Development of Certified Reference Material Solutions for qNMR and Instrument Performance Qualification

Christine Hellriegel, Alexander Rueck, Kathrin Breitruck, and Markus Obkircher

Abstract Over the last decades quantitative NMR (qNMR) spectroscopy has become an important tool for the content determination of organic substances and the quantitative evaluation of impurities. Since the signal intensity is directly proportional to the number of protons contributing to the resonance, qNMR is considered as a relative primary method [1, 2, 3]. Quantitative NMR in combination with metrological weighing was optimized to show the power of the measurement method [4]. Traceability to the SI is achieved using primary Reference Materials from the National Institute of Standards and Technology (NIST) [5] and the National Metrology Institute of Japan (NMIJ) [6]. Neat material CRM for qNMR is already well established, and here we present the development of new CRM in deuterated solvent, which are ampuled and ready-to-use. Furthermore, a comparison of the results between qNMR measurements and gravimetric results of the preparation of the solutions under an ISO 17034 and ISO/IEC 17025 accreditation is presented.

Keywords qNMR · CRM (Certified reference material) · Metrological traceability · Accreditation · Certification · Instrument performance qualification · ISO 17034 · ISO/IEC 17025 · Gravimetry · Quantitative nuclear magnetic resonance · Measurement uncertainty

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Introduction

In recent years, quantitative Nuclear Magnetic Resonance (qNMR) has become widely accepted as a very efficient and precise method for the quantification of organic compounds in many fields. Quantitative NMR has several outstanding advantages compared to other analytical techniques regarding purity determination, [4] especially the indifference towards impurities. An analyte can be measured directly against a reference and impurities do not affect the result as long as one single signal from sample and reference is pure enough for integration. This also includes non-detectable impurities such as salts and residual water. Jancke et al. proposed qNMR spectroscopy as a relative primary analytical method for measurement results at the highest metrological level since it can be described completely by mathematical equations [2, 7, 8].

The signal integrals are in direct proportionality with the number of protons or other nuclei contributing to the resonances [1]. Hereby, the structures of the chemical substances are fully irrelevant. Hence, a direct metrological traceability to the "système international "(SI) through internationally accepted primary reference standards can be achieved by comparing signal intensities of analyte and reference substance, which is usually not possible with chromatographic techniques [3, 9]. Since qNMR is increasingly used in the pharmaceutical industry and analytical testing laboratories, there is a growing demand for certified reference materials (CRM) for applications in different markets and fields. Sigma-Aldrich Production GmbH is holding an ISO/IEC 17025 [10] and ISO 17034 [11] accreditation as a manufacturer of certified reference materials for quantitative NMR since 2009. Through an accredited certification process, several CRM for the intended use in qNMR as well as for chromatographic applications were generated. In this workflow, various preliminary tests are first carried out since it has to be ensured that internal reference and analyte do not react and that peaks used for integration do not overlap. Subsequently, high-precision weighing is carried out, followed by the actual qNMR measurement, determination of the homogeneity, and setup of a full uncertainty budget. For these measurements, internationally accepted references (for example NIST SRM), which ensure metrological traceability to the SI, are crucial. At present, there are primary calibrants available from the National Metrology Institute of Japan (NMIJ)[1] and the National Institute of Standards and Technology (NIST),[2] which are designed for the intended use in qNMR. For one of those materials, NIST collaborated with Sigma Aldrich Production GmbH and the National Research Council Canada (NRC), to produce and certify

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[1] NMIJ CRM 4601-b No. +++ 3.5-Bis(trifluoromethyl)benzoic Acid for Quantitative NMR (^{1}H, ^{19}F). https://unit.aist.go.jp/nmij/english/refmate/crm/cert/4601b_en.pdf.

NMIJ CRM 4602-A No. +++ 1.4-Bis(trimethylsilyl)-2,3,5,6-tetrafluorobenzene for Quantitative NMR (^{1}H, ^{19}F). https://unit.aist.go.jp/nmij/english/refmate/crm/cert/4602a_en.pdf accessed May 25th, 2021.

[2] NIST PS1 Primary Standard for quantitative NMR (Benzoic Acid). Certificate of analysis. https://www.nist.gov/system/files/documents/2020/07/16/NIST_PS1_COA.pdf accessed May 25th, 2021.

ultra-pure benzoic acid (PS1) as primary material with metrological traceability to the SI [5].

After the measurements for the certified value and homogeneity are completed, stability studies, data analysis and calculation as well as further characterization of the material are carried out. Finally, a certificate is issued which must contain a statement on metrological traceability guaranteeing the link to the SI unit as well as an expiry date, which indicates the period in which the certified value is valid within the stated expanded overall uncertainty. By following this workflow, multiple CRM neat materials with diverse solubilities and signals in different regions of the spectrum were developed, by using three NMR active nuclei ^{1}H, ^{31}P, and ^{19}F [12]. Those CRM can be used in testing or QC laboratories either for performing qNMR or in chromatographic analytical measurements. For chromatographic techniques, a broad range of CRM from different fields were developed, e.g. pesticides, extractables & leachables, flavors & fragrances, amino acids, antibiotics, fatty acid methyl esters (FAME), organic pollutants, polyaromatic hydrocarbons (PAH), phthalates, substances of very high concern (SVHC), toxins, vitamins, and many more. There is also an increasing interest in qNMR and demand for qNMR standards in the pharmaceutical industry, as the technique offers a straightforward alternative to the time-consuming mass balance approach for purity determination, but also for comparison to mass balance results, or to complement other methods [13, 14]. Compounds certified in this segment comprise Active Pharmaceutical Ingredients (API) and pharmaceutical impurities.

The large range of CRM for chromatographic applications is important, since in HPLC or GC scientists have to calibrate with an identical reference in order to receive quantitative results. In contrast, qNMR laboratories cannot use the same molecule as reference and analyte due to signal overlap, at least when using the internal quantification method. Therefore, a small set of CRM with a choice between different nuclei, chemical shifts and solubilities is sufficient for the qNMR user.

In addition to using CRM for quantification, it has to be demonstrated especially in regulated environments that the NMR instrument itself performs according to requirements given by the authorities or guidelines. After setup of the instrument by the supplier, typically an installation qualification (IQ) and operational qualification (OQ) is performed. While IQ evaluates the means of accommodating new equipment and testing its material, OQ is essential in challenging equipment parameters. Finally, Performance Qualification (PQ) is used to verify that the instrument is running according to the specification under real conditions. Users must perform PQ of their instrument, as directed by various authorities. A popular way is using a standard tube for NMR referencing, as it is regarded as supplying a particularly relevant test for the samples that are typically analyzed, in addition to standard NMR tests like line shape or ^{1}H sensitivity based on certified reference samples.

Most of our CRM qNMR standards have so far been provided in neat form to offer a maximum of flexibility. However, to facilitate the sample preparation and add convenience for the CRM user, a series of CRM solutions have been developed. Because for this type of CRM, the time-consuming dissolution step and part of the weighing is already done, the user is able to save laboratory time. The available CRM solutions comprise some of the most used calibration standards for ^{1}H and

^{31}P quantitative NMR dissolved in a deuterated solvent and filled into ampules. The certification procedure of qNMR CRM in deuterated solution is described here in detail.

Materials and Methods

The certification procedure for CRM solutions in amber glass ampules starts with the selection of highly pure starting material, which usually is well-characterized neat CRM. In this case, *Trace*Cert® materials from Sigma-Aldrich were used: Phosphono acetic acid, 96708; Fumaric acid, 76635; Calcium formate, 03826; Benzoic acid, 06185; Dimethyl sulfone 41867, Dimethyl terephthalate, 07038; 1,2,4,5-Tetrachloro-3-nitrobenzene, 40384; 1,3,5-Trimethoxybenzene, 74599. The following deuterated solvents were used: D_2O, Sigma-Aldrich, 151882; $CDCl_3$, Sigma-Aldrich, 151823; DMSO-d_6, Armar AG, 015000.2050. For this purpose, several pretests must be done in order to exclude solutions which are not suitable as CRM for qNMR measurements, since they have to be stable over a defined period of time. The selected CRMs are dissolved in various deuterated solvents and NMR spectra are recorded. The solutions are then stored at different temperatures (4 °C - 45 °C) and further NMR spectra are recorded periodically to determine the most stable combination.

Then, a high-precision weighing step on an analytical balance (XP205DR, Mettler Toledo) is used to create the solution of the starting material in a deuterated solvent according to the target value. This gravimetric step is also performed under ISO/IEC 17025 and ensures metrological traceability to the SI. During each weighing step, climate conditions are tracked for subsequent air buoyancy correction. Afterwards, the solution is dispensed in ampules in a defined and validated process under inert gas following the guidelines of ISO 17034 accreditation.

As outlined in ISO Guide 35:2017, [15] a sampling step is performed by taking representative ampules from the entire batch for homogeneity testing. In addition to the gravimetric value received through the weighing step, the certified value of the analyte in solution is determined by qNMR, as this is later the intended use.

For this qNMR measurement it is not only necessary to set the NMR parameters correctly, but important attention has also to be paid to the sample preparation. Of highest importance is the weighing step, because the weighing results directly influence the measured content and the measurement uncertainty. Therefore, the next steps in the quantification of the material are metrological weighing with an ultra-micro balance (XP6U or XPR6U, Mettler Toledo) of the solution and a suitable reference material including air buoyancy correction and subsequent qNMR measurements for the determination of the mass fraction.

All NMR experiments were performed on a Bruker Avance 600 MHz NMR instrument equipped with a 5 mm PABBO BB-^{1}H/D Z gradient probe. In most cases 16–64 scans were recorded for every sample with a ^{1}H relaxation time of $d1 = 60$ s and a ^{31}P relaxation time of 30 s, respectively. Quantification of the analyte content was directly calculated from the NMR peak areas, the number of nuclei, the molecular masses, the content of the reference material, and the weighing values of the analyte and reference substance. Those measurements are also used for the calculation of homogeneity of the material via analysis of variance (ANOVA) [15].

Extensive stability tests were taken into account during the certification process. [16] For this purpose, a representative amount of the finished ampules was used in accelerated stability tests (stress tests) at elevated temperature for a shorter time period as well as long-term stability tests at storage temperature for a longer time period. This step is followed by data analysis, uncertainty calculations, finalization of the certificate and review by quality assurance [17].

Metrological Traceability to the SI was achieved using primary reference material from the National Institute of Standards and Technology (NIST).

Results

By following the entire accredited process as outlined above, it was possible to certify suitable standards for intended use in ^{1}H or ^{31}P qNMR (see Fig. 1).

The overall uncertainty for the gravimetric preparation of these solution CRM are illustrated by the following cause-effect diagram in Fig. 2. It contains contributing factors from the weighing processes, the purity of the reference, as well as influencing factors from the ampule filling and homogeneity testing, which are determined and validated independently for each solvent.

The certified mass fraction values were determined by qNMR measurements. Calculation of the result is done by applying the following Eq. 1 [2]:

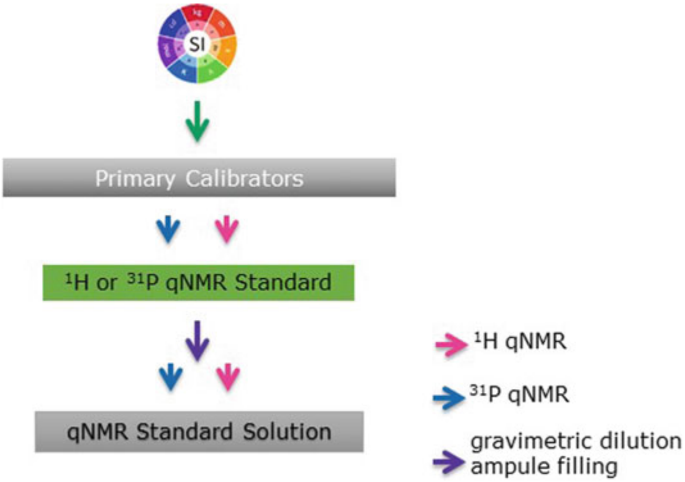

Fig. 1 Metrological traceability concept for qNMR standard solutions

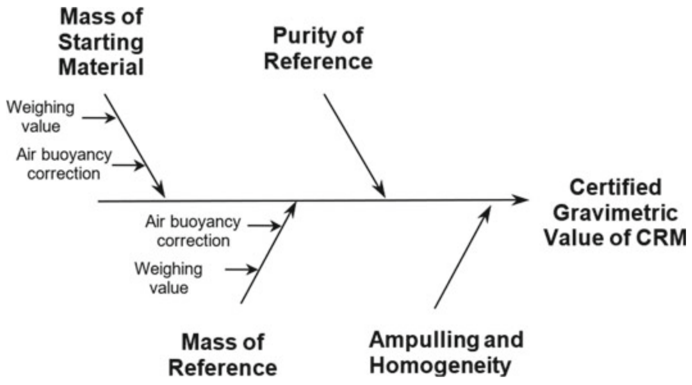

Fig. 2 Example Ishikawa diagram illustrating the potential sources of uncertainty in the reference value for a CRM solution, characterized by gravimetry

$$w_{sample}\left[\frac{mg}{g}\right] = \left(\frac{I_{sample}}{I_{Ref}} \times \frac{N_{Ref}}{N_{sample}} \times \frac{M_{sample}}{M_{Ref}} \times \frac{m_{Ref}}{m_{sample}} \times w_{Ref}\right) \times 1000 \tag{1}$$

w_{Sample}	mass fraction of the sample/[mg/g]	M_{sample}	Molecular mass of the sample/[g/mol]
w_{Ref}	mass fraction of the reference/[g/g]	M_{Ref}	Molecular mass of the reference/[g/mol]
I_{sample}	Integral of the sample signal/[-]	m_{Sample}	Mass of sample/[mg]
I_{Ref}	Integral of the reference signal/[-]	m_{Ref}	Mass of the reference/[mg]
N_{sample}	Number of nuclei generating the sample signal/[-]		
N_{Ref}	Number of nuclei generating the reference signal/[-]		

Additional factors need to be considered for the expanded uncertainty of the material, to fully comply with ISO/IEC 17025. The combined standard uncertainty is calculated by combination of the standard uncertainties of the input estimates according to Eurachem/CITAC Guide “Quantifying Uncertainty in Analytical Measurement.” [18]

To fulfill the requirements of ISO 17034 [11], the full uncertainty budget includes in addition to the characterization measurement contributions from homogeneity assessment and stability studies, as mentioned before. The overall uncertainty can be described by the following Eq. (2) and results are shown in Table (1): [15]

$$u_{CRM} = \sqrt{u_{char}^2 + u_{hom}^2 + u_{lts}^2} \tag{2}$$

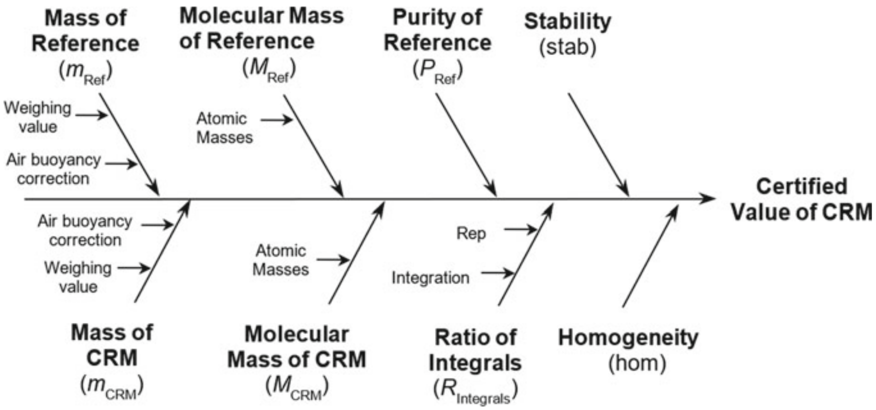

Fig. 3 Example Ishikawa diagram illustrating the potential sources of uncertainty in the reference value for a CRM solution, characterized by qNMR measurements

u_{CRM}	standard uncertainty associated with property value of the CRM
u_{char}	standard uncertainty associated with a value assigned in a characterization study
u_{hom}	standard uncertainty associated with heterogeneity
u_{lts}	standard uncertainty associated with long term stability

The certified value is ultimately given as mass fraction (mg/g) allowing the user to do gravimetric sample preparation, which gives the most accurate results. The uncertainty is calculated for a shelf life of 2 or 4 years, depending on the stability data. A certified value for the density is also provided on the certificate of analysis, to enable volumetric sampling as well if desired.

The uncertainty contributions for the qNMR certification of a solution CRM are illustrated by the following cause-effect diagram (Fig. 3):

Even though it is the value from the qNMR and not the gravimetric weighing measurement that appears in the certificate, the certified values derived from qNMR characterization are additionally compared to the gravimetric values, taking into account the uncertainties described above. The results were compared by applying the following Eq. (3) from ISO Guide 35 [15].

$$|x_{CRM} - x_{meas}| \leq k\sqrt{u_{CRM}^2 + u_{meas}^2} \tag{3}$$

x_{CRM}	property value of the CRM
x_{meas}	value from the qNMR measurement
u_{CRM}	standard uncertainty associated with property value of the CRM
u_{meas}	standard uncertainty associated with a value assigned by qNMR measurement

(continued)

(continued)

x_{CRM}	property value of the CRM
k	coverage factor ($k = 2$)

Table 2 shows the comparison of the value obtained during the production process (i.e. gravimetric step) with the certified value obtained by another measurement (i.e. qNMR), under consideration of the respective standard uncertainties and an appropriate coverage factor. The two results are matching with each other according to Eq. (3) for every single solution and thus the certified value can be considered confirmed. It is important to consider effects from ampule filling (for example possible evaporation of solvents, duration of the process especially with varying batch sizes) into the uncertainty budget, when determining a value by gravimetry. Those effects depend on the solvent and become more important with more volatile matrices.

The results of the determination of the mass fraction from qNMR experiments and gravimetry are illustrated in the following graphic (Fig. 4). The high volatility of deuterated chloroform is also illustrated by the higher level of uncertainty in the gravimetric production of the solution whereas the higher measurement uncertainty in the quantitative determination by means of qNMR of phosphono acetic acid can be attributed to the use of the method with ^{31}P. The ^{31}P 1D-NMR experiment is much less sensitive compared to the ^{1}H experiment, because of this the spectra are noisier and therefore a homogeneous evaluation of the experiments of the individual samples is more challenging resulting in higher measurement uncertainties.

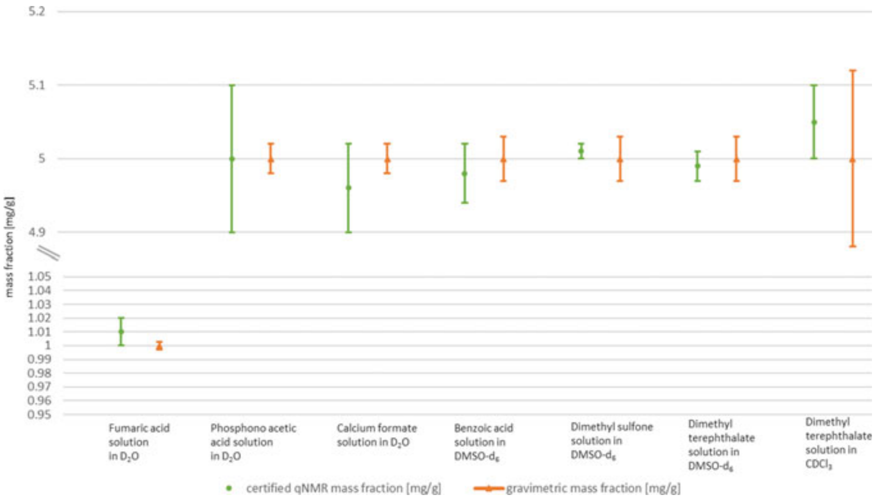

Fig. 4 Comparison of the mass fraction determined by qNMR (green) and by gravimetric production (orange) with their respective expanded measurement uncertainties. The scale had to be adjusted due to the lower mass fraction of the Fumaric acid solution

In order to demonstrate that instruments perform according to requirements given by the authorities, guidelines or internal SOPs, valid test samples have to be used.

There are clear advantages in using a performance qualification reference material that has been manufactured by a recognized high-quality manufacturer of such materials. This is partly because of convenience and speed. The ability to generate, test and certify high quality reference materials for the purposes described is not normally available in a typical NMR laboratory. Additionally, separation of the development process of the reference standard from the testing process provides a clear degree of independence.

A collaboration between an instrument manufacturer and Sigma-Aldrich Production GmbH has led to the development of certified reference material (CRM) tailored to quantitative performance qualification for NMR.

Even though the CRM for the quantitative Performance Qualification (qPQ) of NMR and potency determination has been developed using the same techniques that were already described above for the realization of the qNMR standard solutions by applying an internal standard certification method, there is one important difference. Since the end user must be able to directly assess the performance of the instrument, the CRM consists of a binary mixture of two well-characterized constituents. After a series of evaluations regarding applicability and stability 1,2,4,5-Tetrachloro-3-nitrobenzene (TCNB) and 1,3,5-Trimethoxybenzene (TMXB) were found to be ideal candidates in a DMSO-d_6 solution. By introducing a binary mixture, NMR users can compare the specific peaks of these two components against each other and use the mixture as a system suitability test.

The certification of this two-component mixture is an advancement of the process described for the single solution CRM. All steps that have already been described for the single solution CRM before, were also followed for the Performance Qualification mixture: characterization of the starting materials, choice of solvent, choice of separated peaks for quantification, determination of homogeneity and stability.

Equal to the qNMR CRM solutions described in Tables 1 and 2, the certified values of the qPQ CRM measured by qNMR can also be compared to the gravimetric results by applying Eq. (3). The results of the two different methods are shown in table 3 and show good correlation with each other, considering their respective uncertainties.

Contributions to the overall measurement uncertainty from the qNMR measurement, homogeneity and stability are in the same range as mentioned above (Table 2).

Discussion and Conclusion

The process of certifying neat materials by qNMR under ISO/IEC 17025 is well established at Sigma-Aldrich, Buchs, Switzerland for many years and in this paper, the successful certification of CRM solutions by qNMR was demonstrated, while

Table 1 Overview of the various CRM solutions including detailed uncertainty contributions from the qNMR measurement [u_{char}], homogeneity, and stability. An example of the influence of the various uncertainty contributions on the total measurement uncertainty was presented elsewhere [19]

Substance/ product no.*	Solvent	u_{char} [mg/g]	u_{hom} [mg/g]	u_{stab} [mg/g]	Certified qNMR mass fraction [mg/g] $\pm U$ ($k = 2$)
Fumaric acid solution/ 76201	D_2O	0.003	0.000	0.001	1.01 ± 0.01
Phosphono acetic acid solution/ 79251	D_2O	0.034	0.000	0.003	5.00 ± 0.10
Calcium formate solution/ 39606	D_2O	0.011	0.001	0.016	4.96 ± 0.06
Benzoic acid solution/ 39457	DMSO-d_6	0.009	0.000	0.016	4.98 ± 0.04
Dimethyl sulfone solution/ 16736	DMSO-d_6	0.017	0.016	0.004	5.01 ± 0.01
Dimethyl terephthalate solution/ 39387	DMSO-d_6	0.006	0.002	0.010	4.99 ± 0.02
Dimethyl terephthalate solution/ 39751	$CDCl_3$	0.021	0.012	0.003	5.05 ± 0.05

* For more information, please visit www.sigmaaldrich.com

complying to all steps mandatory for CRM production under ISO 17034 accreditation. Both types of qNMR CRM standards, neat material, and solution, have certain advantages depending on the application.

For the qNMR CRM in solution, it was demonstrated that metrological traceability can be achieved following two different routes, either through gravimetric dilution of well-characterized starting materials, or through qNMR measurements of the final product after the production process. Both routes are complying with the ISO 17034 guidelines and the respective results are in agreement with each other taking into account their corresponding measurement uncertainties.

The qNMR solution concept was in addition applied for a binary solution, containing two certified analytes. This qPQ (quantitative Performance Qualification) sample offers customers a ready-to-use solution to verify and control the performance of the NMR instrument, which is a crucial factor in order to receive reliable and accurate quantitation results.

Table 2 Key attributes and overview of the different qNMR solutions for ^{31}P and ^{1}H qNMR including chemical shift and individual relaxation time that was measured for each solution separately. The metrological traceability to the stated NIST SRM is realized either through a direct comparison or through an unbroken chain of measurements [19]

Substance/product no	Solvent	Intended use	Chemical shift δ [ppm]	Relaxation time T_1 [s]	Certified qNMR mass fraction [mg/g] ± U ($k = 2$)	Gravimetric mass fraction [mg/g] ± U ($k = 2$)	Metrological Traceability
Fumaric acid solution/ 76201	D_2O	^{1}H qNMR	6.5	12	1.01 ± 0.01	1.00 ± 0.01	NIST SRM 84 l (KHP) and NIST SRM 350b (Benzoic acid)
Phosphono acetic acid solution/ 79251	D_2O	^{31}P qNMR	15.7	5.3	5.00 ± 0.10	5.00 ± 0.02	NIST SRM 84 l (KHP)
Calcium formate solution/ 39606	D_2O	^{1}H qNMR	8.1	19.8	4.96 ± 0.06	5.00 ± 0.02	NIST SRM 84 l (KHP)
Benzoic acid solution/ 39457	DMSO-d_6	^{1}H qNMR	7.9 7.6 7.5	3.5 3.8 2.9	4.98 ± 0.04	5.00 ± 0.03	NIST PS1 (Benzoic acid)
Dimethyl sulfone solution/ 16736	DMSO-d_6	^{1}H qNMR	3	2.4	5.01 ± 0.01	5.00 ± 0.03	NIST PS1 (Benzoic acid)
Dimethyl terephthalate solution/ 39387	DMSO-d_6	^{1}H qNMR	8.1 3.9	3.4 1.2	4.99 ± 0.02	5.00 ± 0.03	NIST SRM 350b (Benzoic acid)
Dimethyl terephthalate solution/ 39751	$CDCl_3$	^{1}H qNMR	8.1 4	4.0 2.0	5.05 ± 0.05	5.00 ± 0.12	NIST SRM 350b (Benzoic acid)

Table 3 Key attributes and measurement values of the qPQ qNMR including chemical shift and individual relaxation time that was measured

Substance	Solvent	Intended use	chemical shift δ [ppm]	Relaxation time T_1 [s]	Certified qNMR mass fraction [mg/g] ± *U* (*k* = 2)	Gravimetric mass fraction [mg/g] ± *U* (*k* = 2)	Metrological Traceability
TCNB	DMSO-d_6	PQ	8.5	15	23.51 ± 0.18	23.50 ± 0.06	NIST PS1 (Benzoic acid)
TMXB	DMSO-d_6	PQ	6.1 3.7	3.5 1.5	5.00 ± 0.04	5.00 ± 0.03	NIST PS1 (Benzoic acid)

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