved that NMR spectroscopy is a good analytical technique for quantitative estimation. Unlike other techniques, qNMR spectroscopy has certain acquisition and processing parameters and referencing techniques that need careful consideration in order to achieve a high degree of accuracy and precision. Sample preparation and experimental methods used may also introduce significant errors into qNMR analysis, thereby reducing the

accuracy and the precision of the resulting data. The analyst should therefore be well acquainted with acquisition and processing parameters, referencing techniques and other analytical steps for careful optimization prior to qNMR analysis.

This review aims to extend awareness of experimental protocols for accurate quantification of analytes and their application to the analysis of mixtures in metabolomics, natural products and pharmaceutical applications. We comprehensively discuss all parameters associated with qNMR analysis, their effects and their

optimization for better accuracy and precision. The review also covers basic principles of qNMR and their applications in various scientific disciplines.

We discuss in detail all the latest techniques used for referencing in qNMR, along with their advantages and disadvantages. In this review, we optimized and validated all the experimental parameters for proton NMR spectroscopy. However, qNMR may also be applicable to all other NMR-sensitive and naturally abundant nuclei. The results, methods and conclusions drawn from the literature are adequately cited. All the experimental results given in this review were performed on 400-MHz or 800-MHz NMR spectrometers. We utilized standard NMR test samples, synthetic mixtures of amino acids and single-compound solutions to demonstrate the experimental results.

2. Basics of qNMR spectroscopy

The most important fundamental relationship of qNMR is that the signal intensity in the NMR spectrum is directly proportional to the number of nuclei responsible for that particular resonance:

$$I_x \propto N_x, \text{ so } I_x = K_s \cdot N_x \quad (1)$$

where K_s is the spectrometer constant and remains the same for all resonances in a NMR spectrum. However, factors that affect K_s are:

- pulse excitation (that should be uniform throughout the spectral width);
- repetition time (should be 5 times T_1); and,
- broad-band decoupling, which causes inherent distortion in intensity due to the Nuclear Overhauser Effect (NOE).

We discuss these parameters in detail below. There are two ways of quantifying analytes using NMR spectroscopy – relative quantitation and absolute quantitation.

2.1. Relative quantitation method

Relative quantitation is one of the easiest methods for NMR. The molar ratio M_X/M_Y between two compounds X and Y can be calculated by employing the following expression [6]:

$$\frac{M_X}{M_Y} = \frac{I_X}{I_Y} \cdot \frac{N_Y}{N_X} \quad (2)$$

This expression is devoid of K_s because it will be same for all the resonances in a spectrum provided all the factors affecting K_s are optimized.

Similarly, the fraction of compound A in a mixture of Z components can also be calculated using the following formula [6]:

$$\frac{M_A}{\sum_{i=1}^Z n_i} = \frac{I_A N_A}{\sum_{i=1}^Z I_i / N_i} \cdot 100\% \quad (3)$$

2.2. Absolute quantification method

There are two analytical procedures for determining absolute concentration of analytes:

- If all the impurities (or other components) present in the NMR spectrum can be assigned structurally and measured quantitatively, then the assay is just a difference from the 100% value. This method is limited when resonances from the impurities overlap in the spectrum with molecules of interest or impurities (e.g., carbonates, chlorides, salts, metal ions, phosphates, moisture) present in the sample.
- In this procedure, the purity of main component X can be calculated directly from the NMR spectrum using the following formula [6]:

$$P_x = \frac{I_x}{I_{std}} \cdot \frac{N_{std}}{N_x} \cdot \frac{M_x}{M_{std}} \cdot \frac{W_{std}}{W} \cdot P_{std} \quad (4)$$

where, I, N, M, W and P are integral area, number of nuclei, molar mass, gravimetric weight and purity of analyte (X) and standard (STD), respectively.

3. Experimental parameters affecting the accuracy and the precision of qNMR

There are several experimental parameters (e.g., delay, pulse sequence, and acquisition time), which affect the quantitative accuracy and precision, so careful optimization is necessary prior to quantitative analysis.

3.1. Selection of pulse sequence for quantitation

Single-pulse NMR experiments have been widely used for quantitative analysis of small molecules. A single-pulse sequence comprises a relaxation delay (RD), sometimes referred to as a waiting period, and a 90° hard pulse followed by acquisition of signals (Fig. 3). If the analyte of interest is very low in concentration (within the LOD), then the dynamic range problem could affect the quantitative accuracy and precision due to the presence of a very strong solvent signal, even in the case of a deuterated signal (residual proton signal in deuterated solvents). In such conditions, solvent-suppression techniques are used to achieve a good signal-to-noise ratio (S/N) for better quantitative accuracy and precision. Solvent-suppression techniques require some modification in the pulse sequence. The simplest method of solvent suppression is pre-saturation of the solvent frequency before the 90° hard pulse.

3.2. Acquisition parameters

3.2.1. Excitation pulse. A 90° pulse angle or lower can be used for recording the spectra. The excitation bandwidth of the hard pulse should be uniform throughout the entire spectral width. The effectiveness of the 90° pulse may vary at points too far from offset because it

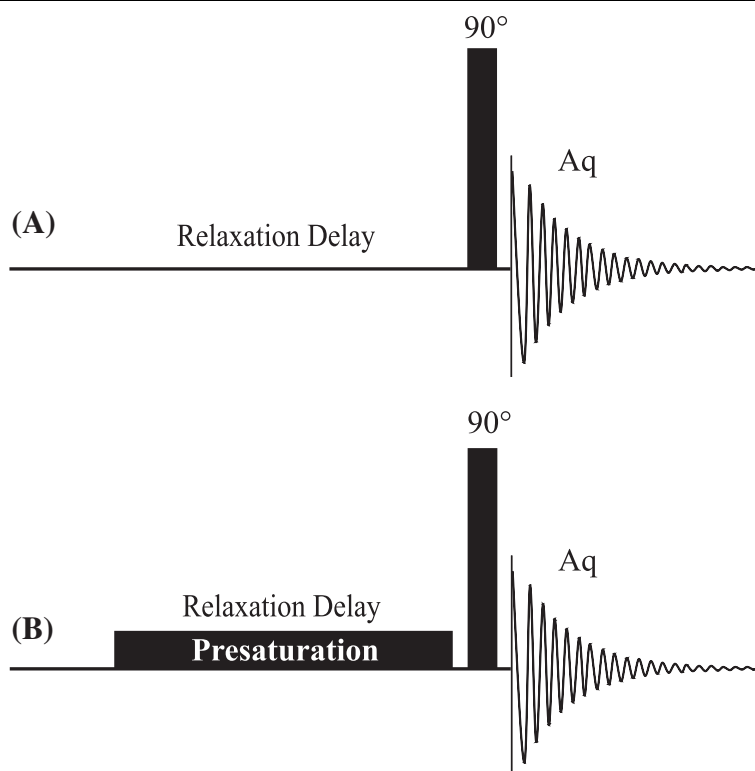


Figure 3. Graphical representation of basic (A) single pulse NMR experiments comprising a relaxation delay and 90° hard pulse followed by acquisition (B) single pulse sequence with the pre-saturation technique used for suppression of strong signals arising due to solvents. In the pre-saturation technique, a selective low-power pulse was used during relaxation delay.

depends upon the design of low-pass frequency filter (eclipsed or brick-wall shaped) used in the NMR system, resulting in erroneous signal intensity [7]. The effectiveness of 90° pulse may also vary from sample to sample, depending upon the physico-chemical properties of the sample. To achieve better precision and accuracy, the pulse length should therefore be calibrated for each sample. Normally, a 90° pulse provides maximum intensity, but a lesser pulse angle can also be used for complete relaxation of resonances, thereby reducing the total experimental time. To achieve a good S/N with reduced repetition time, an “Ernst angle” pulse can also be used, with Ernst angle being calculated using the following formula:

$$\cos \alpha = e^{-\left(\frac{T_R}{T_1}\right)} \quad (5)$$

where α , T_R and T_1 denote optimum pulse angle, repetition time and relaxation time, respectively [8]. Ernst angle depends upon T_1 , which varies for every resonance and hence its application is limited to complex mixture analysis.

3.2.2. Repetition time (T_R). T_R is defined as the total time spent to acquire a single-scan spectrum. This specifically includes RD and acquisition time. Other minor delays can be ignored. T_R depends upon the longest T_1

present in the sample and ideally, it should be five times the longest T_1 to measure 99% of the equilibrium magnetization [8,9]. Percentage relaxation of magnetization for a particular resonance at a fixed T_1 can be calculated by using the following expression.

$$M_z = M_0 \cdot (1 - e^{-t/T_1}) \quad (6)$$

where, M_z and M_0 are the magnetization in z-axis following the repetition time ‘t’ and at full relaxation respectively. T_1 is the longitudinal relaxation time of resonance.

Quantification of metabolites recorded at a shorter repetition time can be achieved by applying a T_1 correction factor to compensate for the relaxation effects, using the following formula [9]:

$$\text{Conc}_{(\infty)} = \text{Conc}_{(t)} \left[\frac{(1 - e^{-t/T_1^S})}{(1 - e^{-t/T_1^M})} \right] \quad (7)$$

where, $\text{Conc}_{(t)}$ and $\text{Conc}_{(\infty)}$ are the concentrations of metabolite at repetition time ‘t’ and at full relaxation. T_1 is longitudinal relaxation time for metabolite resonance (M) and standard resonances (S).

3.2.3. Broad-band decoupling. Hetero-nuclear NMR experiments for X nuclei with ^1H broad-band decoupling using composite pulses (or ^1H experiments with

broad-band decoupling of X nuclei) cause inherent distortion in intensity due to the NOE.

To reduce the intensity distortion below 1%, the following criteria must be fulfilled:

- (1) broad-band decoupling should be applied during the acquisition time (Fig. 4);
- (2) repetition time must be 5–7 times T_1 [8]; and,
- (3) 90° pulse should be used for excitation.

In dynamic mixtures, ^{13}C satellites of highly concentrated metabolites may be equivalent to the intensity of low-concentration metabolites, and that causes ambiguity in qNMR analysis. In such conditions, broad-band decoupling should be used to eliminate ^{13}C satellite signals [3].

3.2.4. Acquisition time. Sufficient acquisition time should be used to avoid truncation of FID, which can lead to the presence of “wiggles” in spectra, resulting in inaccurate intensity measurements.

3.2.5. Time domain (TD). Time-domain data points also affect qNMR analysis. In modern NMR spectrometers with high-memory/high-speed computers, 64 K data point can be used for quantitation. Very low TD values do not provide better resolution for quantitation of partially overlapped signals.

3.2.6. Signal-to-noise ratio (S/N). To achieve precise quantitative results, S/N should be in the ratio of 250:1 for ^1H , 300:1 for ^{19}F and 600:1 for ^{31}P [10].

The S/N may vary for different resonances of a molecule depending upon the number of protons per resonance line. If the concentration of analyte is very low, then the number of scans can be increased to achieve an

acceptable S/N. The sensitivity of NMR experiments is given by the S/N, which can be calculated using Equation (8). However, in modern NMR spectrometers, sensitivity also depends upon the type of probe used (e.g., CryoProbe, inverse probe, or nuclei-specific probe).

$$S/N = \frac{N\gamma_{\text{exc}}T_2(\gamma_{\text{det}}B)^{3/2}\sqrt{ns}}{T} \quad (8)$$

where, N = number of spins in the system (sample concentration/number of protons), γ_{exc} = gyromagnetic ratio of the excited nucleus, γ_{det} = gyromagnetic ratio of the detected nucleus, ns = number of scans, B_0 = external magnetic field, T_2 = transverse relaxation time (determines the line width), and T = sample temperature.

The S/N also varies exponentially as a function of the repetition time when other parameters are kept constant. S/Ns are calculated at different repetition-time periods for the $-\text{CH}_2-$ signal of 0.1% ethylbenzene (standard sample), as shown in Fig. 5. The variation of S/N with repetition time as shown depends upon the T_1 of the $-\text{CH}_2-$ resonance, so the repetition time can also be manipulated to get an appropriate S/N for quantitation in order to improve the accuracy.

3.2.7. Receiver gain (RG). An optimal RG setting must be maintained as it is very important because:

- (1) too high a setting causes baseline distortion of the signal, while
- (2) too low a setting causes loss of analyte signal or a very low S/N.

Modern spectrometers by default have an *auto receiver gain* setting, in which a pulse, at a selected pulse angle, is first delivered with full RG. If the pulse causes receiver

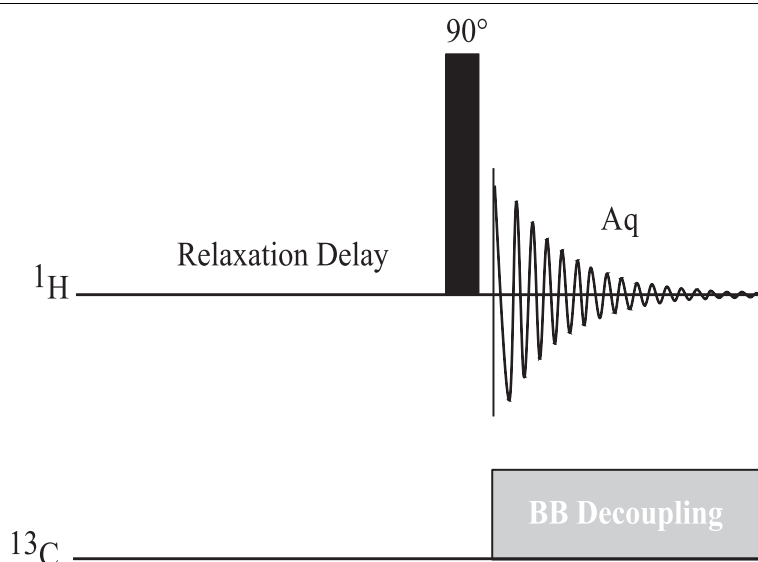


Figure 4. Graphical representation of ^1H detected NMR pulse with inverse-gated decoupling scheme to eliminate ^{13}C satellite from one-dimensional ^1H NMR spectra.

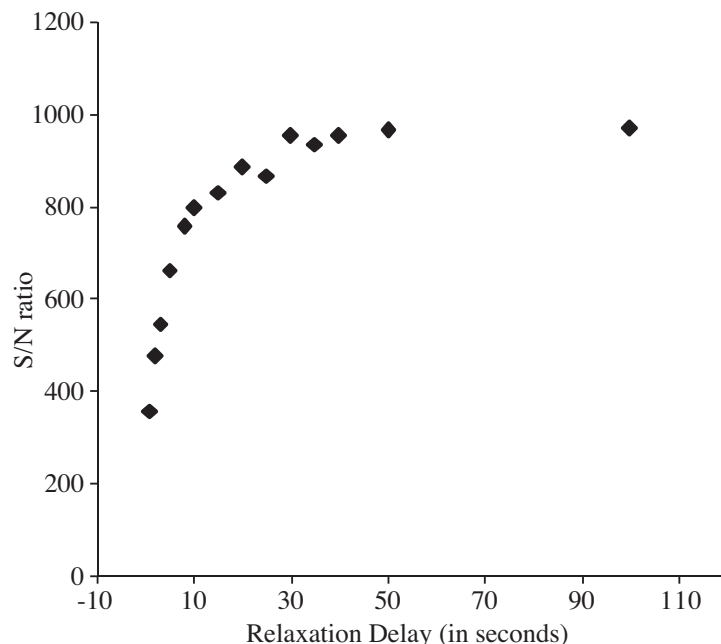


Figure 5. Showing variations in signal-to-noise ratio as a function of repetition time calculated for $-\text{CH}_2-$ resonance of 0.1% ethylbenzene in CDCl_3 .

overload, the gain is decreased by 10% and the spectrometer is pulsed again. The process is repeated until a suitable gain that does not result in receiver overload is obtained by the spectrometer. The effect of RG values on signal intensity and S/N has been studied and the corresponding result is shown in Fig. 6.

To identify the correlation between signal intensity and S/N with respect to RG, 12 experiments were carried out using a solution of glycine in deuterated water at different RG values, while keeping all other parameters unchanged. A simple linear regression test between intensity and RG value provides a very good correlation with the correlation coefficient 0.997. Fig. 7A demonstrates that the signal intensity is highest at the optimum RG value and consequently yields a better S/N. The S/N varies exponentially as a function of the RG value (Fig. 7B), so the optimum RG value should be used for better quantitative accuracy and precision.

3.2.8. Digital resolution. Quantitative analysis also requires that the spectra should be measured with adequate digital resolution. Digital resolution of the final spectrum in Hertz/point is equal to the spectral width divided by the number of data points. Modern NMR spectrometers provide high digital resolution with improved peak dispersion.

The NMR data acquired at 32 K data points are sufficient for quantitative analysis (usually 0.1–0.5 Hz/point). This implies that doubling the data points in TD

(FID) will enhance the digital resolution in the frequency domain.

3.2.9. Shimming. Magnetic field inhomogeneity creates signal distortion or inappropriate peak shapes, which result in poor resolution and low S/N of the spectra. Magnetic field homogeneity (shimming) should be optimized to achieve a highly homogenous magnetic field around the sample. During optimization, an increase in homogeneity can be determined by any one of the following ways:

- (1) increase in the height of lock signal;
- (2) shape of the FID; and,
- (3) shape and line width of a resonance line in the sample.

3.2.10. Tuning and matching. Improper tuning and matching of the spectrometer frequency may affect the effectiveness of the 90° pulse, resulting in variations in the intensity of the signals [11], so, to achieve high reproducibility, all the samples require correct tuning and matching before conducting the experiments.

3.2.11. Temperature. Temperature is also an important factor that affects the reproducibility of quantitative results. It should be kept constant throughout the study, even during the calibration of the external reference used for quantitation. Temperature variation also affects the relaxation properties of the molecules.

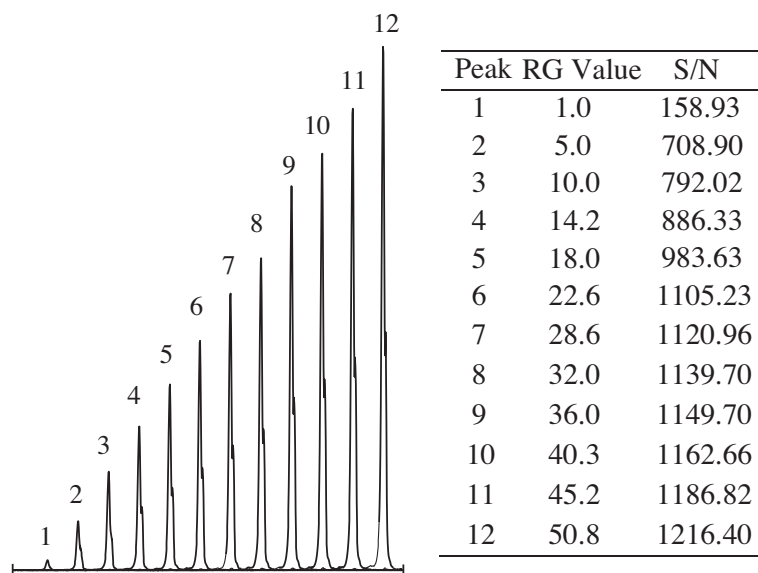


Figure 6. Variations in the intensity/area of resonances as a function of receiver gain (RG) shown using glycine resonance at 3.56 ppm recorded at different RG values.

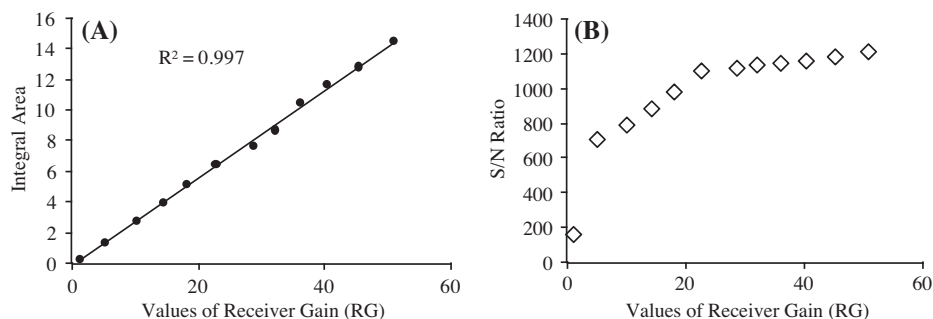


Figure 7. (A) Linear least square regression performed to find out the correlation between signal intensity and receiver gain. (B) Calculation of variation of peak area and S/N for glycine resonance as function of receiver gain.

Air flow during temperature regulation may cause “wiggles” – artifacts at the baseline of strong signals at high flow rates – so the airflow should be optimal and constant during the acquisition time for all the samples to be used for comparative analysis (e.g., metabolomics studies).

Sample temperature also plays a critical role in quantification of metabolites in intact tissues during HR-MAS NMR analysis. A temperature of 4–10°C is recommended for carrying out HR-MAS analysis of tissue samples to minimize the metabolic activity of tissue specimens.

3.2.12. Precision of NMR tube and capillary diameter. When a large set of samples have to be analyzed for comparison, variation in the diameter of NMR tubes also contributes to an error during qNMR analysis [11,12], so NMR tubes with very precise diameter should be used. Before purchasing the NMR tubes, the user should check the manufacturer’s

specifications. When co-axial inserts are used for external reference filling, the same capillary should be used throughout the study to ensure the high reproducibility of the results.

3.2.13. Solvent-suppression techniques. Also a pre-requisite for accurate quantitation is selection of an efficient solvent-suppression technique, which can change depending upon the metabolites of interest.

Pre-saturation of solvent resonances cause partial saturation of the signals of exchangeable protons present in the analyte, so quantification of such signals cannot be carried out by a pre-saturation method. Techniques other than pre-saturation (e.g., excitation sculpting and WATER-GATE) can be used for quantification of such metabolites.

The metabolite signals that are close to solvent signal (e.g., anomeric protons of sugars) should be considered first to see the effect of solvent-suppression techniques. If

the solvent content in the samples is not too high, the band-width of the pre-saturation irradiation (optimally 50–100 Hz) can also be reduced to lower the effect of pre-saturation.

3.3. Post-processing parameters

3.3.1. Windowing. Prior to Fourier transformation, a window (or filter) function is applied to the TD data to improve the S/N and the resolution of spectrum. This technique is routinely used and is called Windowing. Usually, in a 1D spectrum, the S/N is improved by multiplying the FID with an exponential window function. The decay rate of the exponential function determines the line broadening of signals. This rate is adjusted by the processing parameter line broadening (lb). An lb value of 0.3–1.0 Hz is recommended.

3.3.2. Zero filling. After applying the window function, the next step in data processing is to zero fill the FID to a factor of 2. If in data, the number of points in the FID is 32 K, zero filling with factor 2 will provide 64 K data points after Fourier transformation, so the number of points that describes the real spectrum is 64 K rather than 32 K. The sole experimental requirement for zero filling is that the FID must have been decayed near to zero by the end of acquisition time, so final digital resolution will be equivalent to 64 K real data points rather than half that without zero filling.

3.3.3. Phase correction. Proper signal phasing is necessary for accurate intensity measurement. Phase error

may cause significant error in peak-ratio measurement, and consequently in absolute or relative concentration during qNMR analysis of compounds. Manual phase correction is preferred over automatic phase correction in metabolomic studies because small signals get distorted in automatic phase correction. A negative deviation in the signal of a molecule, x%, will produce an area error of 2x% when compared with other signals (Fig. 8). If optimum phase precision is required, vertical expansion should be increased as much as possible with the implementation of manual phasing.

3.3.4. Baseline correction. An incorrect baseline introduces significant error during integration of peak area and subsequently in quantitation. Automatic baseline correction is generally performed and is easier to execute than manual baseline correction. There are a number of algorithms built into NMR-analysis software that can be used for baseline correction to facilitate accurate quantitation further [13].

3.3.5. Integration. Integration of peak area is one of the most crucial steps in qNMR analysis. The range of the integral region and the setting of the slope and the bias (0th and 1st order coefficient of straight line defining the baseline in the integral region, respectively) greatly affect the quantitative accuracy. At this point, error can be easily introduced by the analyst during integration, especially during slope and bias setting.

To include 100% of the peak area, integration would need to extend to infinity in both directions. In order to

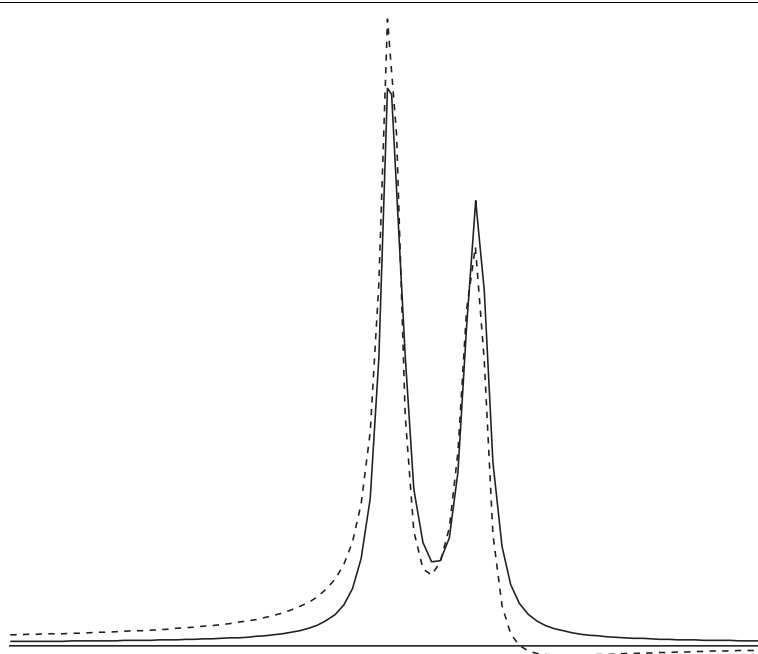


Figure 8. Proper phase correction (solid lines) and variation in intensity due to inaccurate phase correction (dotted line) of a simulated signal. This figure demonstrates that inaccurate phase correction, which increases the intensity of one signal while reducing the intensity of other one, may affect the peak-ratio measurement.

cover 99% of the total area, the integral region should be extended to 20 times the peak width in both directions. Slope and bias corrections are unnecessary if baseline correction is adequately performed. The integrals are more sensitive to baseline imperfection. Manual slope and bias corrections are required for accurate quantitation. It is also better to perform integration 5–10 times and to take an average of all the values to reduce the analyst error that could arise during slope and bias corrections [14].

3.3.6. Deconvolution. Area determination of overlapping peaks cannot be obtained accurately using routine integration or bucketing methods, so line-fitting [15] or, more commonly, the deconvolution method is used. Deconvolution is performed to determine the contribution of an individual peak to the total area.

4. Physico-chemical properties that affect qNMR analysis

4.1. pH of sample

The pH of the sample also plays a significant role in quantitative analysis. Change in sample pH may cause a drift in the chemical shift of the desired signal, which may lead to overlapping of signals in the spectrum and alteration in the relaxation time. To evaluate the effect of pH on T_1 relaxation, a mixture of amino acids having the same concentration was prepared at three different pH values ranging from acidic to basic, and the T_1 was evaluated as shown in Fig. 9. In metabolomics, where a

large sample size is required, pH buffers are recommended to eliminate the effect of pH variations [16].

4.2. Ionic strength

Ionic strength (i.e. salt concentration of the samples) also affects T_1 relaxation and drifts in the chemical shift, which, in turn, affects quantitative analysis. A high salt concentration affects probe performance, causing difficulty in setting up tuning and matching.

In order to demonstrate the effect of ionic concentration on T_1 relaxation, mixtures of amino acids of the same concentration and pH were prepared while varying the ionic strength. Significant changes were observed in the T_1 relaxation of metabolites (Fig. 10). A drift in the chemical shift of the metabolites due to ionic strength affects the statistical pattern-recognition techniques {e.g., Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA) [17]}. To overcome the effects of salt concentration, deuterated chelating agents can be used [17]. Dilution of the sample may also help to reduce the effect of salt concentration [16].

4.3. Sample storage

The pH of biological samples (e.g., serum, bile and urine) alters upon storage for a long time and during sample processing [18]. These samples should be checked first for any pH variation and an appropriate buffer solution should be used to lower the pH effect.

Multiple freeze and thaw processes also affect the signal intensity and cause line broadening, while stable, pure samples (e.g., isolated natural products, active

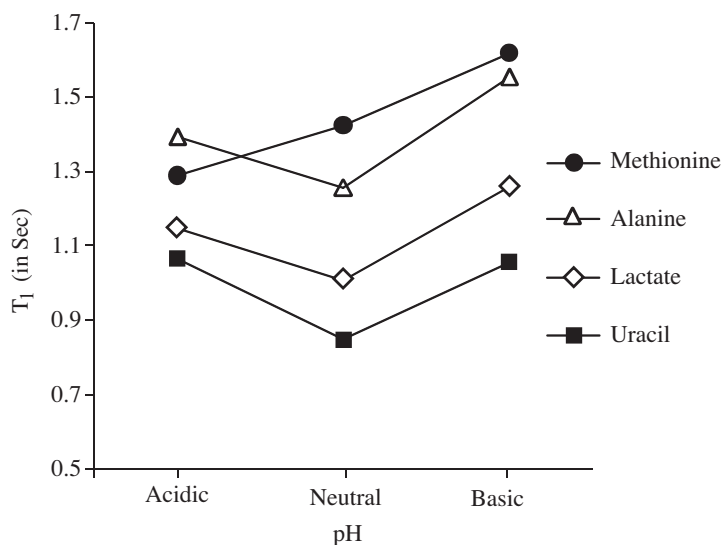


Figure 9. Variation of T_1 as a function of pH was evaluated for methionine (2.63 ppm), alanine (1.48 ppm), lactate (1.33 ppm) and uracil (5.80 ppm) at different pH values ranging from acidic (4–5) to neutral (7.4) to basic (9–10).

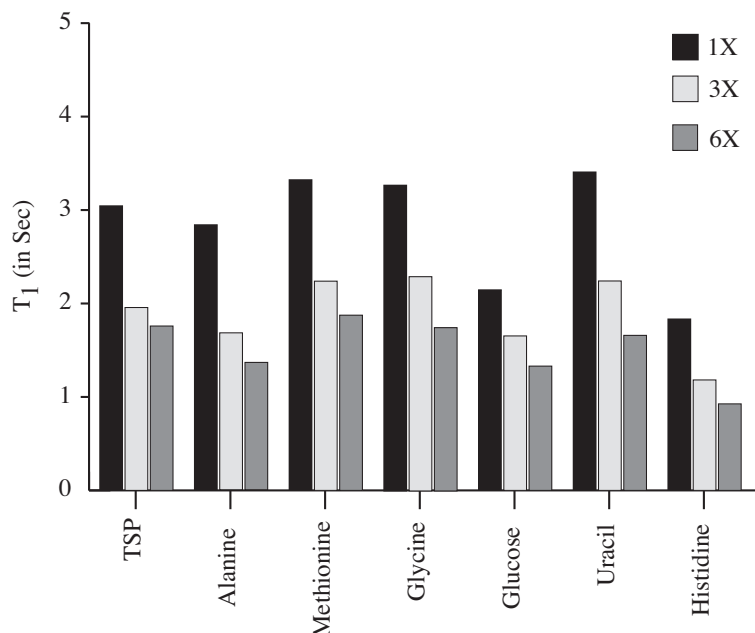


Figure 10. Changes in the T_1 (in s) of TSP (0.0 ppm), alanine (1.48 ppm), methionine (2.63 ppm), glycine (3.56 ppm), glucose (4.65 ppm), uracil (5.8 ppm) and histidine (7.02 ppm) resonances are shown at different ionic concentrations. Concentration and pH of the amino-acid mixture were maintained constant.

drugs and synthetic samples) have no effect on storage and freeze and thaw.

4.4. Concentration of analyte and reference

Concentrations of analyte and reference compounds should be comparable in range. If the difference in concentration between analyte and reference is too large, it will cause overflow of information, resulting in distortion of the signal, or low S/N for the lower concentration. Too high a concentration of either analyte or reference compounds will cause overflow of signals and result in distortion of the spectra. During manual integration, a slight variation in the area of the highest signal will also cause significant quantitative error in the smaller ones.

4.5. Solubility

The analyte should be completely soluble in the solvent and should not form suspension or precipitate, so, before preparing the analyte solution, its solubility needs to be checked to see whether it is sparingly soluble (1 g in 100 mL), soluble (1 gm in 30 mL), freely soluble (1 g in 10 mL) or very soluble (1 g in 1 mL) [British Pharmacopoeia]. The amount of analyte that should be dissolved in a fixed volume of the solvent will be decided by its solubility.

4.6. Chemical interaction

Analytes and reference compounds present in the sample should be inert to chemical interactions [e.g., a few metabolites (e.g., lactate, histidine, phenylalanine, tryptophan, and citrate) and reference compound

3-(trimethylsilyl) propionic acid sodium salt (TSP) are known to bind with serum proteins]. Ammonium chloride or any binding-releasing agents can be used to release such metabolites prior to qNMR analysis [19].

5. Referencing techniques in qNMR

Quantitative analysis by NMR requires a reference compound for calculating the concentration of an analyte. An ideal internal standard for assay purpose would be one that is readily available in a highly purified form, less expensive, stable, chemically inert, non-volatile, non-hygroscopic and soluble in most of the NMR solvents that are being routinely used. The reference signal should be well separated and preferably be a singlet. Relaxation time of the reference compound should also be in the range of the analyte relaxation time in order to avoid unnecessary increase in experimental time. Based on their usage, reference compounds can be classified as internal and external standards. We discuss below details of their advantages, disadvantages and applications.

5.1. Internal standard

A known concentration or a weighted amount of a reference compound is dissolved in a known volume of analyte solution for quantitative estimation.

A number of reference compounds are available for qNMR analysis. Tetra methyl silane (TMS, organic

solubility), 3-(trimethyl-silyl)-1-propane sulfonic acid sodium salt (DSS, aqueous solubility) and TSP (aqueous solubility) are the most widely used reference compounds for chemical-shift referencing and quantitative analysis. TMS is highly volatile, so it is unsuitable for quantitative analysis. Other reference compounds used for qNMR include maleic acid, p-toluenesulfonic acid, tert-butyl alcohol, 1,3,5-trioxane, 1,4-dioxane, sodium acetate, sodium maleate and formic acid [3].

The most important conditions for an internal standard is its solubility and its chemical interaction with the analyte. In the case of biological samples (e.g., serum, plasma, ascetic fluid, pus, and CSF), where proteins, lipoproteins and fatty acids are abundantly present, the internal standard should be selected very carefully.

DSS and TSP are known to bind with proteins and fatty acids, thus affecting the quantitative accuracy, so knowledge of the nature of the analyte solution is also a very important factor in the selection of the reference compound in qNMR. Reference compound 4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA, deuterated form, $(\text{CH}_3)_3\text{SiCD}_2\text{CD}_2\text{CD}_2\text{NH}_3^+\text{CF}_3\text{COO}^-$) can be used in proteinaceous sample since it does not interact with peptides, proteins and lipids [20].

5.2. External standard

Contamination of a valued analyte sample by the reference compound can be avoided by using a co-axial stem insert (Fig. 11). A co-axial stem insert is generally filled by reference compound dissolved in a suitable deuterated solvent (not necessarily according to analyte solubility) and inserted in NMR tubes containing analyte solution. The height of both the analyte solution and reference solution filled in co-axial stem insert should be equal. Here, the concentration of the reference-compound solution filled in the capillary cannot be directly used for quantitation.

The effective concentration of reference should be calibrated by placing the sealed capillary in the NMR tube containing a solution of the primary standard (known solution of any compound) and a quantitative NMR spectrum is recorded. The effective concentration of reference can be calculated by the equation used for quantitative analysis [Equation (4)]. After standardization of the external standard solution, the capillary is removed, rinsed, dried and placed in an NMR tube containing the analyte solution, and a reverse calculation is performed to quantify the analyte. This capillary can be reused for quantitative analysis of a variety of samples.

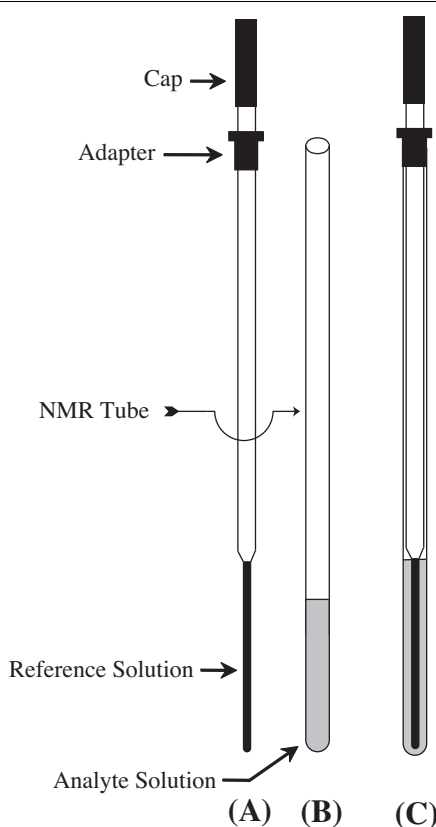


Figure 11. A stem co-axial insert with its plastic cap and NMR sample-tube adapter, the co-axial insert (A) and NMR sample tube with sample (B) assembled for analysis (C).

There are a few disadvantages of this method, namely:

- (1) shimming of the sample becomes a bit difficult, especially when the reference and the analyte are having different solvent systems;
- (2) the sensitivity is reduced due to there being less unit volume of analyte in the radio frequency coil region.

About 30% reduction in S/N was observed for a standard WILMAD co-axial insert (outer diameter 1.8 mm) in 5 mm NMR tube. This reduction depends upon the capillary diameter, so a very thin capillary should be used to occupy less sample volume in the NMR tube.

The external standard method provides better precision than the internal standard method.

TSP has been reported to stick to the glass wall of capillary after prolonged usage, so, after 1–2 months, a new capillary has to be used. Other reference compounds (e.g., maleic acid and formic acid) can also be used in the capillary instead of TSP, because they do not usually stick to the capillary wall [10]. In principle, the same compounds that are used as an internal reference can also be used as an external reference. The external reference, when dissolved in a deuterated solvent, also provides a field/frequency lock for the spectrometer. If

required, a paramagnetic relaxation agent can be added to shorten the T_1 of the external reference considerably, provided the T_1 of the analyte is too short for quantitative estimation.

5.3. Standard-addition and calibration-curve methods

These methods use a specific standard compound (i.e. the pure form of the analyte instead of non-specific internal or external reference).

In these methods, a calibration curve is developed using least square linear regression, and the NMR integral area is obtained from serial dilutions of stock solution of the reference compound. Analyte test samples are then recorded using the same experimental parameters and the integral area is compared with the calibration curve to calculate the concentration. If 10 metabolites are to be quantified in a mixture, then 10 calibration curves (or standard-addition curves) will have to be measured using their respective reference compounds. In such conditions, the standard-addition method requires more sample solution for analysis.

In these methods, some other techniques are used to normalize the area of analyte signal after each addition [19]. Even a simulated spectrum can also be used for normalization of the area. A simulated spectrum can be

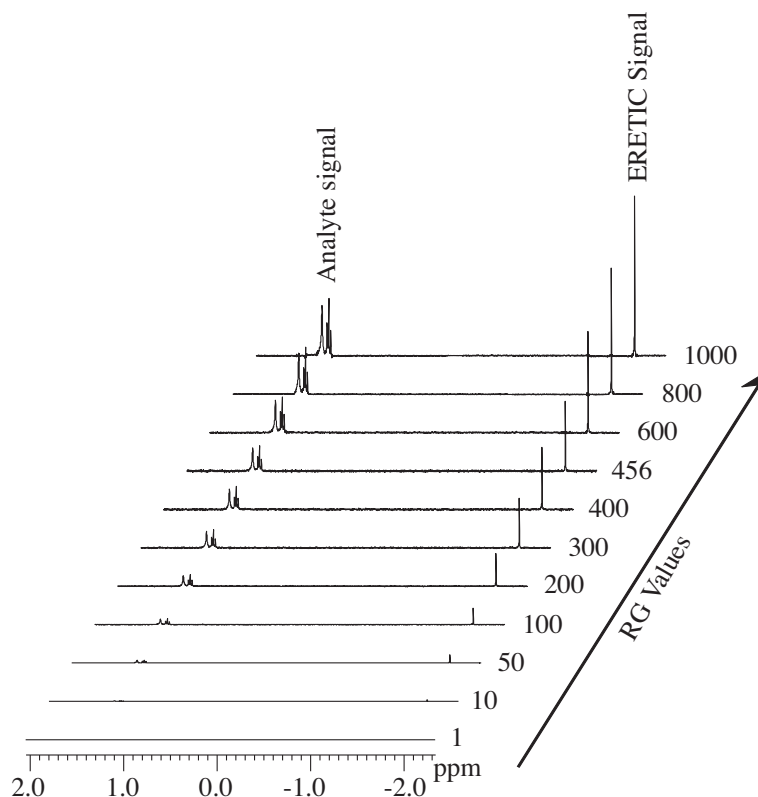


Figure 12. Effect of receiver gain (RG) on ERETIC signal and analyte signal. The factors of amplification for ERETIC and the analyte signal are similar.

incorporated into the original spectrum (e.g., QUANTAS methods by simple addition of two spectra).

We describe the details of the procedure and the basic concepts of these methods in Sections 6.1 and 6.2.

5.4. Electronic reference method (ERETIC)

Akoka et al. developed a method for quantification of analytes using an electronically-synthesized reference signal, called Electronic Reference To access In-vivo Concentration (ERETIC) [21]. The ERETIC signal can be calibrated using a solution of known concentration, so it can also be used for quantitative analysis of test samples.

The ERETIC method provides a pseudo-FID that has all the characteristics of a real NMR signal, and its parameters (frequency, magnitude, and phase) can be controlled from the workstation. The pseudo FID is manipulated by multiplying an exponentially decreasing signal and a sinusoidal signal at the observed frequency.

In all modern spectrometers (e.g., Bruker DRX and Avance series), the ERETIC signal can be generated by second channel (usually the ^{13}C coil) of the probe. The ERETIC signal is derived after frequency modulation and prior to the amplifier. During amplification, the ERETIC signal is treated as real NMR signal (Fig. 12), so that there will be no discrepancies in comparing quantitative results, even if they are recorded at different RG values.

The ERETIC signal should be first calibrated by a solution of known concentration using the following formula:

$$[\text{ERETIC}] = [\text{REF}] \cdot \frac{A_{\text{ERETIC}}}{A_{\text{REF}}} \quad (9)$$

where [REF] and [ERETIC] are the concentrations of the calibration solution and the ERETIC signal, respectively. A_{REF} and A_{ERETIC} are the areas of the calibration peak and ERETIC peak, respectively.

After calibration of the ERETIC signal, the concentration of any other compound can be quantified using the following expression:

$$[\text{Comp}] = K[\text{ERETIC}] \cdot \frac{A_{\text{Comp}}}{A_{\text{ERETIC}}} \quad (10)$$

where K takes into account the number of protons in the peak of interest and the molecular weight of that compound.

There is no need to calibrate the ERETIC signal on a daily basis. If the instrument power level, room temperature, pulse length and other conditions are kept constant, then variations in the area of the ERETIC signal will be insignificant and calibration will be required only monthly [22].

NMR pulse sequences need a little modification to generate the ERETIC signal. The phase of the ERETIC signal needs to be adjusted separately with a computer

program. Normally, these pulse programs are available with the instruments.

5.5. Amplitude-corrected Referencing Through Signal Injection (ARTSI)

Avizonis et al. developed a method that was similar to ERETIC but had some more advantages [23]. If the quality factor (Q-factor) of the probe changes from one sample to another, then the quantitative accuracy of ERETIC signal will be affected. Samples that have dielectric properties similar to the reference standard do well with the ERETIC techniques, while those in different solvents or salt compositions resulting in different dielectric properties can introduce significant errors in quantitation. As each sample may change the receptivity of the probe coil and alter the intensity of NMR signal received, the electronic signal intensity must be scaled accordingly. The Q-factor of a probe is inversely proportional to the square of 90° pulse width. Thus, for a given probe and sample, if the 90° pulse is shorter, the Q-factor of probe will be larger and vice versa. Based on this relationship, a power correction for ERETIC signal is made according to the pulse width of the reference sample and the electronic referencing power. This method is more robust for the samples with large variations in salt concentrations [23].

5.6. QUANTification by Artificial Signal (QUANTAS) or digital referencing

Digital referencing refers to the software-generated signal for quantification of analytes. Two such methods are available for qNMR analysis. One was developed by Bruker Biospin and is incorporated in TOPSPIN 3.0 as ERETIC2, and Farrant et al. developed another method [24]. This method involves adding a standard artificial signal and compensating its scaling according to the changes in experimental parameters. This synchronizes the intensity of NMR signals relative to a digital reference signal. The integral values are then automatically converted into their mmol equivalents.

A simple program can be written to add a simulated signal to a real spectrum (Appendix A) using available NMR software. The added signal needs to be calibrated like ERETIC and can further be used for qNMR analysis. The linearity test for this added signal was carried out by a standard solution of alanine and the results were found to be excellent, as shown in Fig. 13. The experimental and processing parameters for an analyte solution should be the same as those used during calibration of the added signal. Sometimes, analyte concentrations to be measured are either too low or too high; in such cases, spectra cannot be recorded at the desired RG value. In metabolomic analysis, where variations in the concentration of metabolites are too high, a constant RG cannot be set for all the samples. As was already demonstrated in subsection 3.2.7, the intensity of a resonance is linearly

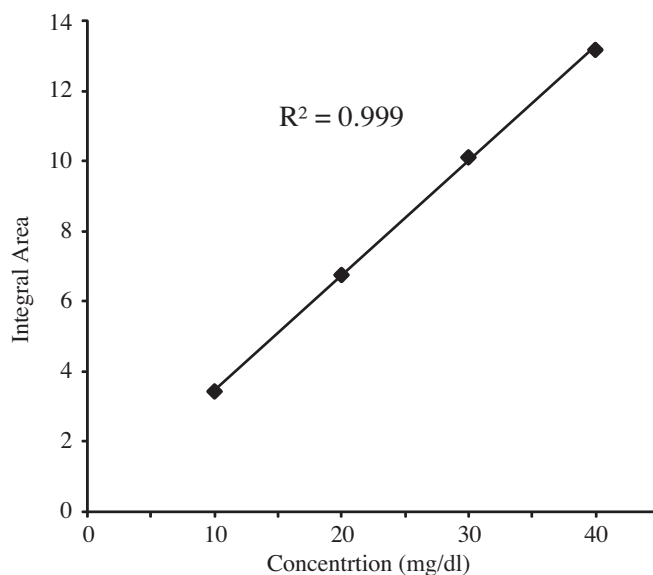


Figure 13. Linearity of artificial reference signal (QUANTAS) calculated for alanine (1.48 ppm).

correlated with RG ($R^2 = 0.997$), so the signal intensity of metabolites recorded at different RG values can be extrapolated to the desired fixed RG value used in majority of the samples with a standard error of about 0.5%.

5.7. Pulse Into Gradient (PIG)

Ziarelli et al. developed a method for quantitative analysis that is similar to the ERETIC method [25]. In this PIG method, using a secondary RF channel and a gradient coil, a low-level, exponentially-damped RF signal is introduced near the frequency of the primary RF channel to serve as an external concentration standard.

The PIG method was reported to be more robust during variations in the ionic concentration of the samples (within 5% for 0.0–2.0 M NaCl). This method also provides a better option of gradient-tailored water suppression, which is more effective than the pre-saturation technique. The method was reported to be stable for 14 h.

5.8. Pulse length based concentration determination (PULCON)

Wider et al., published a method for quantitative analysis of protein samples based on changes in pulse length [12]. In this method, a reference-protein sample with known concentration is needed to calculate the concentration of the test-protein sample. The method was reported to be most robust during variations in ionic concentration and easy to implement, as one reference sample covers a wide range of analyte concentrations. It is very sensitive to the RF power delivered to the coil and setting up the tuning and matching. Samples having high salt concentration need careful setting of tuning and matching to avoid errors in concentration measurements.

6. Other analytical procedures for qNMR

6.1. Standard-addition method

The standard-addition method involves adding two or more increments of standard solution to sample aliquots having the same volume. Each solution is then diluted and made up to a fixed volume before it is measured. When the sample amount is limited, standard addition can be carried out by successive increments of the standard to a single measured aliquot of an unknown sample. Measurements are carried out on the original sample and also after each addition of the standard solution. A curve is plotted using peak area and concentration of standard added in the unknown sample. Extrapolation of the plot will provide the concentration of the analyte in an unknown sample [26].

To demonstrate the standard-addition method, an unknown coded glycine solution was used and the spectrum was recorded using single-pulse experiment at RD of 4.0 s. Further, NMR experiments were recorded with the same parameters after successive additions of standard glycine solution to four aliquots of the same unknown sample. A linear regression was carried out and extrapolation of curve crossing at the negative portion of the concentration axis (x-axis) provided the concentration of glycine in the test sample (Fig. 14).

6.1.1. Theory. Assuming that several identical aliquots, V_X mL, of an unknown solution of analyte X having concentration C_X are transferred to volumetric flasks with a volume capacity of V_T mL. To each of these flasks increasing volume V_S mL of standard solution of the analyte X with a known concentration C_S is added. Next, the solution in each flask is diluted and its volume is

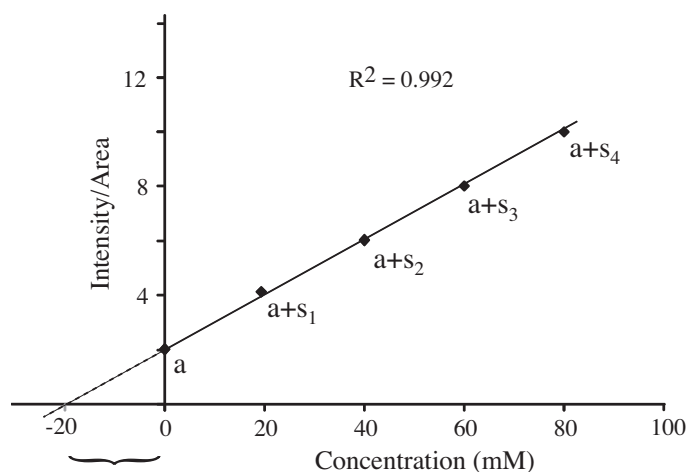


Figure 14. Quantitative analysis by the standard-addition method. Extrapolation of the calibration curve gives the concentration of the analyte on the negative side of the X-axis. This curve was generated for quantitative analysis of glycine solution. Here, 'a' represents the intensity due to the actual concentration of the test solution, 'a+s₁' and other intensities are due to successive additions of standard solution to the test solution of the analyte. The concentration values of successive additions are as follows $s_1 < s_2 < s_3 < s_4$. The quantitative inaccuracy of the method was found to be less than 1%. Calculated and actual concentrations of coded glycine solution were 20.07 mg/dL and 20.0 mg/dL, respectively.

made up to V_T mL using the respective pure solvent. The total integral area (I_T) of the analyte depends upon the concentration of unknown and standard solutions and volume of solution, which can be described by the following expression [27]:

$$I_T = \frac{kV_S C_S}{V_T} + \frac{kV_X C_X}{V_T} = KV_S C_S + KV_X C_X$$

where K is a constant $= k/V_T$.

Plotting I_T as a function of V_S should yield a straight line of the form:

$$I_T = mV_S + b$$

where the slope 'm' and the intercept 'b' are given by $m = KC_S$ and $b = KV_X C_X$.

A linear least square regression analysis can be used to determine 'm' and 'b', after which C_X can be determined from the ratio of these two quantities and the known values of C_S , V_X and V_S , so:

$$\frac{m}{b} = \frac{KC_S}{KV_X C_X}$$

On rearranging, this gives:

$$C_X = \frac{bC_S}{mV_X} \quad (11)$$

6.2. Calibration-curve method

To use the calibration-curve technique, a series of standard solutions containing known concentrations of analyte are used and the response of the instrument (i.e. intensity/area) is recorded. These solutions should span the concentration range of interest and should have a matrix composition as close as possible to that of the analyte solution. The resulting data are then plotted to

produce a graph of intensity/area versus analyte concentration.

Normally, an equation for the calibration curve is developed by least square linear regression so that the sample concentration can be computed directly [27]. Fig. 15 shows a typical calibration curve used for calculating alanine concentration in two test solutions. The calibration curve was prepared earlier using eight standard solutions of alanine with known concentrations. Using the linear least square regression method, an equation (Fig. 15) was formulated and used for computing the concentration of unknown solutions (samples T-1 and T-2). Concentrations of the test solutions were calculated with a maximum inaccuracy of about 1%. The repetition time required for relaxation of magnetization was relatively much shorter for these experiments. This demonstrates that the calibration curve nullifies the effect of relaxation on quantitative accuracy. All the parameters were kept constant for standard and test solutions.

7. Two-dimensional qNMR spectroscopy

One-dimensional NMR techniques are generally used for the quantitative estimation of small molecules or simple mixtures of low-molecular-weight substances. The qNMR analysis of complex mixtures is often hampered by overlapping signals. Two different approaches (viz theoretical and experimental) have been used to overcome this problem.

The theoretical approach is used to fit the overlapping signal in 1D NMR spectra with modeled peaks [28]. In the experimental approach, 2D NMR spectra collected

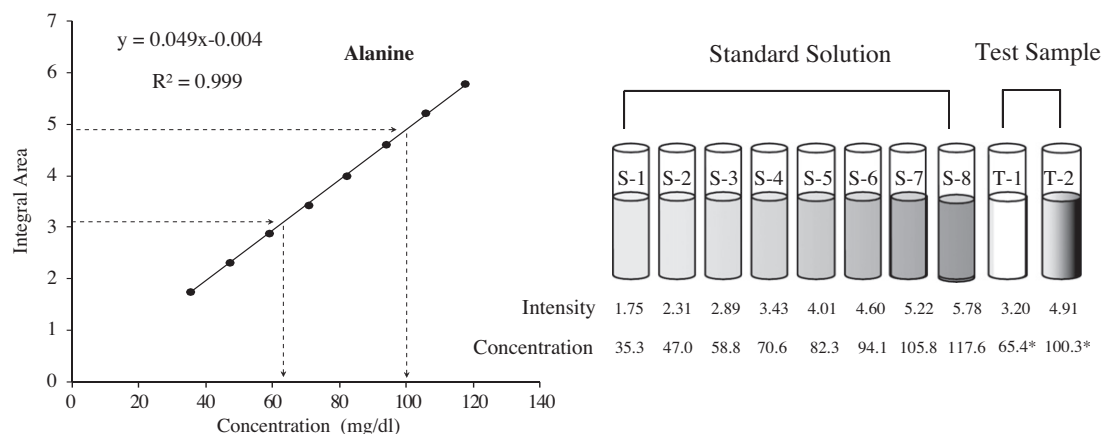


Figure 15. Quantitative analysis by the calibration-curve method for alanine generated using eight standard solutions. Dotted lines indicate the quantitation of two unknown test solutions of alanine. Standard and test solutions, denoted by 'S' and 'T' along with their intensity and concentrations are shown.

disperse overlapping peaks into an indirect dimension. ^1H - ^{13}C HSQC is one of the commonly used methods to resolve the peak overlap, as it provides better dispersion in the ^{13}C dimension [29].

Despite its great potential, quantitative 2D NMR has two major limitations. First, because of low sensitivity, it is hampered by lengthy acquisition (several minutes to several hours), due to the necessary t_1 increments in the second dimension to get better resolution. Second, 2D peak volumes are influenced by several factors {e.g., uneven excitation, non-uniform relaxation, J-couplings or transverse relaxation times (T_2) [29]}.

The first limitation of 2D qNMR can be solved by using an ultrafast single-scan 2D method, developed by Frydman et al. [30].

Shanaiah et al. have proposed a chemical-derivatization method for enhanced ^{13}C NMR detection of amino-acid metabolites in biological mixtures [31].

In future, methods based on dynamic nuclear polarization (DNP) for metabolomics applications will help by increasing the sensitivity by several orders of magnitude (~ 1000 times) [32].

To overcome the second limitation of 2D qNMR, the calibration-curve method has proved to be very useful. This method involves preparation of a standard-addition curve or a calibration curve using pure compounds of selected metabolites. This method is effective when a few metabolites are to be quantified, but it becomes quite tedious when a large number of metabolites are to be quantified.

Rai et al. published a method that can compensate for all types of errors that can arise (e.g., due to T_1 , T_2 , INEPT transfer, and uneven excitation) [29].

Hu et al. published another method, in which a series of HSQC spectra were acquired with incremented repetition times (the time between the end of the first ^1H

excitation pulse to the beginning of data acquisition) and extrapolated back to zero time to yield a time-zero 2D ^1H - ^{13}C HSQC spectrum (HSQC₀), in which signal intensities were proportional to concentrations of individual metabolites [33]. Relative concentrations determined from cross peak volumes can easily be converted to absolute concentrations by reference to an internal standard of known concentration.

8. Validation of qNMR

8.1. Accuracy and precision

Accuracy is defined as the closeness of agreement of a measured value with known true values, whereas precision is concerned with the ability to reproduce the same values in a series of experiments.

Malz and Jancke reported that the accuracy of qNMR for model mixtures is more than 98.5% [6], which implies an uncertainty of 1.5% in each measurement. To achieve accuracy with an error of less than 1.0%, all the parameters discussed above should be optimized with the utmost care. Sample properties (e.g., chemical interaction, and pH variation) will also affect the accuracy of qNMR, so these should also be taken into consideration. Quantitative results will be more precise when all the parameters are kept constant during measurements.

8.2. Linearity

In the process of calibrating the instruments, linearity should be tested every six months. Linearity is defined as the ability to obtain results that are directly proportional to the concentration of analytes. Linearity of any spectrometer can be tested using serial dilutions of a standard stock solution and recording their NMR spectra at identical conditions. Then least square linear regression

of signal response (Intensity/Area) with respect to the concentration of the standard stock solution is determined to tell how good the linearity of the method is. Linearity of all the modern NMR spectrometers is usually ≥ 0.999 [34].

8.3. Reproducibility

The electronic performances of NMR spectrometers are very stable. Spectra of a stable or standard sample stored in a sealed tube shows a reproducible integral area for a few years with a variation of less than 1.0% [35]. During an NMR experiment, there is no chance of any contamination, dilution, or interaction with the detector, so the probe-head permits repetition of experiment on a sample after a long time with excellent reproducibility. These factors allow reduction in expenditure on validation measurements when using the NMR method.

For testing reproducibility, a mixture of amino acids was prepared and sealed in an NMR tube. NMR spectra were recorded four times in a year. Before each experiment, pulse-length calibration was performed and only 0.39% variation in the peak area was observed.

8.4. Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD is defined as the minimum concentration or mass of analyte that can be detected at a known confidence level. The LOD depends upon the S/N. The LOQ is the lowest amount of analyte (concentration) in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

In qNMR spectroscopy, the experimental parameters can always be adjusted in such a manner that the desired S/N for detection and quantification of any component can be achieved. The LOD and the LOQ also depend upon the strength of the magnetic field, the sharpness of the peak and the coupling pattern of a signal. New developments [e.g., higher magnetic field (800–1000 MHz), CryoProbe and DNP] have to a great extent reduced the LOD and the LOQ.

8.5. Robustness

The robustness of an analytical method is a measure of its ability to remain unaffected by small but considerable variations in the analytical procedures (e.g., extraction techniques used for sample preparation, or changing the instrument, the place, or the analyst), and it provides an indication of reliability during normal practice.

In metabolomics or collaborative studies, robustness plays a vital role. Ward et al. conducted an inter-laboratory study to see the robustness of ^1H NMR spectroscopy and they reported that it is a highly robust technique for collaborative metabolomic data collection [36]. The robustness of an analytical procedure should

therefore also be checked before performing a qNMR analysis.

8.6. Specificity and selectivity

The International Conference on Harmonization (ICH) guidelines define specificity as the ability to assess analytes accurately in the presence of impurities, degradation products, and matrix components. Selectivity of a method is given by the ability to determine the analyte of interest in a complex mixture without interference from other components of the mixture.

In analysis of a mixture by NMR, at least one resonance from the metabolite of interest is required for quantitative estimation. The specificity and the selectivity of NMR are better because it provides structural information of analytes along with their concentration. These parameters may be affected by sample properties (e.g., chemical interaction between analyte and sample matrix), so specificity and selectivity of qNMR should be tested prior to analysis.

9. Application of qNMR Spectroscopy

In the past three decades, several articles have been published on quantitative or semi-quantitative NMR and its applications in the field of chemistry, biology and medicine [3]. We describe below some of the major applications of qNMR.

9.1. Pharmaceutical analysis

Turczan et al. published a number of articles on the quantitative analysis of pharmaceutically active drugs and excipients in their formulation and their purity determination using qNMR spectroscopy [4,37]. Their results suggested that qNMR is a very good method for quantitative analysis of drugs and drug excipients with an average of 0.5–2.0% error in the assay.

Similarly, other researchers also published several articles on quantitative analysis of active drugs and excipients by qNMR [6], which was also applied in quantitative analysis of the enantiomeric purity of pharmaceutically-active drugs [38].

qNMR has found use in the British Pharmacopeia (BP), US Pharmacopeia (USP) and European Pharmacopeia (EP) monographs for qualitative and quantitative analysis of several compounds [39].

9.2. Natural products

NMR spectroscopy is known to be one of the most suitable techniques for carrying out comprehensive qualitative and quantitative analysis of natural products. CryoProbes and microcoil probes offer significant advantages for the measurement of natural products at nmol concentration [40]. Analysis of natural products

by NMR spectroscopy provides the following information:

- (1) chemical structure and structure-activity relationships;
- (2) structural equilibria (e.g., tautomerism);
- (3) purity determination of bioactive agents;
- (4) determination of impurities; and,
- (5) exploration of the structural analogues and the metabolomics composition of mixtures [41].

NMR-based metabolomics has also been applied to plant sciences for various studies (e.g., analyzing fruits from different origins, the effect of drought, environmental stress, pathogenic stress, wild and transgenic types, developmental stages, cultivation and effect of soil on growth, classification of different species, quality assessment, genetic modifications in species, and fruit ripening) [3].

9.3. Organic synthesis

It is rather difficult to determine the concentration and the purity of new molecular species in a mixture synthesized by organic reaction. Concentration or purity determination of such molecules by analytical procedures other than NMR (e.g., HPLC, HPTLC, or UV) requires a specific reference compound (i.e. analyte in its pure form), which is generally available for well-known compounds only. Organic synthesis (i.e. combinatorial, parallel, high throughput synthesis) requires separation of components of the mixture for quantitation. NMR can easily be used for the determination of absolute purity (concentration) of individual components in a mixture along with their structural information.

9.4. Metabolomics

Metabolomics is the systematic identification and quantitation of all metabolites in a given organism or biological samples. The main analytical techniques used for metabolomics studies are based on NMR spectroscopy and mass spectrometry. Metabolomics is one of the major applications of quantitative or semi-quantitative NMR spectroscopy. NMR spectroscopy provides detailed information on the molecular structure of a wide range of compounds along with information about their concentration with high analytical precision.

Currently, qNMR-based metabolomics is widely applied (e.g., pharmaceutical research, animal-model study, toxicological studies, environmental analysis, nutrition and disease diagnosis) [5]. As metabolomics involves large sample sizes, subtle differences in pathological states can be identified by generating a disease-specific statistical model using chemometric methods. In this regard, supervised and unsupervised chemometric methods (e.g., PCA and PLS-DA) applied to the NMR spectra of biological samples utilize the quantitative information (semi or relative quantitation) of the spectra and help to differentiate between different groups.

9.5. Estimation of metal ions

Metal ions are analyzed in two ways by NMR:

- (1) first is by implementing metal NMR spectroscopy (if metal ion is NMR active); and,
- (2) second is by chelating the metal ion by ^1H -containing chelating agents.

Somashekar et al. published a method for analysis of calcium, magnesium and sodium in human serum, in which calcium and magnesium were analyzed by ^{23}Na NMR spectroscopy using chelating agent ethylenediaminetetraacetic acid (EDTA) and sodium [42].

9.6. Reaction monitoring and kinetic analysis

Since NMR spectroscopy can be used for qualitative and quantitative analysis of compounds, it can easily be implemented in on-line monitoring of chemical equilibrium and kinetic analysis, including rate of reaction, rate of product or impurity formation, and structural information based on the chemical shift, coupling pattern and coupling constants [43].

Monitoring of a chemical reaction using NMR does not need sampling in between the reaction (if performed in an NMR tube). This is one of the biggest advantages of using NMR to monitor chemical reactions.

Solution-state NMR (with static or slow spinning) cannot be applied for chemical reactions involving solid catalyst or reagents that settle down quickly. This slows down the reaction and does not allow mimicking the reaction as performed in the wet laboratory. Even in spinning conditions in solution-state NMR, isotropic mixing of the reaction mixture cannot be achieved for a reaction involving heterogeneous catalyst or reactants. In such cases, high-resolution magic angle spinning (HR-MAS) NMR spectroscopy is a better option, in which sample can be rotated at a desired speed (1–10 kHz). Abhijeet et al. optimized HR-MAS-NMR spectroscopy for kinetic analysis of the H_2SO_4 -silica-promoted reaction of anthranilamide with acetone and reported the rate constant for the reaction [44].

9.7. Flux analysis

Metabolic flux is the flow of matter in a metabolic network (i.e. rate kinetics of metabolites in metabolic pathways with respect to time) [45]. Each flux (specific to a biochemical reaction of conversion) reflects the function of a specific pathway within the network, and all the metabolic activities related to that pathway.

Metabolomic analysis of the composition of metabolites may be insufficient for complete understanding of system biology, metabolic phenotype or metabolism, but flux measurements could provide useful complementary information to characterize the metabolic networks and to understand their control and regulation [46].

NMR spectroscopy offers a very simple procedure for simultaneous measurement of multiple fluxes without separation.

Stable-isotope, tracer-based approaches (particularly ^{13}C) also offer excellent possibilities for tracking the metabolic fluxes and the conversion rates of metabolic precursors.

Recently, Albert et al. reported a very good method to study bacterial metabolic fluxes and conversion kinetics of specific biochemical reactions using ^{13}C uniformly-labeled glucose [47].

10. Discussion

NMR spectroscopy offers a good platform for the simultaneous quantitative estimation of multiple metabolites and their structural information. ^1H NMR has significant advantages over other quantitative analytical techniques (e.g., HPLC and GC). Chromatographic techniques depend upon the physical properties of molecules rather than their structural complexity. Using chromatography, it is difficult to conduct a straightforward analysis of compounds having low boiling point, low molecular weight, or lack of chromophores or that are too polar. Quantitative analysis of mixtures having medium levels of impurities with similar physical properties becomes a challenging task, whereas qNMR analysis of compounds with all the above-mentioned properties is usually simple.

Quantification of metabolites or molecules in a complex mixture can readily be accomplished and it requires only a single well-resolved signal. Highly complex mixtures create difficulties in quantitative analysis because of the high degree of spectral overlap. In such cases, nuclei other than proton (e.g., ^{13}C , ^{19}F , and ^{31}P) can be used to disperse the chemical shift, depending upon the sample concentration, the metabolites of interest and the sensitivity of the instrument used.

In metabolomic studies, up to 50 metabolites from urine spectra were quantified using NMR, and that would otherwise have been quite cumbersome by chromatographic techniques [28].

The major disadvantage of qNMR is the dynamic range of the analytes (i.e. two or more molecules present in the sample can have a very high concentration difference). However, this situation can be handled and absolute quantitation can be obtained in two steps:

- (1) first, quantitation of analyte having higher concentration can be carried out at auto RG value; and,
- (2) second, single or multi-solvent suppression techniques can be used to suppress resonances with a higher intensity, and precise quantitation of the analyte present in lower concentration can also be achieved.

Some of the internal standards used in qNMR create problems because of their chemical interaction with

analytes. In such conditions, reference compounds in capillary, ERETIC, QUANTAS and ARTSI methods are widely used to avoid the chemical interaction and to improve the quantitative accuracy.

Quantitative analysis by NMR using standard-addition or calibration-curve methods nullifies the effects of T_1 and several other experimental parameters on quantitative accuracy. This is the major advantage of calibration-curve and standard-addition methods, which can be used even at a shorter repetition time with a quantitative accuracy of 99% (Fig. 16). Fig. 16 demonstrates that the intensity of the signal varies – depending upon the pulse program and the experimental parameters. Nevertheless, the concentration calculated using the calibration-curve method has been found to be similar in all cases.

The only condition for calibration-curve and standard-addition methods is that all the experimental parameters should be kept constant during the establishment of the calibration curve and the analysis of test samples.

The major problem with these two methods is the establishment of multiple calibration curves when a number of metabolites need to be analyzed. The standard-addition method requires more sample volume for analysis of multiple metabolites, so it becomes difficult to analyze samples with less volume, especially in the case of valued or biological samples.

In qNMR spectroscopy, the acquisition and processing parameters that affect the quantitative accuracy require careful optimization before the quantitative analysis. There are several points where the analyst can introduce errors (e.g., manual phase correction, integration of peaks and other processing steps), so the analyst should be aware of all the experimental parameters and their appropriate settings for better accuracy and precision.

2D qNMR (^1H - ^{13}C HSQC) suffers from additional experimental parameters (e.g., INEPT transfer, offset, uneven excitation, non-uniform relaxation, J-couplings or transverse relaxation times). Standard-addition and calibration-curve methods have been used by many researchers to remove the quantitative inaccuracy that arises due to the above parameters [48].

In the case of highly overlapped spectra, integration of peak area requires a good knowledge of curve-fitting methods. Software packages (e.g., Chenomx) are routinely used to analyze NMR spectra. Chenomx, which has inbuilt curve-fitting methods, is easy to use for quantitative analysis of molecules in overlapped spectra (e.g., spectra of biofluids) [15,28]. Deconvolution programs that can also be used for integration of overlapping peaks, are usually available in all NMR software.

Comparing qNMR and chromatography in targeted quantitative analysis of pharmaceutical products (e.g., syrups, tablets, capsules, and injections) shows that chromatographic techniques are better. Only a few active drugs have been recommended by USP, BP and EP

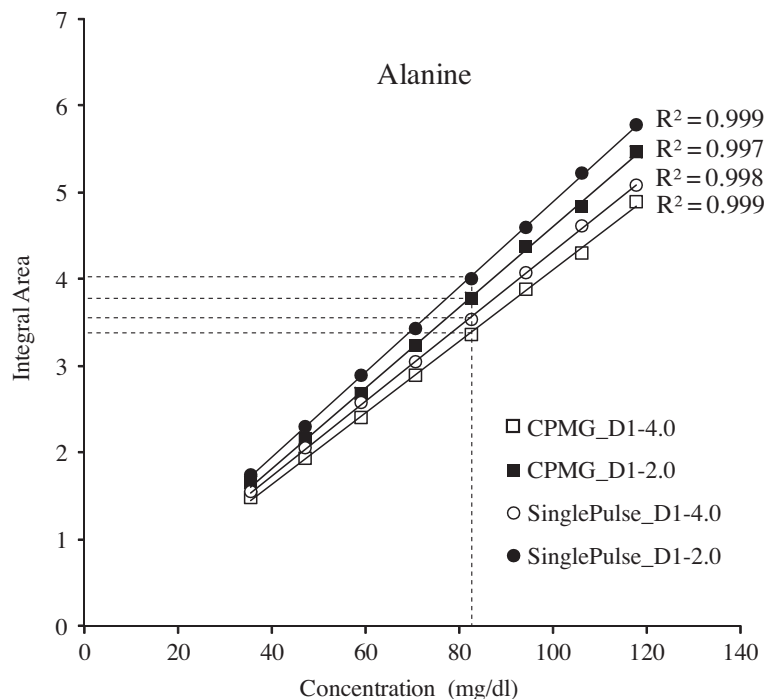


Figure 16. Quantitative analysis of alanine using calibration-curve methods generated at different experimental parameters. Calibration curves were generated by single pulse and CPMG with water pre-saturation at relaxation delay of 2.0 s and 4.0 s. Echo time for CPMG experiments was 270 ms. Alanine concentration was calculated for $-\text{CH}_3$ signal at 1.48 ppm and was found to be 82.25 ± 0.13 mg/dL (actual concentration was 82.32 mg/dL).

for qualitative and quantitative analysis using NMR spectroscopy [37]. In pharmaceutical analysis, well-developed, optimized and molecule-specific methods are used for the quantitative estimation of active drugs or formulation excipients.

Although NMR is a non-selective, non-specific standard, non-specific methods and optimized parameters can be used for all compounds. Due to these advantages, qNMR spectroscopy has a wide range of applications in analysis of pharmaceuticals in order to identify a drug and its embedded impurities. The major applications of qNMR in this area are to evaluate the level of impurities along with their structural information, course of degradation, related impurities, content of residual solvent, isomeric composition and molar ratio [37].

NMR spectroscopy is the most suitable technique for the identification and the qualitative analysis of mixtures, where some of the compounds are unknown. However, quantitative and qualitative analysis of an unknown mixture by chromatographic methods is a challenging task. Optimization of the chromatographic method and quantitation of all the components in such a mixture require extensive time and effort. They also require all the components in their pure form as a reference standard for quantitative analysis. In qNMR, a single reference molecule can be used for quantitative

analysis of all components present in the sample, so, for qualitative and quantitative analyses of natural products (plant extract) or highly complex mixtures (e.g., biofluids), NMR spectroscopy is a better option.

An NMR spectrometer with 300–400 MHz frequency is sufficient for the quantitative analysis of samples in the mmol range [41]. Increase in the magnetic field strength dramatically reduces the LOD to μmol concentration. Currently, commercial magnets up to 1 GHz are also available and developments in the inverse and Cryo-Probe technologies have further increased the sensitivity by a factor of four, so quantitation of metabolites, drugs, drug impurities, and natural products, which are in very low concentrations (even at the nmol scale) is easy by NMR spectroscopy [40]. CryoProbe also offers a better facility for the quantitation of analytes at μmol concentration using nuclei other than proton (e.g., ^{13}C and ^{31}P) [28]. It disperses the chemical shift in the ^{13}C dimension for quantitation of metabolites in the NMR spectra of biofluids (e.g., urine), whereas ^{31}P NMR quantifies phosphorous-containing metabolites.

In addition to other technologies, DNP technology has dramatically increased the sensitivity of NMR by up to ~ 1000 times [49]. It requires additional accessories for nuclear polarization that add more sophistication to the NMR system, so it is not viable for routine metabolomics

or pharmaceutical analysis. Oxford Instruments has tried to reduce the sophistication of DNP and made it more compact than the manual set-up. Currently, it is commercially available under the name “HyperSense” (Oxford Instruments, UK) with automatic, fast sample transfer to the NMR magnet [50].

Appendix A. Simple program for addition of QUANTAS signal in real spectrum of analyte

This program can be implemented in any version of XWINNMR as well as TOPSPIN at LINUX/WINDOW workstations. The program is described below.

```

/******
/* add_signal
/* adds signal at the given ere_offset
/* the signal strength (height) is scaled by the parameter ere_scale
/* in third line from last after ppm, 1 1 are experiment, process no.,file
/* name, directory and then disk directory of simulated signal.
/******
FLOAT ere_scale, ere_offset;
char ere_dat [0];
ere_offset=-1.0
ere_scal=0.005
GETFLOAT ("Signal offset : ", ere_offset);
GETFLOAT ("Scaling factor : ", ere_scale);
sprintf (ere_dat, "accumulate %f %f ppm 1 1 signal_name directory_name d:\\", ere_offset, ere_scale);
XCMD (ere_dat);
QUIT

```

11. Conclusion

Quantitative NMR is relatively rapid and easy to implement for analysis (e.g., mixtures, metabolomics, pharmaceutical preparations, and natural products) because of the specific advantages it offers, namely:

- (1) determination of molecular structure (i.e. verification of the authenticity by direct structural evidence);
- (2) short measuring time;
- (3) non-destructive nature, so that samples can be used for other analysis;
- (4) no necessity for isolation and purification of analyte from its mixture (i.e. easy sample-preparation steps);
- (5) quantitation of multiple components using single reference compounds (i.e. no specific reference required);
- (6) ratio-determination step that does not need any intensity calibration; and,
- (7) better accuracy and precision.

Method-validation parameters (e.g., precision, accuracy, linearity, and reproducibility) qualify NMR as a quantitative analytical technique.

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In the above program, lines starting from /* give a brief description of the program and the other lines that define the program. The entire program can be copied and saved in a program directory as a text file (in the directory: /opt/xwinnmr(TOPSPIN)/exp/stan/nmr/au/src/user). The third line needs editing to define the path of simulated signal where it is located with the other NMR data.

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