QUANTITATIVE NMR ASSAYS (qNMR)

Accuracy and Long-Term Stability of qNMR Methods with External Calibration

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Innovation with Integrity

Quantitative Nuclear Magnetic Resonance spectroscopy (qNMR) is a powerful technique that can be used to determine the concentration of analytes in pure compounds as well as in mixtures. qNMR methods are well established with many applications in fields like pharmaceuticals, natural products, metabolomics, food science, material science etc. and their measurement uncertainty performance is fully understood. Depending on the analytical question to be addressed the methods can be designed without calibration (relative (100 %) qNMR), internal calibration (IC-qNMR) or external calibration (EC-qNMR) (Figure 1). This paper aims to present that external calibration offers unique virtues especially for the quantification of intrinsically mass-limited samples. The analytical performance is convincing and is easy to implement. The basic principle of EC-qNMR breaks down to at least two independent measurements. The first sample contains the reference compound with known concentration. It is analyzed in quantitative conditions to establish the initial calibration. After that an arbitrary number of analyte samples can be quantified based on the initial calibration. The quantification tool ERETIC2 available within TopSpin supports the automated calculation of the analyte concentrations and helps to manage several EC-qNMR references in parallel. In discussions with customers, we have learned that the choice of an appropriate reference sample can be a hurdle which may render it difficult to implement EC-qNMR. In this study we demonstrate that the Bruker standard sample Trifluorotoluene (TFT) in CDCl3 can serve as a viable reference to get started with this powerful method. It is readily available on Bruker NMR Systems equipped with the BBFO type "workhorse" probes. In this study we use TFT as a reference to measure the concentrations of a simple mixture sample containing two analytes. The results of the quantitative analyses show the excellent performance of the method with a trueness significantly lower than 5 % and a precision well below 0.5 %.

Introduction

The wide applicability and potential of qNMR applications has been summarized in the several recent perspective articles [1,2]. The method fulfils highest metrological standards, the results are SI-traceable, and it is recognized as a primary ratio analytical method by the *Commité Consultatif pour la Quantité de Matière* (CCQM) [3]. This infers that qNMR does not require analyte specific reference compounds. For other widespread analytical technique such as HPLC, infrared spectroscopy or mass spectrometry the analyte itself must be available as a certified reference material (CRM) to calibrate the instruments. For NMR on the other hand, in principle any CRM can be used as a calibrant. Conceptually qNMR methods can be divided into three different approaches: (i) ratio measurements (relative (100 %) qNMR), (ii) potency determination (IC-qNMR) and (iii) content measurements (EC-qNMR) (Figure 1). All methods have in common that the ratio of two signal integrals is measured which allows — in combination with further data like sample weight, stoichiometry, molar mass, etc. — to determine quantitative results.

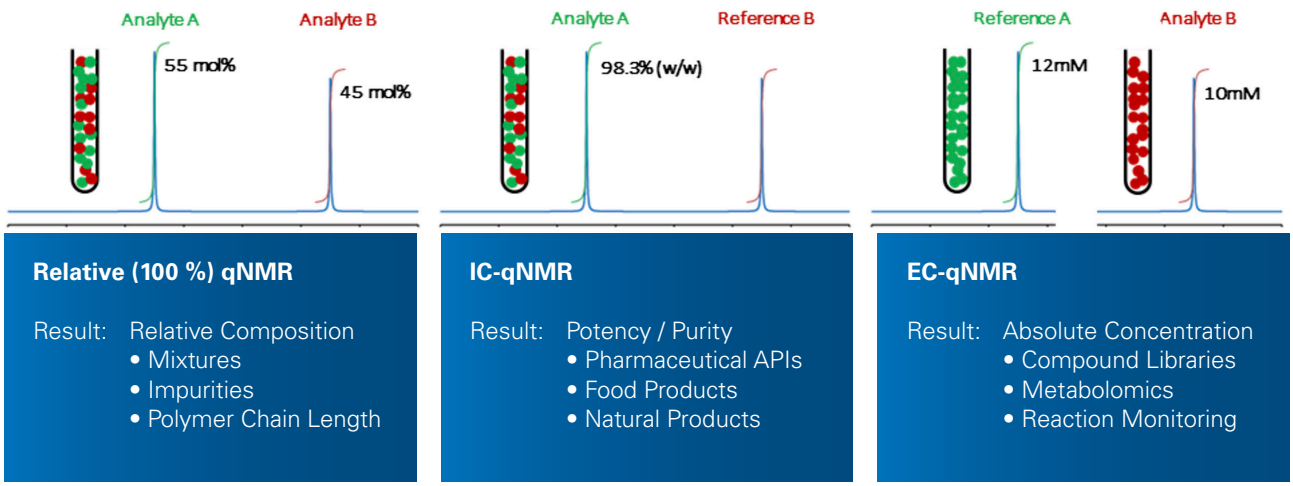

Figure 1 Concept of relative (100 %) qNMR without reference, IC-qNMR with internal reference and EC-qNMR with external reference.

This paper is focused on content measurements based on the EC-qNMR method. In contrast to the other qNMR methods, separate samples will be analyzed. One sample containing the reference compound in a solution of known concentration, the others containing the analytes. The EC-qNMR approach can be further subdivided as follows. (a) The reference solution is kept in a coaxial insert and is measured together with the analyte solution. (b) The reference signal is electronically generated [4]. (c) Direct measurement of concentrations based on the PULCON principle [5, 6]. The latter approach does not require the special hardware or software needed for electronic referencing and the sensitivity of the measurement is not reduced by a coaxial insert. The PULCON EC-qNMR is therefore just as easy to implement as IC qNMR, which make them both excellent candidates in the framework of routine quantitative analyses. Although both methods are very similar the IC-qNMR method is considered the gold standard for quantification because it eliminates certain error sources (variation of the sample volume or the flip angle, long term stability of the instrument, etc.) by simply measuring analyte and reference standard at the same time in the same sample. Nevertheless, EC-qNMR is capable to avoid typical drawbacks of internal referencing like signal overlap and chemical reactions between analyte and standard. Since the reference standard and the analyte are prepared as separate samples the EC-qNMR method offers more flexibility with respect to the choice of the NMR solvent. In addition, the method is more cost effective, because smaller amounts of the CRMs are needed. The aim of the paper is to investigate the capabilities of PULCON EC-qNMR, to provide guidance on how to reduce potential bias and to demonstrate how convenient the process can be organized by the help of the TopSpin component ERETIC2.

The ERETIC2 workflow

As already suggested the typical quantitative analysis workflow using ERETIC2 is divided into two separate parts each based on three steps.

A – Establishment of the Concentration Reference

Step A1: Preparation of the external reference sample (Reference A in Sample Tube 1). This requires precise weighing of the CRM and precise determination of the solvent volume to obtain a well-defined concentration of the reference solution. It is recommended to seal the sample tube so it can be used for repeated calibration of the NMR system.

Step A2: qNMR measurement of the external reference sample. The acquisition parameters required for quantitative measurement conditions will be discussed below.

Step A3: Processing of the qNMR spectrum and integration of the signals of interest. The processed spectrum will then be registered as a concentration reference in the ERETIC2 tool. This will require the number of nuclei contributing to the signals used for quantification and the CRM's molar concentration.

The reference spectrum can be acquired once a week or even once a month and saved in the ERETIC2 database. It is not required to repeat the steps A1 to A3 every time prior to an Analyte Concentration Measurement The frequency with which the calibration should be repeated is discussed in the results section.

B – Analyte Concentration Measurement

Step B1: Preparation of the analyte sample (Analyte B in Sample Tube 2). Precise weighing of the CRM and precise determination of the solvent volume is only required if the potency of the analyte shall be determined. For the measurement of the analyte's molar concentration the sample weight is not needed, only the number of nuclei contributing to the signals used for quantification.

Step B2: qNMR measurement of the analyte sample. It is recommended to use the same parameters for the acquisition of the analyte data as had been used for the reference sample.[1] The possible error sources associated with certain acquisition parameters will be discussed below.

Step B3: Processing of the qNMR spectrum and integration of the of the signals of interest. This is followed by the quantitative analysis in ERETIC2 which typically results in the analyte's molar concentration.

The detailed procedure on how to perform a quantitative analysis using ERETIC2 is explained in the PDF document "ERETIC2" [7] among the Acquisition – Application Manuals on TopSpin's manual. It is recommended to refer to this document before performing any quantitative analysis using this method.

Accuracy

According to ISO 5725-1 the general term *accuracy* is used to describe the closeness of a measurement to the true value [8]. Deviations are attributable to systematic errors (*trueness*) and statistical errors (*precision*).

The trueness describes the closeness between the average value obtained from a large series of test results and an acceptable reference value and is defined as

$$\Delta(\%) = \frac{c - c_{ref}}{c_{ref}} \cdot 100 \qquad (1)$$

where *c* represents the measured concentration (experimental value) and c_{ref} the actual concentration of the sample e.g. derived from the analyte weight, its potency according to the manufacturer's certificate of analysis and the solvent volume.

The precision can be measured by repeating n times the same experiment on a single NMR spectrometer using the same sample and then determining the relative standard deviation (RSD) expressed as

$$RSD(\%) = \frac{\sigma}{\mu} \cdot 100 \qquad (2)$$

Where σ and μ are the standard deviation and the mean of the measured peak areas (or concentrations), respectively.

[1] The PULCON equation allows to correct for changes in the NS and the receiver gain. D1, AQ and TD can be modified, if necessary, without compromising the accuracy of the result. The digital resolution in the frequency domain (as defined by SWH and SI) is critical, though and should be kept constant.

Quantitative Acquisition Conditions

The NMR experiments of an EC-qNMR measurement have to be performed under certain conditions which will ensure accurate quantitative results.

Repetition Time TR

Full relaxation is one of the most important premises for qNMR experiments. The repetition time *TR*, which is the sum of the relaxation delay (*D1*) and the acquisition time (*AQ*), shall be set to at least $5 \cdot T_1^{max}$ if a 90° hard pulse is applied or at least $3 \cdot T_1^{max}$ for a 30° hard pulse to allow the longitudinal magnetization to return to its initial state. T_1^{max} being the largest longitudinal relaxation time of the analyzed molecule. This ensures a maximum potential bias of less than 1 % (Table 1). The actual bias depends on the ratio between T_1 of the CRM and T_1 of the analyte. If both T_1 values are of similar magnitude, the bias is less pronounced even if *TR* is quite short. *TR* can be optimized for each spectrum individually.

	30°	90°	ε^{max}
TR/T_1 ratio required as a function of the flip angle of the hardpulse and the maximum accepted error ε^{max}	1,3	3	5 %
	2,7	4,6	1 %
	3	5	0.7 %
	4,9	7	0.1 %

Table 1 For a given flip angle of the hard pulse, the choice of TR/T_1 determine the maximum potential bias ε^{max} on the measurement of an NMR integral.

Sample Tube Diameter

Since the intensity of any NMR signal is directly proportional to the number of nuclei present in the active volume of the NMR probe, the diameter of the sample tube is a crucial parameter for the accuracy of EC-qNMR measurements. Depending on the quality of the NMR tubes the variations of the sample tube diameter may vary and it is recommended to avoid the usage of economy grade tubes.

Sample Preparation

Obviously, the sample preparation has an important impact on the accuracy of any EC-qNMR experiment. The weighing of the analytes shall be performed with a calibrated analytical balance and the minimum weight, and the measurement uncertainty of the balance must be considered. Also, it is important to ensure full solubility of the analytes before beginning the NMR experiments.

SW, SI, Digital Resolution

The digital resolution in the time domain is determined by the number of time domain data points (TD) and the inverse of the spectral width (SW). They should be optimized to record a fully relaxed FID. In the frequency domain it should be taken care that at least ten data points above the half maximum are present for the narrowest signal which is used for the quantification. This can be adjusted with the size of the real spectrum (SI).

Receiver Gain

The leverage of the receiver gain (RG) is fully accounted for by the ERETIC2 tool. Therefore, it is not strictly required to perform the reference and the analyte measurement at the same RG level. Below we will present data to assess the actual impact of RG on the overall measurement uncertainty.

Signal to Noise Ratio, Number of Transients (NS)

The precision of the qNMR experiment is strongly correlated with the SNR of the spectrum. Therefore, the SNR of the signals which are targeted for quantitative integration should be at least 150:1 [9]. Higher SNR will further improve the measurement uncertainty. In the context of EC-qNMR it is straight forward to adjust the SNR by choosing an appropriate number of transients (NS). If a repetition time larger than $5 \cdot T_1^{max}$ is chosen, dummy scans are not necessary, unless ^{13}C decoupling is used.

Experimental

Hardware and Samples

The NMR experiments were performed on a Bruker AVANCE NEO 400 MHz equipped with a 5 mm broadband observed probe (SmartProbe iProbe) at 298 K. the Bruker standard sample 0.05 % (4.072 mM) aaa-Trifluorotoluene (TFT) in Chloroform-d (CDCl3) was used as the EC-qNMR reference. The analyte was a mixture of 23.501 mg/g (110.340 mM) of 1,2,4,5-Tetrachloro-3-nitrobenzene (TCNB) and 4.999 mg/g (36.407 mM) of 1,3,5-Trimethoxybenzene (TMXB) in a DMSO-d6. This mixture is available from Supelco named as *Bruker quantitative PQ CRM* (42350). Three aliquots of each sample were analyzed in separate sealed NMR tubes. Two TFT samples had been produced in 2021 (TFT1 and TFT2) and the third in 2018 (TFT3). All qPQ samples were from 2019 (qPQ1, qPQ2 and qPQ3). The targeted signal of TFT is a multiplet between 7.61– 7.71 ppm (figure 2) while the targeted signals of TMXB and TCNB are singlets at 6.1 ppm and 8.5 ppm (figure 3). The signal of TMXB at 3.70 ppm has not been quantified since it is partially overlapped by the signal of residual water from DMSO-d6 thus affecting the accuracy of the result.

Although the Bruker standard sample TFT was used here as an external reference for ^{1}H qNMR, it could also be used for ^{19}F qNMR. The preconditions required for sample to be used as an external standard are as follows: a known concentration, a known purity and an NMR signal being well resolved. Hence, besides being simple to implement, this method allows a wide range of reference samples, making it very useful to quantify different nuclei (^{1}H, ^{13}C, ^{19}F) and thus a diverse set of samples and applications.

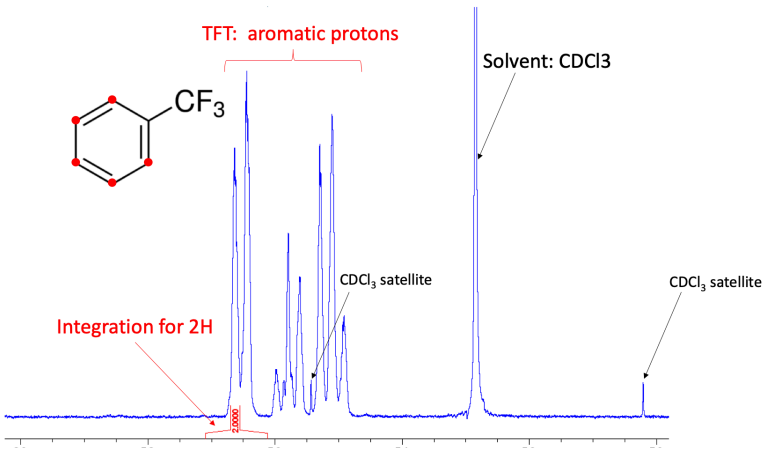

Figure 2: 1D ^{1}H spectrum of the TFT sample acquired using the P_PROTON parameter set on an AVANCE NEO 500 MHz NMR spectrometer. TFT presents several signals between 7.61 and 7.71 ppm. All those signals are accounting for 2H and were integrated within a single integration region.

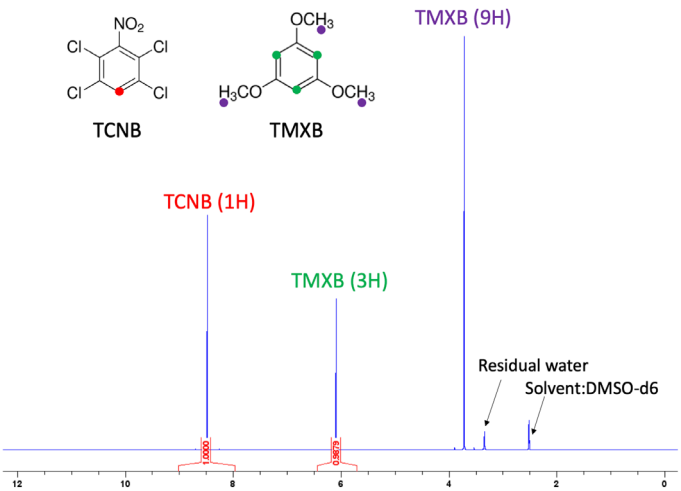

Figure 3: 1D ^{1}H spectrum of the qPQ sample acquired using the P_PROTON parameter set on an AVANCE NEO 500 MHz NMR spectrometer. TCNB presents one signal at 8.1 ppm while TMXB presents two signals at 3.7 ppm and 6.1 ppm.

Optimization of the Experimental Parameters

The acquisition and processing parameters for the EC-qNMR experiments were set-up by using the Bruker library parameter set P_PROTON. This parameter set has been created particularly for qNMR measurements. The large number of points in the time domain (TD=256 k) and the large spectral width (SW=20 ppm) ensures that the acquisition time (AQ >15 s) is long enough that the FID will not be truncated. Since the concentrations of typical qNMR samples are large enough the experiments are not critical with respect to SNR and optimization of AQ for analytes with shorter relaxation times is not required. The Bruker library contains several variants of this parameter set (e.g with 30° flip angle or adiabatic ^{13}C decoupling). Details about how to use it can be found in the PotencyMR manual [10]. For this study the following parameters have been optimized: (i) the relaxation delay *D1*, (ii) the transmitter frequency offset O1p and (iii) the receiver gain RG. The repetition time *TR* is equal to *D1+AQ*. *AQ* was kept as the default value from the parameter set P_PROTON and *D1* was set to $5 \cdot T_1^{max}$. The longitudinal relaxation time T_1 has been measured for each NMR signal using the inversion recovery method. The maximum T_1 values for the TFT samples were 10 s and for the qPQ sample 22 s which corresponds to the T_1 of TCNB. Therefore, *D1* was set to 50 s or 110 s, respectively. The results are given in Table 2.

	T_1 ^1H (s)	D1 (s) (P_PROTON)
TFT	10	50
TCNB	22	110

Table 2 $T_1(^1H)$ values of TFT and TCNB for ^1H quantitative NMR. To be in quantitative conditions, a TR/T_1 ratio of 5 has been used in this study.

The receiver gain (RG) value is calculated automatically on TopSpin using the command "rga". Although, different values of the receiver gain (RG) will be accounted for in the ERETIC2 tool, it is advised to work with similar or better identical RG values, because it is a potential source of error for EC-qNMR analysis which can easily be avoided. The acquisition and processing parameters for the quantitative NMR experiments in this study of TFT and qPQ are detailed in Table 3.

BRUKER AVANCE NEO 400 MHz	
P_PROTON	
PulseProg	zg
NS	16
DS	0
AQ (s)	15.99
T1	TFT = 10 s / TCNB = 22 s
D1 (s)	50 (TFT) / 110 (qPQ)
TD	256 k
SW (Hz)	20
O1p (ppm)	5
RG	101
DW (µs)	50
SI	512 k
LB (Hz)	0.1
t_{exp} (min)	17 (TFT) / 33 (qPQ)
WDW	EM

Table 3 Selected experimental parameters. t_{exp} : corresponds to the duration of a single 1D ^1H experiment (i.e. one spectrum).

Results

Quantitative analyses of three qPQ samples with respect to three TFT samples: assessment of the accuracy and robustness of the EC-qNMR method

For each individual sample tube (three TFT and three qPQ) five consecutive spectra were recorded with experimental conditions summarized in Table 3. All 30 spectra were processed with the same workflow.

- Zero-filling, apodization and FT according to Table 3
- Phase correction
- Baseline correction
- Definition of integral ranges
- ERETIC2 analysis
- Values exported to Excel for statistical analysis

For the ERETIC2 analysis the qPQ samples were considered as the analyte while the TFT samples served as the reference. Each of the three qPQ samples were quantified with respect to each of the three TFT samples. Since each qPQ sample contains two distinct components (TMXB and TCNB) this setup results in 18 analyte-reference combinations. For each of these combinations five times five spectra were submitted to the ERETIC2 quantification, resulting in 25 concentration values for TMXB and TCNB, respectively. Considering each of the analyte-reference combinations this resulted in 450 concentrations values in total. Based on this data set, the trueness, and the precision of the EC-qNMR method were estimated according to equation (1) and (2), respectively.

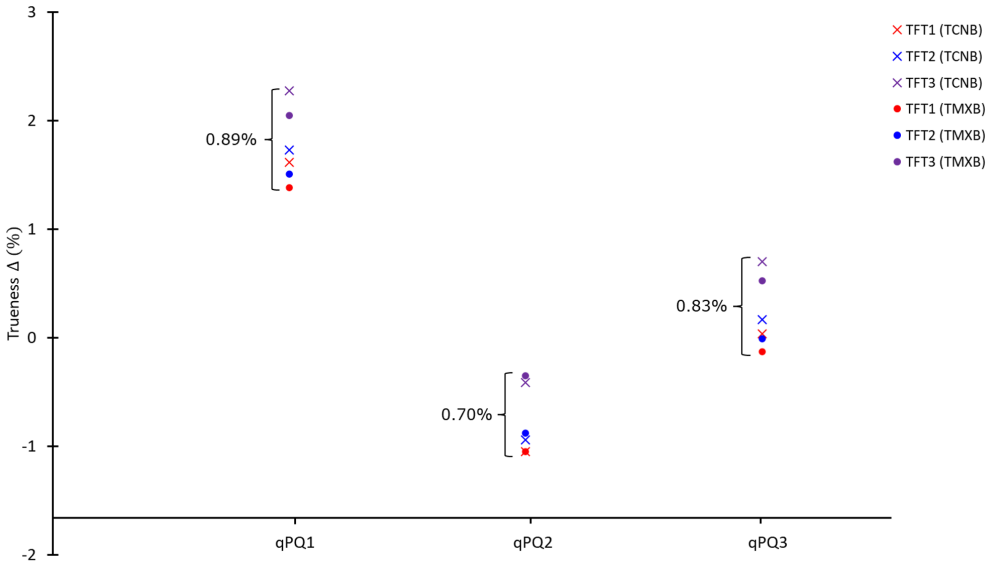

Figure 4: Quantification of TMXB and TCNB from three different qPQ samples using three different TFT samples as external reference. ^{1}H NMR analyses were performed using the P_PROTON parameter set on a 400 MHz NMR spectrometer. The data points indicated as crosses represent the results for TCNB and those indicated as filled circles for TMXB, respectively. The color coding indicates the reference sample (red: TFT1, blue: TFT2, purple: TFT3).

The results from the quantitative analyses are shown in Figure 4. Each dot on the graphic represents the trueness found for a particular analyte-reference combination. Comparing the results for TCNB and TMXB from one analyte sample (e.g. qPQ1) with respect to one reference sample (e;g. TFT1) shows deviations ranging from 0.05 % to 0.23 %. Within these combinations several error sources of EC-qNMR measurements like variations of the sample diameter are excluded and the spread of the data is most likely dominated by the short-term to mid-term stability of the hardware. The results, which are in the regime of IC-qNMR measurements, demonstrate the excellent performance of Bruker NMR spectrometers.

If the comparison is extended to one analyte sample (e.g. qPQ3) vs. all the three reference samples (TFT1, TFT2 and TFT3), the trueness value Δ(%) cover a range of 0.89 % for qPQ1, 0.70 % for qPQ2 and 0.83 % for qPQ3. The range of 0.70 % - 0.89 % is presumably due to variations of the sample diameter but also differences in the actual concentrations of the TFT reference samples which originated from different production batches.

Comparing the trueness values found for the three qPQ analyte samples with respect to one TFT reference sample show a maximum difference of approximately 2 % with the highest values found for qPQ1, the lowest values for qPQ2 and the intermediate values for qPQ3. The ranges are larger as for comparing one qPQ sample vs. three TFT samples. Assuming that the variations of the sample tube diameters are in the same order of magnitude it would mean that the variations of the sample concentrations within the qPQ samples would be larger than within the TFT samples.

Long-Term Repeatability

A significant advantage of EC-qNMR method is that an arbitrary number of analytes can be quantified based on the initial calibration of one standard sample used as external reference. It means that, it is not necessary to calibrate the external reference right before each quantification of the analyte. Nevertheless, the validity of the initial calibration depends on the stability of the NMR hardware. Hence, there is much debate in the qNMR community about the frequency at which the external calibration has to be renewed. Following the principles of analytical quality by design the decision shall be based on a general risk assessment and continuous advancement. To demonstrate this approach, a long-time repeatability of the method has been evaluated by performing quantitative analyses of one qPQ sample with respect to one TFT reference sample over one and a half months. The variation of the obtained precision and trueness values has been observed. The initial calibration has been established with sample TFT1 with five consecutive spectra. The sample qPQ1 has been analyzed nine times at t_0; t_0+1d; t_0+2d; t_0+3d; t_0+7d; t_0+10d; t_0+16d; t_0+38d and t_0+43d, respectively. For each analysis, five consecutive spectra were acquired as well. The results were averaged over all combination similar to the procedure described above, resulting in 25 concentration values for TMXB and TCNB per measurement day. The trueness and the precision of the EC-qNMR method were estimated according to equation (1) and (2), respectively.

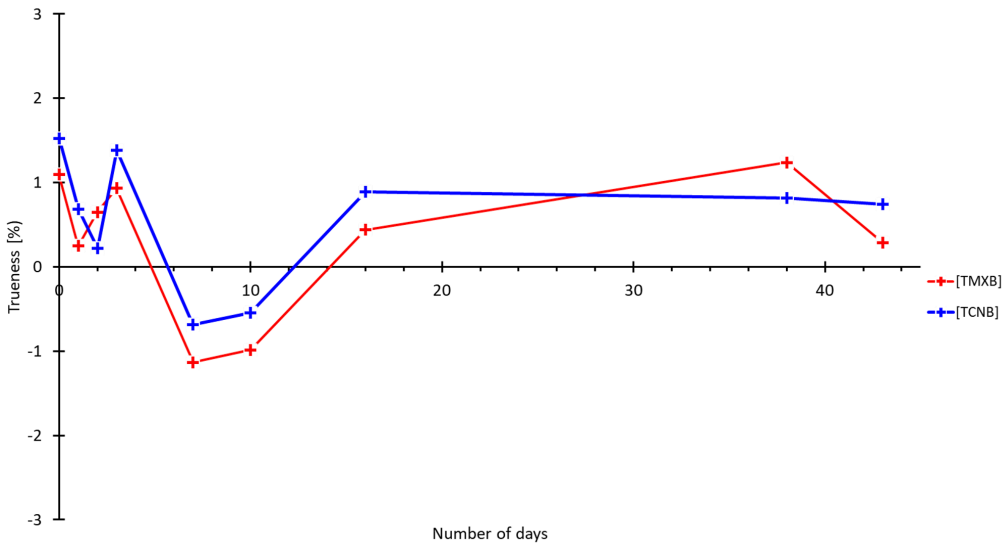

Figure 5: Quantification of TMXB and TCNB from sample qPQ1 using the sample TFT1 as external reference. The sample TFT1 was analyzed once at t0 while qPQ1 was analyzed nine times over one month and a half t_0 to t_0+43 days. The red and blue data points represent the trueness of the quantification of TMXB and TCNB respectively.

The results from the quantitative analyses performed over 43 days are shown in Figure 5. The trueness values Δ(%) of TMXB and TCNB cover a range of 2.4 % and 2.2 %, respectively. Each value was obtained with a precision well below 1 %. This demonstrates that the long-term stability of Bruker NMR systems is compatible with the overall accuracy of the EC-qNMR method. It must be emphasized, though, that the data presented here demonstrates the longterm stability of one particular instrument. Users of the EC-qNMR method are encourage to assess the performance of their own instruments by acquisition of similar time series in order to establish methods with proven confidence.

Receiver Gain

There is common preconception in the qNMR community that it is imperative that EC-qNMR measurements must be performed at identical receiver gain levels. Nevertheless, the correlation between the signal intensity and the RG value is well understood and an appropriate compensation is implemented in ERETIC2. Figure 6 shows that the trueness of EC-qNMR quantifications varies with the receiver gain but not more than 3 % total. If measurements at the same RG level can be carried out without problems this would be desirable, because the receiver gain can be an additional source of error but it is not strictly necessary.

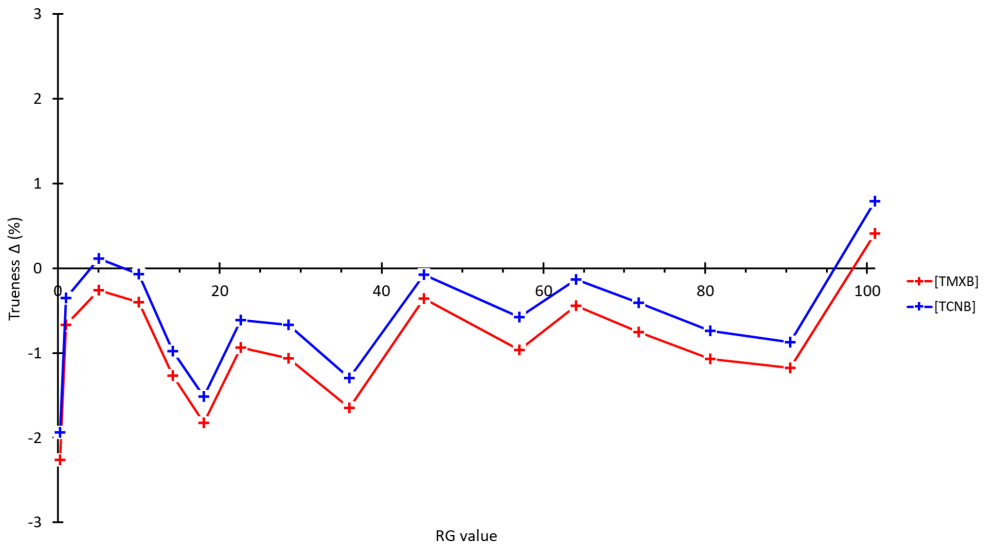

Figure 6: Quantification of TMXB and TCNB at different RG values. The external calibration experiment with TFT had been carried out with RG=101. The RG for the analyte sample has been varied from 0.25 to 101.

Conclusion

The results presented in this study demonstrate that the EC-qNMR method allows to perform accurate quantitative analyses. The workflow can be streamlined by use of the quantification tool ERETIC2. The trueness of the results spans a range from -1.05 % to 2.28 % which is well within the range which is commonly accepted in the qNMR community. The Bruker standard sample TFT which was used as the external standard here, is provided with many NMR systems by default. It is therefore readily available in many NMR labs and Bruker customers can use it to get started with quantitative measurements without the need for additional hardware, software, or expensive CRMs. For laboratories who will perform qNMR according to ISO 17025 or GxP compliant regulations it is nevertheless recommended to work with established CRMs.

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