

Quantitative Nuclear Magnetic Resonance (qNMR), a Metrological Method—Proposed Revisions to the *USP* General Chapters on NMR, Nuclear Magnetic Resonance Spectroscopy (761) and Applications of Nuclear Magnetic Resonance Spectroscopy (1761)

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ABSTRACT

USP general chapters [Nuclear Magnetic Resonance Spectroscopy \(761\)](#) and [Applications of Nuclear Magnetic Resonance Spectroscopy \(1761\)](#) are currently being revised by the quantitative nuclear magnetic resonance (qNMR) Expert Panel. Since the last revision in 2013, there has been a surge in the utilization of NMR as a quantitative tool to determine purity of organic compounds and the composition of mixtures, and there is an urgent need to provide current guidance on the use of qNMR for applications within scope of the USP. The purpose of this *Stimuli* article is to propose the adoption of qNMR as a measurement method that is consistent with the already established principles and guidance framework of metrology—the science of measurement and its application. The concepts of analytical target profile (ATP) and target measurement uncertainty (TMU) are introduced as the principal benchmarks to be used for validation of analytical procedures based on qNMR. A holistic understanding of the measurement procedure and system that accounts for uncertainty contributions from specified sources can be used to qualify the NMR instrument and qNMR method using certified reference materials (CRMs) and thus simplify the validation of the final compound-specific analytical procedure. For example, linearity, a traditional method performance characteristic, can be verified as part of the NMR instrument operational qualification (OQ) and may not have to be verified as part of the qNMR method qualification.

This Expert Panel of the General Chapters—Chemical Analysis Expert Committee is inviting public comment on the following matters:

- Alignment with modern guidance: USP general chapter (761) is currently in line with [Validation of Compendial Procedures \(1225\)](#). (1225), in turn, references [Statistical Tools for Procedure Validation \(1210\)](#), which are validated according to (1225). This qNMR Expert Panel recommends a change to align with the proposed new USP general chapter, [Analytical Procedure Life Cycle \(1220\)](#), which offers an alternative approach to classical analytical validation, including the concepts of ATP and TMU. This change is also anticipated to be aligned with the International Council for Harmonisation Guideline ICH Q14 Analytical Procedure Development and Revision of Q2(R1) Analytical Validation, 14 November 2018 (<https://www.gmp-compliance.org/guidelines/gmp-guideline/ich-q14-analytical-procedure-development-and-revision-of-q2r1-analytical-validation-final-concept-paper>). The final concept paper for this revision explicitly states that the scope of the [Q2(R1)] revision will include validation principles that cover analytical use of spectroscopic or spectrometry data (e.g., NMR).
- A holistic approach to validation of analytical procedures that uses qualified NMR instruments, qualified qNMR methods, CRMs, and assessment of measurement uncertainty to establish fitness for purpose.
- Quality risk management with application of tools such as cause-and-effect diagrams (also known as Ishikawa or “fishbone” diagrams) to determine and prioritize sources of variability (components of measurement uncertainty) that can be attributed to either the NMR instrument or qNMR method or otherwise be separately assessed and controlled.
- The potential application of NMR for direct determination of identity, strength, quality, and purity of drug substances and drug products according to current good manufacturing practice (cGMP) regulations. In particular, the use of “mass fraction” as an expression of “purity” (drug substance) and “strength” (drug product).
- Discussion of the value of the “measurement uncertainty approach” over the “traditional” method qualification using the performance characteristics in (1225).
- Beyond the concepts in this article, the authors seek recommendations for additional proposed revisions for (761) and (1761).

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PRINCIPLE: THE QUANTITATIVE NATURE OF NMR

NMR experiments can be performed such that they are inherently quantitative. The quantitative nature of NMR is based on direct proportionality between the observed signal response and the number of nuclei generating the signal. Peaks in an NMR spectrum represent structurally different molecular moieties. Generally speaking, the use of other spectroscopic techniques to assess chemical quantities requires knowledge of response factors, extinction coefficients, and/or calibration curves using a series of standard solutions. These response factors can vary considerably amongst chemical entities with significantly different structures. In an NMR spectrum, the resonances of all chemically equivalent nuclei will have peak areas that are proportional to their concentration in the test sample. This holds true whether the nuclei are part of the same molecular structure or located in different molecular entities.

Some Physical Background

The intensity of an NMR signal depends entirely on the magnitude of the equilibrium magnetization, M_0 , that arises from placing the sample into a static magnetic field. In order to derive that magnitude for spin $\frac{1}{2}$ nuclei, one may apply the Boltzmann factor for the two possible spin states, α and β :

$$M_0 \propto e^{\frac{E_\alpha - E_\beta}{kT}}$$

According to the "fundamental equation of NMR", the energy difference between the α and β states is given by Planck's constant multiplied by the Larmor frequency of the nucleus, ω_0 :

$$\Delta E = E_\alpha - E_\beta = \hbar\omega_0$$

In order to compare the equilibrium magnetization of one signal, M_0 , with that of a second signal, M'_0 , we consider the ratio of the individual Boltzmann factors, which gives us an expression that depends on the frequency difference of the two NMR signals:

$$\frac{M_0}{M'_0} = \frac{e^{\frac{\hbar\omega_0}{kT}}}{e^{\frac{\hbar\omega'_0}{kT}}} = e^{\frac{\hbar\Delta\omega}{kT}}$$

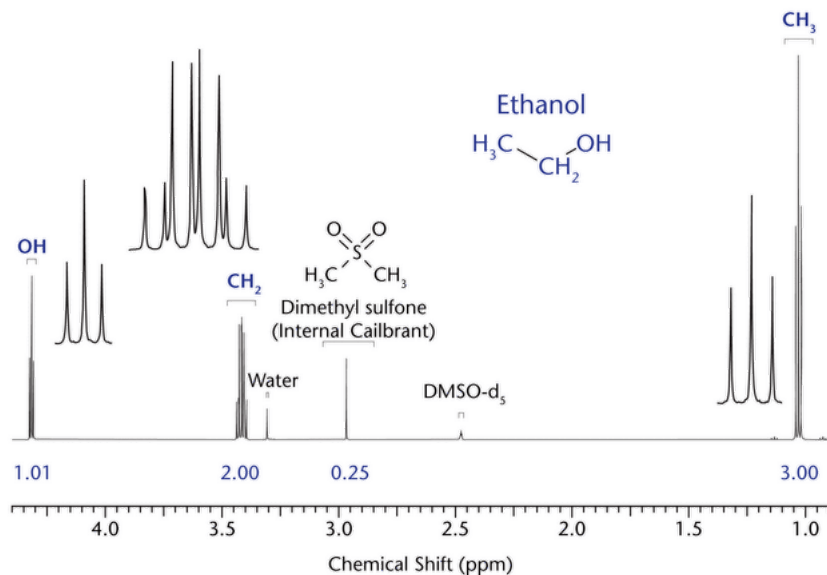
Thus, the equilibrium magnetizations of two nuclei actually do differ, depending on their resonance frequencies.

Mathematical treatment demonstrates the magnitude of this effect. Assume a worst-case scenario and set $\Delta\omega_0$ to 500 kHz and the temperature to 298 K:

$$\frac{M_0}{M'_0} = e^{\frac{\hbar\Delta\omega}{kT}} = e^{\frac{6.62 \cdot 10^{-34} \text{ Js} \cdot 500 \text{ kHz}}{1.38 \cdot 10^{-23} \text{ JK}^{-1} \cdot 298 \text{ K}}} = 1.00000008$$

The "error" is less than 10^{-7} for the worst-case scenario. For typical NMR spectra, it is even smaller, substantiating the use of NMR as part of a primary quantitative ratio method.

Because of the quantitative nature of NMR, a correctly set up NMR experiment allows for the determination of the relative amounts of substances in a mixture; their relative concentrations can be determined by establishing a ratio of the peak areas of their resonances corrected for the number of equivalent nuclei giving rise to each signal. So, in the ^1H qNMR example of ethanol shown in [Figure 1](#), the peak areas of the methyl, methylene, and hydroxyl are normalized by dividing by 3, 2, and 1, respectively. Similarly, the dimethyl sulfone peak area is normalized by dividing by 6. The resultant normalized peak areas are then proportional to their relative molar concentrations. Given the known concentration of the internal calibrant, one can then calculate the molar concentration of the analyte. This simple example can be extended to much more complicated mixtures. In fact, qNMR allows for simultaneous quantitation of multiple constituents in a mixture based on a single reference material. More importantly, the calibrant does not need to comprise the same chemical structures of any of the analytes of interest. This key feature makes qNMR an extremely versatile method and enables quantitation of chemical species that are rare or in short supply.



Click image to enlarge

Figure 1. ^1H NMR spectrum of ethanol with dimethyl sulfone internal calibrant in dimethyl sulfoxide- d_6 ($\text{DMSO}-\text{d}_6$). Note that the residual protio signal of $\text{DMSO}-\text{d}_6$, i.e., from $\text{DMSO}-\text{d}_5$, is observed.

Primary Method of Measurement

A primary method of measurement is a method of the highest metrological quality that can be realized using a completely described and understood measurement procedure to obtain results without relation to a standard for a quantity of the same kind. Results produced by these procedures include complete assessments of the measurement uncertainty. Verified procedures that constitute the general qNMR method can meet these criteria and allow measurement of the ratio of two chemical substances, without the use of a reference standard. As such, qNMR is potentially a primary ratio method.

In addition to quantitation of relative amounts of chemical species, other kinds of quantities, such as the mass purity of substances, can be determined by weighing a reference material of known purity into a solution containing the known mass of the test sample. NMR provides one of the most universally applicable forms of direct purity determination without need for reference materials of impurities or the calculation of response factors. As both materials are in the same solvent, the relative molar concentrations can be determined easily. With the knowledge of the molar masses of both components, determination of the mass fraction of the analyte content can be made. The NMR-determined mass fraction is used as a measure of the purity of the analyte.

The same determination can be made with separate solutions of the reference material and the analyte, but in that case, the concentrations of the two materials must be known. This so-called external calibration method is usually slightly less accurate and precise because, in addition to the errors from weighing, one encounters volume measurement errors.

Are calibration curves ever used in NMR? Yes, there are some special applications when a calibration curve is needed. One example would be when you have an analyte of unknown molar mass, such as a polymer, which is appearing as a contaminant in a solution of another polymer. If a sample of the contaminating polymer is available, a calibration curve made from different masses of this material can be constructed that will give the mass fraction (weight %) of the contaminant. Another example of where calibration curves may be needed is in certain cases of quantitative multidimensional experiments.

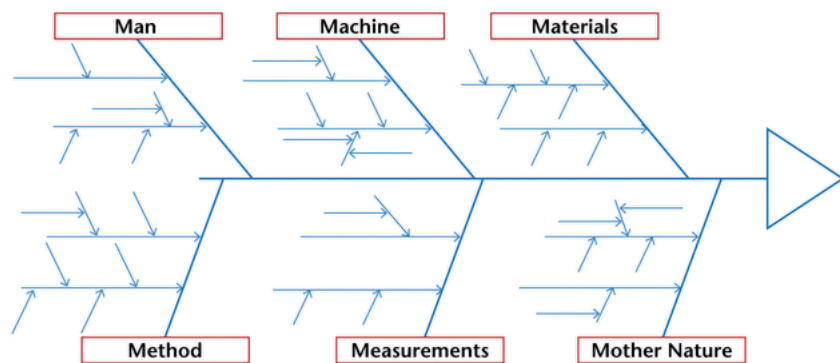
Linearity

The occasional use of calibration curves defines the difference between validation of linearity in the compound-specific procedure versus the validation of linearity of the instrument as part of the operational qualification. Unlike quantitative chromatographic procedures, there is no need for validation of compound-specific linearity. As one does not need to calibrate an analytical balance for each measurement, one does not need to do so for an NMR instrument. The linearity of NMR instruments can be established for all viable analytes as part of the instrument operational qualification (OQ). The results of the OQ also provide information about the range for the development of the procedure itself. For continuous verification of instrument performance, it should then be sufficient to verify that the signal-to-noise ratio (S/N) of the standard ^1H sensitivity test meets the performance qualification (PQ) specifications

VALIDATION OF ANALYTICAL PROCEDURES BASED ON QNMR METHODS

For guidance concerning validation of analytical procedures that are based on qNMR, we intend to align with the proposed USP general chapter [Analytical Procedure Life Cycle \(1220\)](#) and anticipated revision 2 of ICH Q2 "Validation of Analytical Procedures" by adopting the complementary concepts of analytical target profile (ATP) and target measurement uncertainty (TMU) as tools to describe and evaluate fitness for purpose ([1-2](#)).

The "measurement uncertainty approach" requires a fundamental understanding of the analytical procedure that, in combination with experimental evidence and quality risk management, can be presented as "valid" or fit for the intended purpose. This paradigm involves the concept of measurement uncertainty, rather than measurement error, and application of multivariate statistics. Uncertainty is fundamentally an expression of the dispersion of likely values that can be attributed to a measured quantity, typically for a stated coverage probability. Fortunately, this is consistent with metrological principles and concepts in an already well-defined guidance framework ([3-4](#)). Also, in the general field of analytical chemistry, existing guidelines for expression of measurement uncertainty and examples of practical applications are available for reference ([5](#)). In essence, the objective is to use a quality by design (QbD) methodology to reduce the measurement uncertainty (the TMU) to a level that is acceptable for the intended purpose (the ATP). The advantage of qNMR is that it is a method that is based on fundamental scientific principles that can be expressed in universal mathematical equations and it is thus ideally suited for development by QbD and validation by statistical evaluation (e.g., as per [1210](#)). Modern quality management tools, such as cause-and-effect diagrams (Ishikawa diagrams), can be used to identify and analyze sources of measurement uncertainty ([Figure 2](#)). This is a proven concept that also finds application in the DMAIC (Define, Measure, Analyze, Improve, and Control) methodology of Six Sigma and has commonly been used with the 6M model (Machine, Method, Materials, Measurements, Man/Personnel, Mother Nature/Environment). For these reasons, we propose to replace the traditional single-event validation with an approach that uses CRMs (Materials), includes separate NMR instrument qualification (Machine) and qNMR method qualification (Method), and integrates the documented knowledge in the final analytical procedure by adding the compound-specific parameters (e.g., selectivity/specificity, stability, solubility) to demonstrate and document that the procedure is fit for purpose—validated.



Click image to enlarge

Figure 2. Example cause-and-effect diagram (Ishikawa diagram).

ANALYTICAL TARGET PROFILE

The ATP does not prescribe any specific analytical procedure or technology but includes an expression of the required measurement uncertainty of the reportable results and thus translates into a quantitative TMU for use in validation of a specific procedure. For an overview of the concepts of ATP and TMU, please review the series of *Stimuli* articles that were published in the *Pharmaceutical Forum* ([6-13](#)).

Example ATP with TMU

The analytical procedure must be able to determine the mass fraction of compound X in the presence of NMT 0.5% of impurity Y with an expanded measurement uncertainty of NMT 2.0% ($k = 2$, 95% confidence) within a range of 50–150% of the nominal test sample concentration.

As qNMR is already recognized as an analytical method that can yield results with metrological traceability to the International System of Units (SI) and is used to realize mass fractions of certified reference materials (CRMs) (14) within the ISO quality management framework (e.g., ISO/IEC 17025, ISO 17034), this is an opportune time to introduce qNMR as an analytical tool for pharmaceutical quality control and develop a framework for validation that is based on scientific principles and quality risk management. There is an obvious convergence and path forward for qNMR to also be used for direct measurements of mass fractions for assessment of "purity" and "strength" in current pharmaceutical quality control. The framework of the ATP statement given above is applicable to USP-relevant applications such as: 1) NMR may be used to quantitate major components in drug substances or active ingredients in drug products, analogous to (1225), *Validation, Data Elements Required for Validation, Category I* (assay), in which case the mass fraction of the main component of an active pharmaceutical ingredient (API) or drug substance relates to a direct measurement of its purity or strength, and 2) NMR may be used to determine the mass fraction of impurities in drug substances or degradation products (defined in *Impurities in Drug Substances and Drug Products (1086)*) in finished drug products analogous to (1225), *Validation, Data Elements Required for Validation, Category II* (impurities).

DESIGN AND DEVELOPMENT OF QNMR METHODS

Each individual laboratory will usually design and develop an in-house qNMR method that is optimized for the available NMR instrument and fit for the intended purpose. It is recommended to use sound scientific principles and current best practices as guidance. See for example the European Network of Forensic Science Institutes (ENFSI) guidance for qNMR in forensics and customs laboratories and EUROLABS guidance for qNMR method development and validation (intended for accredited ISO 17025 testing laboratories) (15-16). To facilitate standardization and enable interlaboratory proficiency testing, it would be ideal if a standardized qNMR method could be established and verified using commercially available CRMs. However, despite the specific method used, it is always the measurement uncertainty of the result of the analytical procedure that is critical to determine fitness for the intended use.

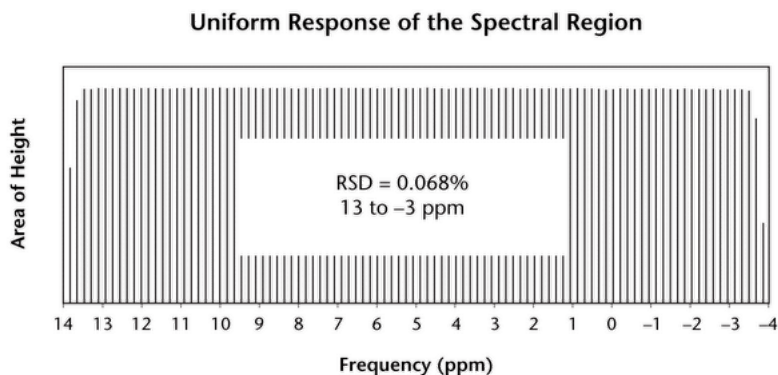
INSTRUMENT QUALIFICATION

Prior to validation of the compound-specific analytical procedure, it is beneficial to simplify the process by determining which parameters are general in nature and can be relegated to the qualification of the instrument and generic method. This allows the contributions of uncertainty from the instrument and method, respectively, to be parametrized as separate contributing factors or "bones" in an Ishikawa diagram.

For example, because the linearity of the NMR signal collected during a properly executed qNMR procedure is based on fundamental physical laws, is not compound-specific, and is expressed in mathematical terms, it is sufficient to demonstrate that the NMR instrument is able to measure the signal with linearity during the OQ.

The proper function of the receiver gain and the elimination of small extraneous artifacts ensure consistent results. The correct selection for these parameters can be demonstrated via two types of tests: uniformity of the spectral region and linearity versus analyte concentration.

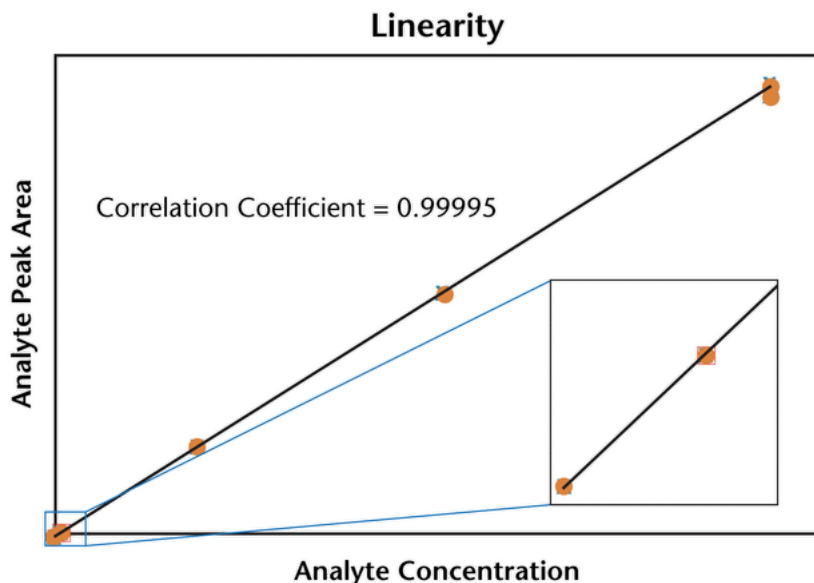
NMR instruments utilize filters in order to exclude signals outside of the chemical shift region of interest. These filters affect the response on either edge of a spectrum. To demonstrate the region of uniform response for a given spectral region, multiple spectra of one sample with a single peak, such as a readily available sample of H₂O in D₂O with GdCl₃ as a relaxation agent, can be acquired where the sample peak is moved throughout the spectrum, as demonstrated for a proton experiment in Figure 3.



Click image to enlarge

Figure 3. Assessment of the signal response across the spectral region.

For a qNMR method, the instrument linearity can be demonstrated by using a small number of samples (e.g., triplicates of at least five concentrations). Note that the linearity test may be optimized using a minimum delay between pulses. The low and high concentrations may be used to indicate the range for the method, including the limit of quantitation (LOQ). For NMR, the LOQ is determined via a minimum S/N and the amount of instrument time available to run the experiment. The S/N depends not only on the concentration but also on peak shape, which can vary significantly due to multiple factors such as exchange rates, relaxation rates, and peak splitting. Given cases where peak shapes are known, one compound can be used to estimate the LOQ of any other entity via the relative peak height and molar mass. S/N is the greatest factor for determining uncertainty within a spectrum. Similar to IR and Raman, the S/N may be increased by collecting additional scans. The linearity test demonstrates the lack of artifacts upon increasing the number of scans as well as frequency locking. Over time, the field strength of the instrument changes, which should be compensated for by locking onto the deuterium signal of the solvent. The maximum number of scans correlates to the maximum amount of time available per sample. The high concentration sample ensures that the receiver gain is adjusting correctly. With some instruments, strong signals can cause intermodulation artifacts or harmonic oscillations in the spectra. If this occurs, it may then be necessary to reduce the pulse angle in these instances. Although the theoretical maximum concentration may be at the solubility limit, saturated solutions result in broadened peaks, which can impact integral set points. It is important to test both the low and high concentrations, so any limitations are demonstrated. Figure 4 demonstrates the linear nature of 5 spectra from 5 concentrations (0.4 to 100 mg/mL).



Click image to enlarge

Figure 4. Linearity demonstrated by analyte peak area as a function of concentration.

The acquisition time is related to the narrowness of the peaks or best magnetic field homogeneity obtained for the instrument. If the acquisition time is too long, additional noise is collected, but if the acquisition time is too short, ringing can appear in the spectrum. The acquisition time must be long enough to prevent artifacts caused by truncation of the free induction decay of strong signals.

In addition to initial NMR system installation, instrumental qualification tests should be performed upon replacement of instrument components that are utilized to transmit or receive spectral signals and as part of operational qualification with some periodicity, e.g., annually.

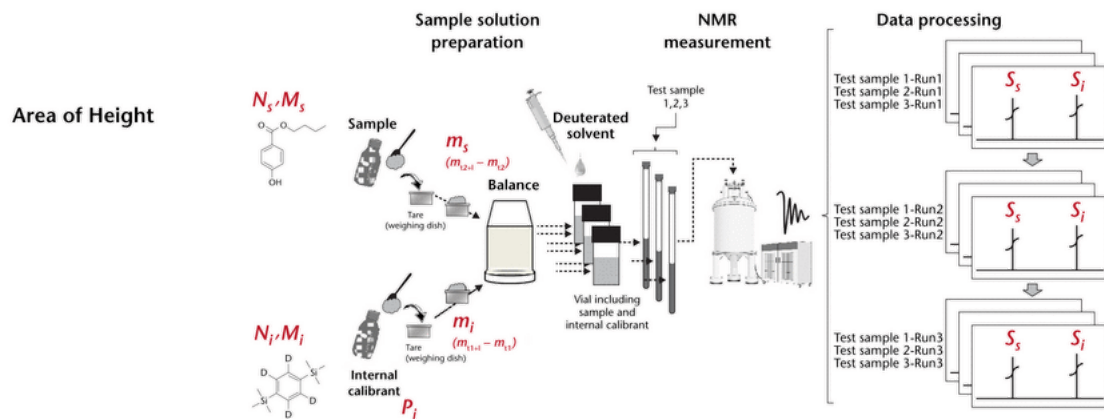
METHOD QUALIFICATION

As an example of method qualification by assessment of measurement uncertainty, the determination of the mass fraction of a pure butyl *p*-hydroxybenzoate substance based on ^1H qNMR with the internal calibration method is used. Currently official general chapter [Nuclear Magnetic Resonance Spectroscopy \(761\)](#) defines this method of quantitation as the "internal standard method", and the intent of the Expert Panel is to revise the chapter to employ the use of the term "internal calibrant" in lieu of "internal standard" to avoid confusion with the use of USP Reference Standards, unless otherwise indicated in a monograph. The ATP in this example could be described as follows: "The analytical procedure must be able to determine the mass fraction of butyl *p*-hydroxybenzoate with an expanded measurement uncertainty of NMT 2.0% ($k = 2$, 95% confidence)". For this particular example, an experiment design implementing a relatively large number of measurements was employed to elucidate likely sources and substantiate the high degree of confidence in results using the procedure. Ultimately, the number of measurements needed (experiment design) will depend on the requirements specified by the ATP. Thus, a suitable corresponding approach to estimating measurement uncertainty as part of method qualification can be simpler (or more rigorous) than that described for this example.

The mass fraction determination of butyl *p*-hydroxybenzoate by ^1H qNMR using 1,4-bis(trimethylsilyl)benzene- d_4 (BTMSB- d_4) CRM [certified mass fraction (%): 100.0 \pm 0.5 ($k = 2$, 95% confidence)] as an internal calibrant, and acetone- d_6 as a solvent is based on *Equation 1*. A schematic illustration of the analytical procedure is shown in [Figure 5](#). In the example, three replicate sample solutions were prepared (test samples 1, 2, and 3 in sequence). For test sample 1, three results (mass fraction, %) obtained from three repeated measurements under optimized data acquisition parameters for quantitative analysis were recorded for each of selected 4 signals. A typical ^1H NMR spectrum is shown in [Figure 6](#). The same procedure was repeated for test samples 2 and 3, to obtain a total of 36 results. These results (36 values) are shown in [Table 1](#).

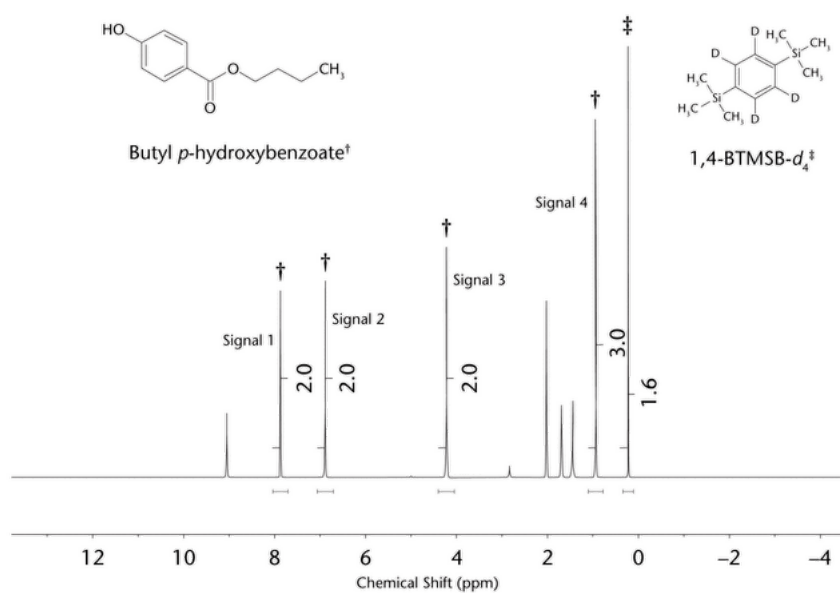
$$P_s = \frac{S_s}{S_i} \times \frac{N_i}{N_s} \times \frac{M_s}{M_i} \times \frac{m_i}{m_s} \times P_i \quad (\text{Equation 1})$$

- P_s = purity (mass fraction, %) of the sample
- S_s = signal area of the analyte
- S_i = signal area of the qNMR calibrant
- N_s = number of resonating hydrogens of the analyte
- N_i = number of resonating hydrogens of the qNMR calibrant
- M_s = molar mass of the analyte
- M_i = molar mass of the qNMR calibrant
- m_s = mass of the sample
- m_i = mass of the internal calibrant for qNMR
- P_i = purity (mass fraction, %) of the internal calibrant for qNMR



Click image to enlarge

Figure 5. Schematic illustration of analytical procedure for ^1H qNMR using internal calibration method (17).



Click image to enlarge

Figure 6. A typical ^1H NMR spectrum for measurement of the mass fraction of butyl *p*-hydroxybenzoate.

Table 1. Analytical Results from Measurements of the Mass Fraction of Butyl *p*-Hydroxybenzoate by ^1H qNMR with Internal Calibration Method

Test Sample	Signal	NMR Measurement (purity as mass fraction, %)		
		1	2	3
1	1	100.45	99.94	100.39
	2	99.59	99.45	99.24
	3	99.18	99.58	99.76
	4	99.49	99.46	99.61
2	1	99.79	99.98	99.43
	2	99.03	99.43	99.52
	3	99.44	99.27	99.14
	4	99.36	99.29	99.10
3	1	100.15	99.74	99.69
	2	99.35	99.28	99.57
	3	99.76	99.84	99.96
	4	99.69	99.48	99.97

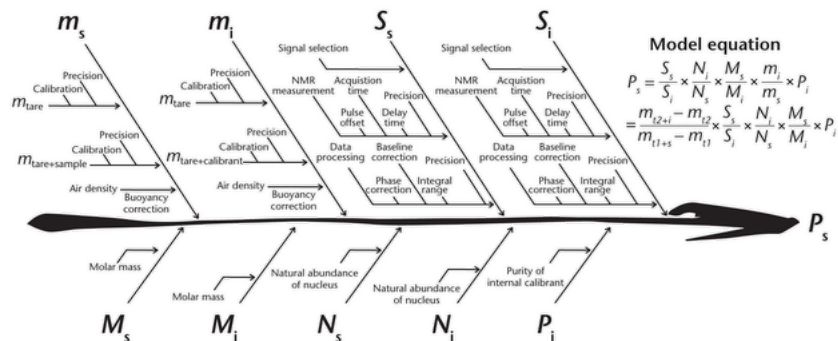
Sources of measurement uncertainty associated with qNMR procedures have been studied and characterized in technical detail for a variety of applications (18–21). These include several factors attributable to instrumentation, analyst execution of procedures, experiment design, data processing, and chemical interferences, among others. Described approaches to the evaluation of qNMR measurement uncertainty account for practically significant sources and are generally in accordance with the “Guide to the expression of uncertainty in measurement” (GUM) (6,22–23) and EURACHEM/CITAC Guide CG 4 (24), as well as other rigorous statistical procedures (18,25).

Using an approach based on GUM, the combined standard uncertainty of the mass fraction of butyl *p*-hydroxybenzoate, $u_c(P_s)$, can be calculated by combining the relative standard uncertainties of each proportionality in Equation 1 according to Equation 2:

$$u_c(P_s) = \sqrt{\left(\frac{u(S_s/S_i)}{S_s/S_i}\right)^2 + \left(\frac{u(N_i/N_s)}{N_i/N_s}\right)^2 + \left(\frac{u(M_s/M_i)}{M_s/M_i}\right)^2 + \left(\frac{u(m_i/m_s)}{m_i/m_s}\right)^2 + \left(\frac{u(P_i)}{P_i}\right)^2} \quad (\text{Equation 2})$$

- P_s = purity (mass fraction, %) of the sample
 S_s = signal area of the analyte
 S_i = signal area of the qNMR calibrant
 N_i = number of resonating hydrogens of the qNMR calibrant
 N_s = number of resonating hydrogens of the analyte
 M_s = molar mass of the analyte
 M_i = molar mass of the qNMR calibrant
 m_i = mass of the internal calibrant for qNMR
 m_s = mass of the sample
 P_i = purity (mass fraction, %) of the internal calibrant for qNMR

Several factors that potentially contribute to uncertainty are shown in Figure 7. This diagram includes effects that directly influence the terms defined in Equation 1.



Click image to enlarge

Figure 7. Ishikawa fishbone diagram for factors of measurement uncertainty in qNMR measurement using internal calibration method

Among those in Figure 7, the following 7 factors of measurement uncertainty were considered for this example.

1. Variability in replicate qNMR sample solution preparations; $u(m_i/m_s)$ evaluated through between-sample (replicates) variance, expressed as $u(S_s/S_i)_a$
2. Variability among the selected signal integrals; $u(S_s/S_i)_b$
3. Repeatability of NMR measurement; $u(S_s/S_i)_c$
4. Uncertainty associated with the balance used; m_i, m_s
5. Uncertainty in the molar masses of the sample and internal calibrant; $u(M_s/M_i)$
6. Uncertainties associated with the natural abundance of ^1H ; $u(N_i/N_s)$
7. Uncertainty associated with the purity of the internal calibrant as mass fraction; $u(P_i)$

The uncertainties associated with factors 1 through 3 can be collectively treated as components of uncertainty inferable through observable peak ratios. These factors are ultimately related to the experiment design and can be assessed through statistical analysis of a series of experimental observations (i.e., Type A uncertainties). For this example, the respective standard uncertainties were calculated through an analysis of variance (ANOVA) of all 36 purity values (Table 2) using the mean square of each factor, in accordance with Equations 3–5. These uncertainty components were treated separately in this example. When significant random effects are not detected among variable factors, the standard uncertainties may be pooled. See GUM and EURACHEM/CITAC CG 4 for more detailed guidance. Additionally, rigorous assessment of correlated observations may also be made using Monte Carlo methods based on random effects models.

[NOTE—The example provided here describes measurement of a high-purity stable substance (butyl *p*-hydroxybenzoate) and consideration of uncertainty of effects such as bias due to impurity peak overlap with the selected signals, $u(S_s/S_i)_x$, or chemical lability, $u(S_s/S_i)_y$, are not significant or explicitly discussed. The presence of

these non-random effects in the measurement of more complex substances would ultimately require validation of an improved procedure or realistic adjustment of the measurement results. These are generally important considerations that analysts should make on a case-by-case basis when qualifying qNMR procedures.]

For a simpler experiment design, such as one that does not include repeat NMR measurements and where there is only one signal integral, $u(S_s/S_i)_a$ can be evaluated simply as the standard deviation of results from sample replicates. For such a scenario, it is also advisable to use expert judgment, prior experimental evidence, or other knowledge (Type B approach) to determine reasonable estimates of $u(S_s/S_i)_c$ and/or $u(S_s/S_i)_x$, $u(S_s/S_i)_y$, or others, when appropriate.

Table 2. The ANOVA Result of Purity Determination with ¹H qNMR for Uncertainty Evaluation

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F	p
Sample preparation	0.6973197	2	0.348660	1.3937	0.2969700
Signal selection	2.2515284	9	0.250170	5.5092	0.0003873
Repeatability	1.0898286	24	0.045410	—	—
Total	4.0386766	35	0.115391	—	—

Equations 3–5. Calculation of relative standard uncertainties of factors 1, 2, and 3:

$$s_p^2 = \frac{MS_p - MS_q}{nq}, \frac{u\left(\frac{S_s}{S_i}\right)_a}{\left(\frac{S_s}{S_i}\right)_a} = \sqrt{\frac{s_p^2}{p}} \quad (\text{Equation 3})$$

$$s_q^2 = \frac{MS_q - MS_e}{n}, \frac{u\left(\frac{S_s}{S_i}\right)_b}{\left(\frac{S_s}{S_i}\right)_b} = \sqrt{\frac{s_q^2}{qp}} \quad (\text{Equation 4})$$

$$s_e^2 = MS_e, \frac{u\left(\frac{S_s}{S_i}\right)_c}{\left(\frac{S_s}{S_i}\right)_c} = \sqrt{\frac{MS_e}{nqp}} \quad (\text{Equation 5})$$

s_p^2 = variability in replicate qNMR sample solution preparations

s_q^2 = variability among selected signal integrals

s_e^2 = repeatability of NMR measurement

MS_p = mean square of qNMR sample solution preparations

MS_q = mean square of selected signal integrals

MS_e = mean square of repeated NMR measurements

p = number of prepared sample solutions

q = number of selected signal integrals

n = number of repeated NMR measurement

For factors 4 through 7, the standard uncertainties were estimated using information other than the values provided in [Table 1](#), as presented and described in the Appendix. The estimate of $u(P_i)$ is provided through certification of 1,4-BTMSB-*d*₄ CRM; $\pm 0.5\%/k = \pm 0.25\%$.

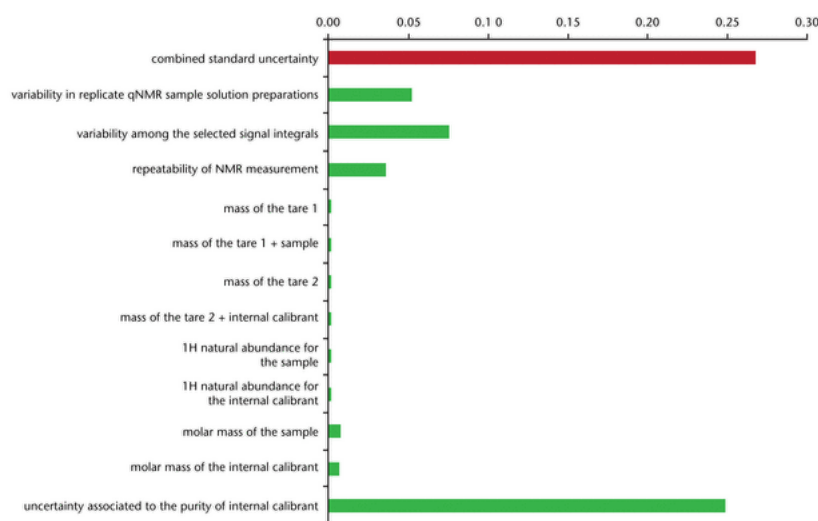
The total measurement uncertainty budget is provided in [Table 3](#). The proportion of each uncertainty as a component $u_c(P_s)$ is plotted in [Figure 8](#). It is evident from inspection of this plot that the uncertainties attributable to factors 1, $u(S_s/S_i)_a$, 2, $u(S_s/S_i)_b$, 3, $u(S_s/S_i)_c$, and 7, $u(P_i)$, are the most substantial components, whereas those attributed to factors 4–6 are relatively small. As such, the standard uncertainties of factors 4–6 were treated as practically negligible and the combined standard uncertainty was calculated using the values in [Table 3](#) and in accordance with [Equation 2](#) as:

$$u_c(P_s) = \sqrt{\left(\frac{u(S_s/S_i)}{(S_s/S_i)}\right)_a^2 + \left(\frac{u(S_s/S_i)}{(S_s/S_i)}\right)_b^2 + \left(\frac{u(S_s/S_i)}{(S_s/S_i)}\right)_c^2 + \left(\frac{u(P_i)}{(P_i)}\right)^2} = \sqrt{(0.052)^2 + (0.075)^2 + (0.036)^2}$$

Table 3. Measurement Uncertainty Budget for the Mass Fraction of Butyl *p*-Hydroxybenzoate Determined by ¹H qNMR with Internal Calibration Method

Quality	Source of Uncertainty	Value	Units	Type	Standard Uncertainty	Sensitivity Coefficient	Uncertainty Component	Degree of Freedom
NMR experiments	Variability in replicate qNMR sample solution preparations	99.60	mass fraction, %	A	0.05231	1	5.23E-02	2
	Variability among the selected signal integrals	99.60	mass fraction, %	A	0.07542	1	7.54E-02	9
	Repeatability of NMR measurement	99.60	mass fraction, %	A	0.03552	1	3.55E-02	24
Balance	Mass of the tare 1	65.7334	mg	B	0.000535	1.5151	8.11E-04	∞

Quality	Source of Uncertainty	Value	Units	Type	Standard Uncertainty	Sensitivity Coefficient	Uncertainty Component	Degree of Freedom
	Mass of the tare 1 + sample	75.33237	mg	B	0.000583	-1.3221	7.70E-04	∞
	Mass of the tare 2	66.1353	mg	B	0.000532	-1.5291	8.14E-04	∞
	Mass of the tare 2 + internal calibrant	66.1667	mg	B	0.000537	1.5052	8.09E-04	∞
Molar mass	¹ H natural abundance for the sample	194.230	g/mol	B	0.01295	0.5128	6.64E-03	∞
	¹ H natural abundance for the internal calibrant	226.502	g/mol	B	0.01433	-0.4397	6.30E-03	∞
Number of resonating hydrogens	Molar mass of the sample	14	—	B	0.000162	-7.1140	1.15E-03	∞
	Molar mass of the internal calibrant	18	—	B	0.000162	5.5331	8.94E-04	∞
Purity of internal calibrant	Uncertainty associated to the purity of internal calibrant	100.0	mass fraction, %	B	0.25	0.9960	2.49E-01	∞
							Combined standard uncertainty 0.268	



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Figure 8. Relative contributions of each component to the combined standard uncertainty of the mass fraction of butyl *p*-hydroxybenzoate determined by ¹H qNMR with internal calibration method.

The estimate $U_c(P_s)$ of was used to calculate the expanded uncertainty of the measurement at the 95% level of confidence, $U(P_s)_{95\%}$.

$$U(P_s)_{95\%} = k \times u_c(P_s), \quad (\text{Equation 6})$$

where k is the coverage factor at the 95% level of confidence and is based on the effective degrees of freedom and assumption that the interval $P_s \pm U(P_s)_{95\%}$ corresponds to a normal distribution. For this example and when the number of effective degrees of freedom is large, it is often considered practical to express the measurement uncertainty under the assumption that $k = 2$.

Therefore, the expanded measurement uncertainty was calculated as:

$$U(P_s)_{95\%} = 2 \times 0.268 \% = 0.536 \%$$

The final result of purity determination (mass fraction, %) of butyl *p*-hydroxybenzoate by ¹H qNMR was expressed as:

$$\text{Purity as mass fraction (\%)} = 99.6 \% \pm 0.6 \% (k = 2)$$

According to these results, the specifications of the ATP are met (measurement uncertainty NMT 2.0%) and the method is fit for purpose (i.e., validated).

Importantly, this process yields an actual value for the measurement uncertainty that can be used for lifecycle management of the method. This example highlights the single-laboratory qualification process, but after this initial step is completed, the method can be subjected to interlaboratory method qualification, a laboratory proficiency testing (PT) scheme, or ongoing instrument PQ that all serve as components of a continuous control strategy to provide assurance that the analytical

procedure is fit for purpose. It is also noteworthy that many of the individual contributions to the measurement uncertainty are not compound-specific and the data can be used as estimates for further development of compound-specific analytical procedures, where only a subset of the variables will have to be assessed (e.g., specificity/selectivity, working range, sample stability) and subject to requalification.

COMPARISON OF TRADITIONAL VERSUS NEW VALIDATION APPROACHES

Establishment of ATP up-front ensures the analytical needs are defined and that the appropriate technique is selected for method development. The example shown above illustrates uncertainty assessment based on triplicate measurements of four NMR signals in triplicate test solution preparations and an assessment of factors contributing to the uncertainty in the NMR measurement. In contrast, the traditional approach to validation based on empirical determination of performance characteristics typically requires more test solution preparations that consume more material, instrument, and analyst time, without an up-front statement to explain requirements for the analytical procedure and without a comprehensive understanding of uncertainty in the measurement. Furthermore, instrument qualification can be used to leverage demonstration of linearity, LOQ, and range so that only a subset of compound-specific variables needs to be assessed, which is another source of time, material, and cost savings.

CONCLUSION

The adoption of the life cycle approach and measurement uncertainty model for applications of qNMR enables the integration of separately qualified instruments and qualified methods. This approach shifts the focus of "validation" to include design and development and uses statistical tools that may be perceived as a paradigm shift. The benefits of not requiring traditional validation for each compound-specific analytical procedure will greatly reduce the overall workload and improve efficiency without compromising quality assurance. The information gathered through multiple iterations of instrument and method qualifications, using CRMs, should be maintained in documented and traceable format to form a knowledge base that can be used to simplify the design, development, and qualification of analytical procedures based on qNMR. The commercial availability of SI-traceable CRMs is a key enabler for the adoption of qNMR as a method for characterization of identity, strength, quality, and purity of drug substances and drug products according to cGMP regulations. For full realization of the benefits, a reconciliation and/or mutual recognition of quality systems and terminologies from standardization organizations (e.g., between ISO and USP) may be required.

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Supplemental Information for the butyl *p*-Hydroxybenzoate Measurement Example

For uncertainty factor 4, the standard uncertainty $u_d(x)$ was calculated by substituting the values of weighed mass of the tares (for the sample and IS), sample, and IS into Equation A-1 (uncertainty formula) specified on the calibration certificate issued from the calibration laboratory of the balance used for the sample preparation.

Calculation of standard uncertainty of factor 4:

$$u_d(x) = \frac{0.00021 + 0.00000857 \times m}{\sqrt{3}} \quad (\text{Equation A-1})$$

m = value of weighed mass

[NOTE—The values substituted in the uncertainty formula above are specified for this example and should not be used to calculate the uncertainty for other balances.]

[NOTE—If the calibration certificate does not have an uncertainty formula, the uncertainty associated with the balance used can be calculated by substituting the uncertainty value of the balance described in the calibration certificate into Equation A-2.]

Calculation of standard uncertainty of factor 4:

$$u_d(x) = \frac{U_{balance}}{k} \quad (\text{Equation A-2})$$

$U_{balance}$ = expanded uncertainty specified in the calibration certificate

k = coverage factor of expanded uncertainty

For factor 5, the standard uncertainty was calculated using Equation A-3. The range of upper and lower limits of the atomic weight of each element specified on International Union of Pure and Applied Chemistry (IUPAC) Technical Report (the latest edition of atomic weights of the elements) were assumed to be a uniform distribution, and the value obtained by dividing this range by the square root of 3 was defined as the uncertainty of atomic weight of each element. Next, the value obtained by multiplying these uncertainties by the number of each atom in a molecule was defined as the uncertainty of each element. Lastly, uncertainties associated to the relative molecular mass of the sample and internal calibrant were calculated by adding together the squares of each of the uncertainties and taking the positive square root of the sum.

Calculation of standard uncertainty of factor 5:

$$u_e(x) = \frac{\sqrt{\sum_{i=1}^n ((A_{ui} - AW_{li}) \times N_i)^2}}{\sqrt{3}} \quad (\text{Equation A-3})$$

A_u = upper limit of the atomic weight of each element

A_l = lower limit of the atomic weight of each element

N = number of each atom in a molecule

[NOTE—There is also an IUPAC Molecular Weight Calculator based on Monte Carlo method that calculates the standard uncertainty of relative molecular mass automatically using standard atomic weights (26).]

For factor 6, the standard uncertainty was calculated using Equation A-4. The range of lower limit and upper limit (assumed to be 1 in this case) of the natural abundance of hydrogen specified on IUPAC Technical Report (the latest edition of isotopic compositions of the elements) were assumed to be a uniform distribution, and the value obtained by dividing this range by the square root of 3 was defined as the uncertainty of natural abundance of hydrogen.

Calculation of standard uncertainty of factor 6:

$$u_f(x) = \frac{NA_u - NA_l}{\sqrt{3}} \quad (\text{Equation A-4})$$

NA_u = upper limit of the natural abundance of hydrogen

NA_l = lower limit of the natural abundance of hydrogen

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