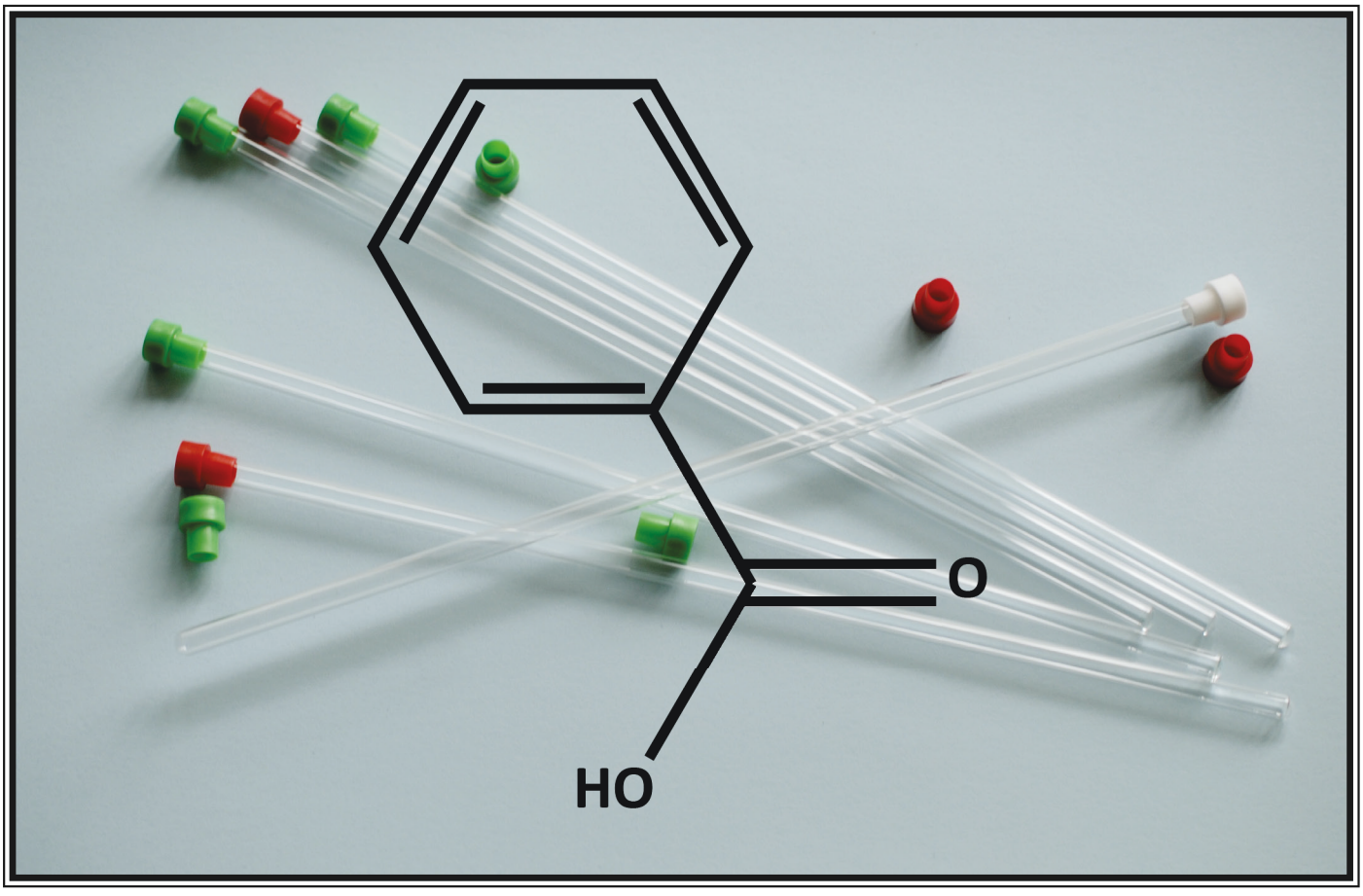
Bureau International des Poids et Mesures

qNMR Internal Standard Reference Data (ISRD)

Internal Standard Reference Data for qNMR: Benzoic Acid [ISRD-08]



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BIPM ISRD-08 : qNMR using benzoic acid

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1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is now well-established as the pre-eminent method for the qualitative structural analysis of organic molecules. The potential for the application of quantitative nuclear magnetic resonance (qNMR) for organic analysis was also recognized soon after the technique became widely commercially available.[1] However it has only been more recently that this potential has begun to be generally realized as instrumental capabilities have achieved a level of accuracy and precision comparable to those attainable by chromatographic techniques. Quantitative methods based on NMR spectroscopy, particularly for the assignment of the purity of individual organic compounds, are now actively and extensively implemented.[2–5] As evidence, an editorial in the *Journal of Medicinal Chemistry*[6] highlighted and recommended the general utility of "absolute quantitative ^{1}H NMR spectroscopy to determine the purity of biologically tested research compounds". Purity assignment by qNMR spectroscopy potentially meets the metrological requirements for a primary ratio measurement procedure.[7] Validated qNMR methods[8] are now being used, often in combination with data obtained by orthogonal chromatographic techniques, to assign the purity of organic materials intended for use as Primary Reference Materials[9] for individual organic analytes.[10-12] The availability of properly characterized Primary Reference Materials is in turn an essential initial step in establishing the metrological traceability for measurement results for an organic analyte linked through a calibration hierarchy to a specific pure material.[13]

The assignment of the mass fraction purity of an organic analyte *A* by qNMR in solution using an internal standard *S* is based on measurement equation (1) below:

$$w_{\mathrm{A}} = \frac{I_{\mathrm{A}}}{I_{\mathrm{S}}} * \frac{N_{\mathrm{S}}}{N_{\mathrm{A}}} * \frac{M_{\mathrm{A}}}{M_{\mathrm{S}}} * \frac{m_{\mathrm{S}}}{m_{\mathrm{A}}} * w_{\mathrm{S}} \qquad \text{Equation 1}$$

w_{A} is the mass fraction of *A* in the material subject to assignment, w_{S} the independently established mass fraction content of the internal standard *S*, I_{A} and I_{S} are the integrals of the quantified signals unique to *A* and *S* respectively, N_{A} and N_{S} the number of ^{1}H nuclei contributing to each quantified signal, M_{A} and M_{S} the molar masses of the analyte and internal standard and m_{A} and m_{S} the masses of the individual aliquots of the analyte and internal standard material used to prepare the solution subject to the qNMR measurement.

In optimal cases and with the data processing carried out by experienced operators the standard uncertainty for purity mass fraction assignments for non-problematic systems has been reported to reach the level of 1 mg.g^{-1} on an absolute basis, equivalent to 0.1 % relative.[14] However this level of uncertainty is difficult to achieve on a routine basis and in addition is limited on a case-by-case basis being contingent on the structural complexity and impurity profile of *A*. Factors including, *inter alia*, the lineshape and multiplicity of the signals integrated, the extent and nature of potential interferences from impurities present in the analyte, the nature of the internal standard and solvent used, the magnetic field strength,

hardware settings and performance characteristics of the spectrometer and the approach taken to transform the time domain free induction decay (FID) signal generated by the NMR experiment and integrate the signals of the resulting frequency domain spectrum can all contribute to the overall uncertainty of the assigned value. Evidently, regardless of the precision of a qNMR measurement, the overall (relative) measurement uncertainty of a qNMR assignment can never be smaller than that associated with the purity of the internal standard used to obtain the result.

The first goal of this document is to furnish general recommendations for the design of a qNMR experiment and for the undertaking of a quantitative ^{1}H NMR measurement using the internal standard approach to provide a measurement result traceable to the International System of Units (SI).[15] It should be noted that although these principles should be applicable in general to quantitative measurement involving any NMR-active nuclei, the specific recommendations in this document are intended only for assignments by ^{1}H qNMR.

The second goal is to describe a set of internal standard reference materials (ISRMs) which the Bureau International des Poids et Mesures (BIPM) in collaboration with the National Metrology Institute of Japan (NMIJ) propose as a "universal" set of higher-order, SI-traceable internal standards for use in routine ^{1}H qNMR measurements. Different groups have proposed specific compounds or sets of compounds suitable for use as qNMR internal standards.[14,16–18] Although there is some commonality between the internal standards recommended in this earlier literature and our proposal, the focus of the earlier papers is the application of the materials for general use in purity assignments rather than, as is the case here, of their suitability as higher-order, SI-traceable primary measurement standards for qNMR. At least one ISRM compound should be suitable for use for the assignment of a given organic compound soluble in a specified NMR solvent. The compounds constituting the "universal" ISRM set together with an overview of their solubility and suitability for use in representative deuterated NMR solvents are described in Table 1 below.

The third goal and the focus of this specific document is to provide guidance regarding the use and limitations of benzoic acid (BA) as an ISRM for qNMR analysis.

Ideally, a qNMR ISRM should consist of a stable crystalline solid which is:

- available as a high-purity Certified Reference Material (CRM) whose value has been assigned by a National Metrology Institute (NMI) using methods independent of qNMR **or** which has been value assigned directly by qNMR using a high purity CRM as the internal standard;
- predominantly a single organic component (w_S > 995 mg.g^{-1});
- value assigned with small standard uncertainty ($u(w_s)$ < 1 mg.g^{-1});
- providing unique NMR signals, preferably as singlet or simple multiplet resonances, having Lorentzian lineshape with a narrow signal width;
- free of impurities interfering with the areas to be integrated;

- inert in solution in suitable NMR solvent;
- soluble in the chosen NMR solvent at a level in excess of 2 $mg.mL^{-1}$;
- readily handled for accurate mass determinations:
  - non-hygroscopic
  - non-volatile
  - not subject to electrostatic effects
- having a relative mass content contribution from the hydrogen atoms giving rise to the quantification signal below 5 %.[a]

It is recognized that these characteristics constitute a "wishlist" rather than prescriptive requirements that must be met by all materials. In fact, BA lacks a number of these characteristics and its global adoption as an ISRM corresponds more to historical reasons. These included the availability of highly pure and stable acidimetric standards traceable to the SI and with low uncertainty that were used, with important caveats, for ^{1}H qNMR applications.[19–21]

The solubility estimates of the ISRMs in the individual solvents listed in Table 1 are indicative of those for solvents having similar solubilizing capabilities. The five solvents shown were selected as the most readily available deuterated solvents. In practice, the majority of the reported applications of qNMR for purity assignment in solution have been undertaken using one of these solvents.

At least three ISRMs are applicable to each solvent class and provide quantification signals distributed across the standard ^{1}H chemical shift range.

BA is suitable for use as an internal standard for qNMR purity assignments of analytes soluble in CD_3OD, DMSO-d_6, $CDCl_3$, acetone and solvents with related solubilizing properties. The following sections of this reference document and the attached annexes describe specific properties and applications of BA for use as an ISRM for qNMR.

[a] When H-content exceeds 5 % by mass, the aliquot size for the internal standard used for a typical analysis is small and the uncertainty associated with gravimetric operations becomes a limiting factor in the overall uncertainty of a qNMR assignment.

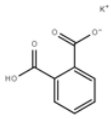
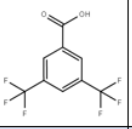
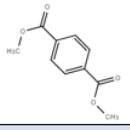
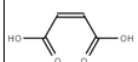
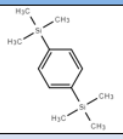
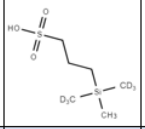
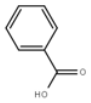
ISRM	KHP	BTFMBA	DMTP	MA	$DMSO_2$	BTMSB-d_4	DSS-d_6	BA
Structure								
δ (ppm)	8.3-7.0 (4H)	8.4-8.5 (2H) 8.2-8.4 (1H)	8.1 (4H) 3.9 (6H)	6.3 (2H)	3.0 (6H)	0.2 (18H)	0.1 (9H)	8.0-8.1 (2H) 7.6 (1H) 7.4-7.5 (2H)
Density ($g \cdot cm^{-3}$)	1.64 ± 0.17	1.72 ± 0.04	1.2 ± 0.24	1.53 ± 0.03	1.4 ± 0.03	1.0 ± 0.02	1.27 ± 0.03	1.266 ± 0.002
H content ($mg \cdot g^{-1}$)	19.6	11.6	20.6 (4H) 30.9 (6H)	17.2	63.8	79.5	44.5	16.5 (2H) 8.3 (1H)
Solvent	**Solubility (mg/mL)**							
D_2O	> 10	< 1	< 1	> 5	> 10	< 1	> 5	< 3
$(CD_3)_2SO$	> 2	> 10	> 5	> 10	> 5	> 2	> 5	> 10
CD_3OD	> 2	> 10	> 2*	*	> 5	> 2	> 5	> 10
$CDCl_3$	< 1	< 1	> 10	< 1	> 10	> 5	< 1	> 10
$(CD_3)_2CO$	> 2	> 10	> 10	> 10	> 10	> 5	> 5	> 10

Table 1: qNMR ISRM Suite[22]

* **soluble but only for quantifications based on the aromatic proton signal. Exchange of the methyl ester with CD_3OD precludes quantification based on the dimethyl ester.**

* **soluble but unsuitable for qNMR due to esterification reaction with CD_3OD**

KEY

KHP: Potassium hydrogen phthalate

BTFMBA: 3,5-Bis(trifluromethyl) benzoic acid

DMTP: Dimethyl terephthalate

MA: Maleic acid

$DMSO_2$: Dimethyl sulfone

BTMSB-d_4: 1,4-Bis(Trimethylsilyl)-2,3,5,6-tetradeutero benzene

DSS-d_6: 3-(Trimethylsilyl)-hexadeuteropropane-1-sulfonic acid
[4,4-Dimethyl-4-silapentane-1-sulfonic acid-d_6]

BA: Benzoic acid

D_2O: Deuterium oxide

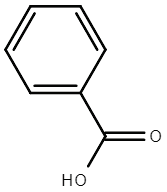
DMSO-d_6: Dimethyl sulfoxide-d_6 / Hexadeuterodimethyl sulfoxide

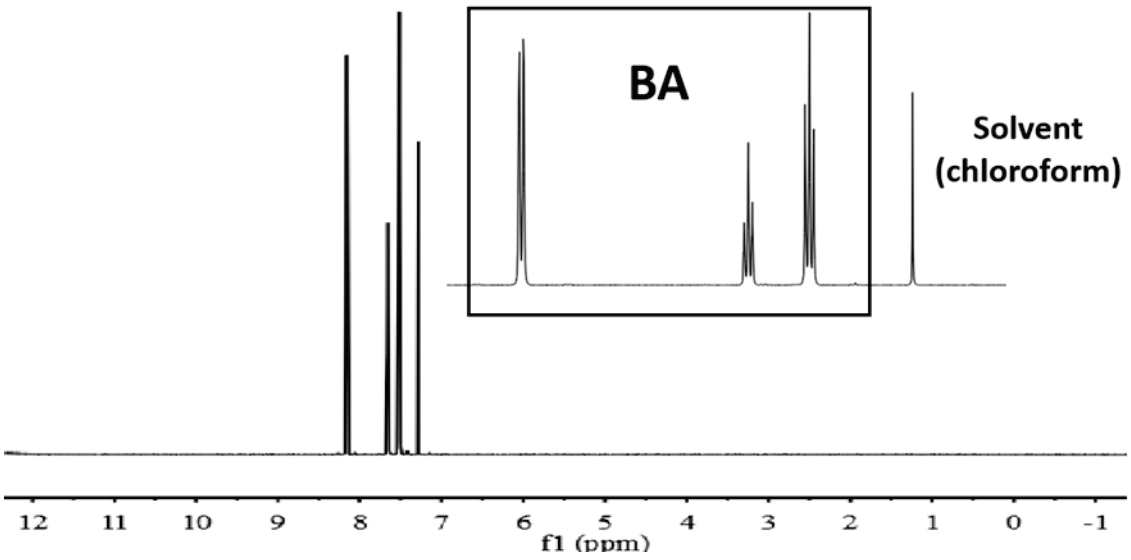
CD_3OD: Methanol-d_4 / Tetradeuteromethanol

$CDCl_3$: Chloroform-d / Deuterochloroform

2. Properties of BA

2.1 Physical Properties

IUPAC Name:	Benzoic acid
Structure:	
CAS Registry Number:	65-85-0
Molecular Formula:	$C_7H_6O_2$
Molar Mass:[23]	122.121 g/mol , u = 0.004 g/mol
Melting point:[24]	122.340 °C, u = 0.005 °C
Density:[24]	1.266 kg/m^3, u = 0.002
Appearance:	White crystalline powder
^{1}H NMR[25]	δ 7.4 - 7.5 (2H) ppm δ 7.6 (1H) ppm δ 8.0 - 8.1 (2H) ppm
^{13}C NMR	δ 172.8, 133.8, 130.3 (2C), 129.4, 128.5 (2C) ppm



^{1}H NMR spectrum of BA in $CDCl_3$: Bruker Ascend NMR spectrometer with 5 mm CPQCI probe.

800 MHz spectra of BA in other solvents are given in Annex 5.1.

2.2 NMR Solvent Compatibility

NMR solvents suitable for use with BA are CD_3OD, DMSO-d_6, $CDCl_3$ and $(CD_3)_2O$, where it is soluble at levels in excess of 10 mg.mL^{-1}. Solubility in D_2O is very limited (about 2.7 mg/mL at 18 °C or 3.4 mg/mL at 25 °C).[26]

2.3 NMR quantification signals

There are two pairs of magnetically equivalent aromatic protons at the 2,6- position and at the 3,5- position of the aromatic ring and one proton at the 4-position of BA. The spins in the aromatic ring present strong coupling and give rise to signals in the chemical shift range 8.0 – 8.1 ppm for the two hydrogen (2,6-position) doublet, 7.4 – 7.5 ppm for the two hydrogen (3,5-position) triplet and 7.6 ppm for the triplet corresponding to hydrogen at position 4. The exact position of the resonance is a function of factors including but not limited to the solvent, temperature and the concentration of BA and other analytes in the solution. In high-field instruments the signal from the protons at 2,6 position is sufficiently separated from the other resonances to be integrated separately (including ^{13}C satellites). However, for instruments operating at 400 MHz or at weaker magnetic fields, resolution may require that all aromatic proton signals be integrated together.

For optimal quantification results the homogeneity of the spectrometer magnetic field should be optimized such that the full width at half maximum (FWHM) of the signal for residual solvent in the solution is less than 1 Hz while the base of each resonance retains a suitable Lorentzian peak shape.

2.4 Impurities and artefact signals

Samples of BA analysed in our laboratory have typically not presented evidence of the presence of significant levels (> 0.1 %) of related structure impurities in the material. In practice the main interferences in a solution containing BA could come from signals due to residual non-deuterated solvent. The chemical shifts of these signals are given in Table 2 below.

2.5 Solvent recommendations and advisories

2.5.1 D_2O and related solvents

BA has a very limited solubility in D_2O. Its solubility has been reported to be 2.7 mg/mL at 18° or 3.4 mg/mL at 25 °C.[26] The addition of NaOD can produce the benzoate ion of increased solubility. Other weaker bases such as $NaHCO_3$ may be added for the same solubilizing purpose at the expense of producing H_2O as by-product, resulting in a broad water resonance peak at 4.8 ppm. Chemical shifts may also change due to pH modifications upon base additions.

2.5.2 **DMSO-d_6 and related solvents**

BA is readily soluble in this solvent. It is recommended for use in qNMR studies where less polar solvents are not suitable for the target analyte. Residual water is often present in DMSO-d6 and may induce a broad peak at 4.3 ppm altering significantly the spectrum baseline.

2.5.3 **Methanol-d_4 and related solvents**

CD_3OD is an excellent solvent for use with BA, with the added advantage that the acidic proton present in BA is exchanged with the solvent and does not interfere with the other signals.

2.5.4 **$CDCl_3$**

$CDCl_3$ is a potential choice as solvent for use with BA. However, there is the potential for signal or baseline interference due to the broad signal from the acid hydrogen. In addition, the residual non-deuterated chloroform signal can interfere with the signal from the BA aromatic protons at positions 3,5. It is recommended to first consider the suitability of another solvent for use with BA.

2.5.5 **$(CD_3)_2O$**

Acetone-d_6 is a very suitable solvent for use with BA. The BA acidic proton is not exchanged with the deuterium from the solvent but it is likely to appear at chemical shifts above 10 ppm, posing no problems of interference.

Table 2 : Solvent Parameters for BA

Solvent	qNMR signal (ppm)*	Integration range (ppm)*	T_1 (s)*	Residual Solvent (ppm)	Comments:
D_2O					Poor solubility
DMSO-d_6	7.9 (2H)	7.8 – 8.0	3.5	2.5	
CD_3OD	8.04 (2H)	7.9 – 8.2	4.6-5.5	3.31	
$CDCl_3$	8.12 (2H)	7.9 – 8.2	3.4	7.25	
$(CD_3)_2O$	8.06 (2H)	7.9 – 8.2	5.0-5.9	2.05	

* Indicative values only. The observed value in a specific qNMR solution will be a function of factors including concentration of BA and analyte, temperature, instrument, etc.

3. Good Practice Guidance for SI-Traceable qNMR Measurement Results

3.1 Introduction

The first step in any purity assignment by qNMR should be the confirmation by qualitative NMR or other techniques of the identity of the analyte subject to purity assessment. In addition to confirming that the molar mass (*M*) and the number of nuclei (*N*) contributing to each signal subject to integration are appropriate, obtaining qualitative NMR spectra also provides a check for the occurrence and extent of any interfering signals in the sections of the NMR spectrum subject to integration.

Once the qualitative identity of the analyte has been appropriately established, the input quantities that influence qNMR measurement results must be evaluated. These are identified from the measurement equation (Eqn. 1, Section 1). The mass fraction purity of the internal standard used for the measurement, which is the source of traceability to the SI for the value to be assigned to the analyte, is established by independent measurements undertaken prior to the qNMR experiment.

The gravimetric procedure used for the preparation of the NMR solution has to be fully validated and fit for its intended purpose,[27,28] and the spectrometer performance, experimental parameters and the protocol for signal processing and integration must be optimized,[4,8,29] in order to produce a result for the ratio of the integral of the analyte and standard signals that accurately reflects the molar ratio of the hydrogen nuclei giving rise to the signals.[30] When these conditions are met the assigned mass fraction purity of the analyte can be regarded as traceable to the SI.[10,31,32] Some general guidance for recommended practice for these critical steps is given in the following sections.

3.2 Internal standard

The internal standard used in a qNMR purity assignment should comply as far as possible with the criteria described above regarding composition, physical characteristics, inertness, solubility, impurity profile and relative hydrogen content by mass. In addition, in order to establish traceability of the result of the qNMR assignment to the SI, the material should comply with the requirements of a reference measurement standard, and in particular a reference material, as defined in the International Vocabulary of Metrology (VIM).[33]

For SI-traceability the internal standard should consist of one of the following:

a. Certified Reference Material (CRM) characterized for its mass fraction purity and value assigned by a National Metrology Institute;
b. CRM provided as a high purity organic material by a Reference Material Producer accredited to ISO 17034:2016[34] requirements;
c. High-purity material subject to a validated measurement procedure for purity assignment by qNMR using as an internal standard a CRM of type (a) or (b).

3.3 Gravimetry

The realization of accurate and precise qNMR measurements relies on the application of a properly implemented gravimetric procedure for the mass determinations of the internal standard and analyte. Recommended practice in this area in the specific context of qNMR sample preparation has been described in the literature.[27] Achieving an overall relative standard measurement uncertainty for a qNMR assignment of 0.1 % requires the relative uncertainty associated with individual gravimetric operations to be less than 0.03 %. If the combined standard uncertainty of a mass determination is 3 µg, a level achievable with modern electronic microanalytical balances, this corresponds to a minimum sample size of 10 mg. Care should be exercised to include an appropriate allowance for the uncertainty of each gravimetric operation within the final uncertainty budget for a qNMR purity assay, that adequately takes into account the aliquot sample sizes and the performance characteristics of the balance used.

In addition to suitable control for each mass determination, if the receptacle used for the final solution preparation is not the same as that used for both mass determinations the procedure for transfer of solids into the solution must address the assumption that the ratio of the gravimetric readings from the balance operations is equivalent to the ratio of the masses of each compound in the solution subject to the qNMR analysis.

For the examples reported in the Annex 5.2 below, gravimetric operations were undertaken using a balance associated with a measurement uncertainty estimate of 0.3 µg for individual mass determinations. In this case, a minimum sample size of 3 mg achieves a relative uncertainty in individual gravimetric operations of 0.01 %. In addition to the measurement uncertainty of the balance reading, for high-accuracy measurements, a correction for sample buoyancy effects and the contribution to the overall measurement uncertainty associated with this correction should also be taken into consideration.[28]

As sample preparation for qNMR involves mass determinations in the milligram range using sensitive balances, the loss of even minute (almost invisible) quantities of powder during the gravimetric procedure will have a measurable influence on the balance reading and hence on the input quantities for the qNMR assignment. Environmental conditions for gravimetry and qNMR sample preparation should be controlled throughout the process, subject to minimum change and kept within the operating range recommended by the manufacturer.[35] It is recommended that mass determinations be performed in an area where the relative humidity is maintained in the range 30 % to 70 %.

The accumulation of surface electrostatic charges is a potential source of bias for mass determinations, particularly for high-polarity, hygroscopic compounds. In these cases, pre-treatment of the sample with an electrostatic charge remover or deionizer is advisable prior to the mass determination. Where possible materials subject to qNMR analysis should be evaluated for their hygroscopicity, for example by measurement of the change in observed

mass as a function of relative humidity using a dynamic sorption balance. This allows for assessment of the likely impact of variation in the relative humidity in the local environment on the results of gravimetric operations for a given compound.

A minimum of two independent gravimetric sample preparations should be undertaken when assigning the purity of a compound by qNMR.

3.4 NMR spectrometer optimization for quantitative measurements

There is no specification of minimum NMR spectrometer field strength for purity measurements. Increasing field strength results provides enhanced signal separation and increases sensitivity, both of which should increase the accuracy and precision of qNMR measurements. Careful optimization of the lineshape (shimming) is mandatory and critical in order to achieve reliable qNMR results.[36] A general guidance is to choose the simplest signal in the sample, often the residual solvent peak, and to optimize the instrument shimming until this signal is symmetrical with a FWHM below at least 1 Hz. Experience has shown that these lineshape requirements are more easily achieved using an inverse probe than a direct type. For lower field magnets (< 300 MHz), this recommendation might not be attainable. If the lineshape is broader the level of measurement uncertainty associated with the assigned value will increase. Under no circumstances should a signal from a labile, exchangeable hydrogen or one subject to dynamic tautomeric exchange be used for quantitative measurements.

Due to the relatively wide Lorentzian signal shape of NMR resonances, the separation of the signals to be quantified from each other and from the remainder of the NMR signals in the spectrum should be considered carefully. Ideally there should be no interfering signals within the range one hundred times the FWHM either side of each signal to be integrated.

3.5 NMR acquisition parameters

The basic experiment to perform quantitative NMR experiments uses a simple 1D pulse sequence designed to minimize differences in the integrated signal intensities due to effects related to incomplete relaxation of the quantification resonances. For highest accuracy assignments use of broadband heteronuclear decoupling should be avoided if possible as it can lead to undesired nuclear Overhauser effects introducing a bias in the intensities of individual measured signals. However, in the common case of ^{13}C-decoupling to remove satellite signals, the potential for bias is greatly attenuated because of the low (1.1 %) natural abundance of the ^{13}C isotopomer. In addition, although the decoupling efficiency for separate ^{13}C satellite signals is generally not equivalent, the combined potential bias introduced due to both effects from the inclusion of ^{13}C-decoupling is negligibly small in most cases.

The recommended basic sequence for a qNMR measurement consists of a “delay-pulse-acquire” experiment. There are critical parameters associated with each phase of the

sequence in order to achieve a reliable, unbiased quantitative signal response. Assuming the experiment starts from an equilibrium magnetization state, the first phase in the experiment is the pulse, which itself is preceded by a delay.

In the pulse phase, the two critical parameters for good qNMR measurement results are the pulse offset and pulse length (also called pulse width or tip angle). When a single "hard" pulse is applied to the bulk magnetization of each compound, off-resonance effects can occur if the frequency offset of the initial pulse is relatively far from that of the signals of interest. Ideally the pulse offset should be positioned as close as possible to the midpoint between the two signals to be quantified. This will not eliminate off-resonance effects but should result in them cancelling out in both signals.

Regarding the pulse length, 90° pulses are recommended for quantitative analyses. A 30° pulse experiment, providing a signal response approximately half that of a 90° pulse, has the potential advantage of needing a significantly shorter relaxation time to re-establish equilibrium magnetization compared with a 90° pulse while requiring only twice as many transients to achieve an equivalent **signal** response. However, this potential practical advantage is offset by the need for four times as many transients as a 90° pulse to achieve the same **signal to noise** ratio. The accuracy (trueness) of the results should not be impacted by the use of different pulse lengths but the acquisition times to achieve equivalent levels of signal precision (repeatability) will.

Additional parameters requiring optimization in the acquisition phase are the spectral window width, the acquisition time, the digital resolution and the relaxation delay time between acquisitions. The spectral window chosen will depend on the design and performance of the instrument used. The theoretical justification for the use of a large spectral window is that oversampling the FID will produce noise filtering. However, the efficiency of digital filters varies by instrument and the appropriate spectral window should be evaluated on a case-by-case basis.

The acquisition time should be at least 2.5 s to avoid truncation of the signals and to allow good digitization of the spectrum. The ideal acquisition time is the smallest time for which no truncation is observed. Use of longer acquisition times than necessary primarily results in addition of noise to the spectrum. The digital resolution should not exceed 0.4 Hz/pt in order to have accurately defined signals that will give meaningful area measurements and suitable repeatability at typical sampling rates.

The relaxation delay between pulses in particular has to be carefully established for each sample mixture. To determine the optimum repetition time for a given qNMR measurement it is critical to determine the longest T_1 time constant of the signals to be quantified. This document presents some observed values measured for BA in different solvents at the concentration and under the specific instrumental conditions used, but these should be regarded as indicative only, and in any event they are not the determining factor in

cases where the T_1 of the analyte quantification signal is longer.

As the T_1 constant arises from a process of spin-lattice relaxation, its values are strongly dependent on the composition of the solution being measured and it should be determined for each signal to be quantified in each mixture on a case-by-case basis. The most commonly used method to determine the T_1 constant is the inversion-recovery sequence generally available in the factory programmed pulse sequences installed with any NMR. The application of the inversion recovery experiment requires knowledge of the optimized 90° pulses for each quantified signal, which should also be determined for each mixture under investigation. The optimized 90° pulse values can be used for both the T_1 determination and the quantitative measurements.

The repetition time between pulses should correspond to the full loop time in the pulse sequence and not simply the relaxation delay. Since most of the time intervals involved in NMR measurement are negligible relatively to the T_1 values, the repetition time (RT) can be estimated as the sum of acquisition time (AQ) and relaxation delay (RD), where the RT is a multiple T_1. After a 90° pulse, if the available instrument time permits, 10 times T_1 of the signal with the longest relaxation time will lead to the recovery of > 99.995 % of the magnetization for all quantified signals. In cases where the T_1 of the quantified signals are similar in magnitude, a shorter relaxation delay will be sufficient for equivalent (even if incomplete) magnetization re-equilibration. At least ten T_1 should be used as a minimum where highest accuracy results are sought.

Thus the pulse RT is given by:

$$RT = RD + AQ = n * T_1 \qquad \text{Equation 2}$$

(n = 10 – 15)

The number of transients (or scans) should be determined according to the concentration of the samples, the nature of the signals and the available instrument time. To achieve a small uncertainty, a signal to noise (S/N) ratio of at least 1000 should be achieved for each signal subject to quantification. Smaller S/N values can still lead to acceptable results, but the reported measurement uncertainties increase as the S/N ratio decreases.

Table 3 – Recommended NMR Parameters for quantitative measurements.

Parameter	Recommended Value	Explanation/Comments
Shimming	FWHM of lineshape signal (eg $CHCl_3$/acetone-d_6) < 1 Hz	Optimization of field homogeneity is critical for uniform response over typical chemical shift range
Pulse Width	90º	Should not change the quality of the results, but the use of a 90º pulse with adequate recovery time leads to a smaller total acquisition time for a target S/N ratio.
Pulse Offset	Midpoint between signals	Theoretically makes off resonance effects equivalent for both signals.
Repetition Time	10 - 15 × T_1	After 90º pulse, a delay of 10 T_1 of the signal with the longest relaxation time necessary for recovery of > 99.995 % of magnetization for all quantified signals.
Number of Transients (scans)	As needed for adequate signal to noise ratio	Evaluate on a case-by-case basis. Minimum requirement is S/N > 1000 for each signal quantified
Spectral Window	> 20 ppm	The use of a wide spectral window for data recording (oversampling) has been reported to yield better results in some instruments because of the noise filtering it produces in the quadrature detection scheme. This is instrument dependent and should be evaluated.
Acquisition Time	> 2.5 s	The correct acquisition time is essential to give the best digital resolution for good quantitative results. If too short, lower digital resolution and truncated signals result. If too long excessive noise is introduced. A minimum of 2.5 s is a useful starting point and 4 s has been found to be suitable for many applications.
Digital resolution	< 0.4 Hz/pt	The digital resolution is the reciprocal of the acquisition time. Suitable signal shape sensitivity requires not less than 0.4 Hz/pt.

Good practice for performing quantitative experiments is to prepare in addition to the sample mixtures one sample consisting of a solvent blank, one with the analyte only and one with the internal standard only in the same solvent. These additional NMR spectra should be acquired prior to the preparation of sample mixtures to check the suitability of the proposed mixture in terms of the absence of interferences from one compound (or impurities present in it) in the other. Other NMR techniques such as 2D HSQC or COSY may be applied to demonstrate the uniqueness of the signals used for quantification and the absence of overlapping contributions from impurities but it is important to be aware that the sensitivity of such techniques is low and the absence of observable interferences does not guarantee a signal free of such interferences.

Each independently weighed analyte/IS mixture (a minimum of two samples) should be measured at least three times in the NMR system. Independent measurements for a particular sample mixture should be non-continuous, where the tube is removed and the measurement process (tuning, locking, shimming) is repeated each time for each sample.

3.6 NMR signal integration

The integration range should extend on each side ideally at least seventy-six times the FWHM of the signal being measured in order to integrate in excess of 99.9 % of the signal. The estimation of signal width should be done for the outer signals if a multiplet signal is subject to integration. A generally acceptable approximation is to use a range extending 30 Hz beyond the furthest ^{13}C satellites as the start and end points for the integration range, as this generally exceeds the above-described width. In a complex spectrum where other signals are adjacent to one or both of the quantification signals and quantification over the full range is not possible, apply a consistent approach to the integration of both signals.

It is important to apply a suitable algorithm for the baseline correction and to check its validity by analysing standard samples. Practical experience has shown that the currently used manual baseline assignment is the most reliable general approach when high-accuracy qNMR results are required.[29,36] A final data treatment parameter that can be applied is an adequate window function. For ^{1}H NMR, exponential multiplication by a factor not greater than 0.3 Hz should be used.

3.7 Measurement uncertainty

Evaluation of the measurement equation previously presented (Equation 1) identifies the factors influencing the input quantities for the measurement uncertainty as shown in the diagram in Figure 2.

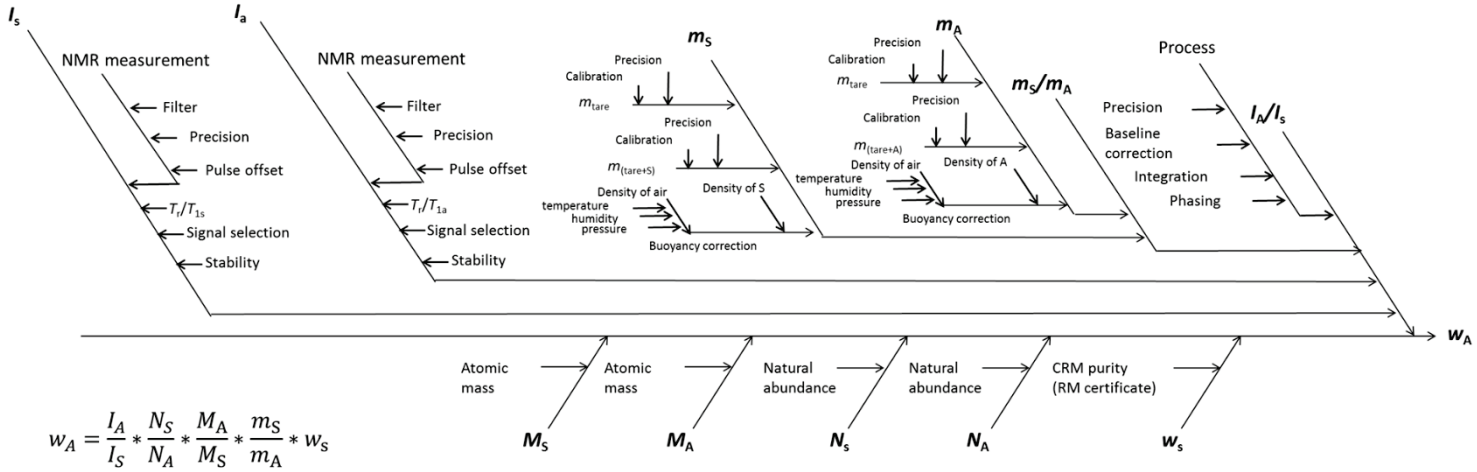


Figure 2 – Ishikawa diagram for input quantities considered for the measurement uncertainty estimation by qNMR

The observed repeatability of the integral area ratios, which incorporates contributions from the input factors for excitation, population, detection efficiency and data processing, is amenable to a type A statistical evaluation.[29,37] Since these measurements come from at least two independent solutions, each containing different sample masses, the observed absolute area ratios will vary on a sample by sample basis.

The measurement uncertainty of the value obtained for each preparation can be evaluated separately and the individual purity results for each sample combined statistically. Another approach is to pool the purity values from the replicate results for the separate samples. Analysis of this combined data by ANOVA produces an assigned value and provides an estimate of the intermediate precision of the overall process. It also identifies if additional variance contributions from sample preparation and signal processing exist in addition to that due to the method repeatability.

The final assigned value will be similar regardless of the approach used, although the contribution to the measurement uncertainty of the result may differ.

The standard uncertainties for the other major input quantities are type B estimates and are straightforward to evaluate. Molar masses and their uncertainties are estimated based on the "conventional" values for atomic weights given in Table 1 of the 2021 revision of the IUPAC Technical report of the standard atomic weights of the elements,[23] the uncertainties of mass determinations are based on balance performance characteristics and are corrected for buoyancy effects[14] and the uncertainty of the purity of the internal standard is assigned by the material provider.

Other approaches to the evaluation of measurement uncertainty for qNMR and the combination of results from qNMR with orthogonal techniques for purity evaluation have also been reported.[20,38] Examples of "best case" measurement uncertainty budgets for qNMR analysis are provided in the examples given in Annex 5.2.

4. Acknowledgements

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DISCLAIMER: Commercial NMR instruments, software and materials are identified in this document in order to describe some procedures. This does not imply a recommendation or endorsement by the BIPM nor does it imply than any of the instruments, equipment and materials identified are necessarily the best available for the purpose.

5. Annexes

5.1 Solution NMR Spectra of BA

5.1.1 BA in CD_3OD

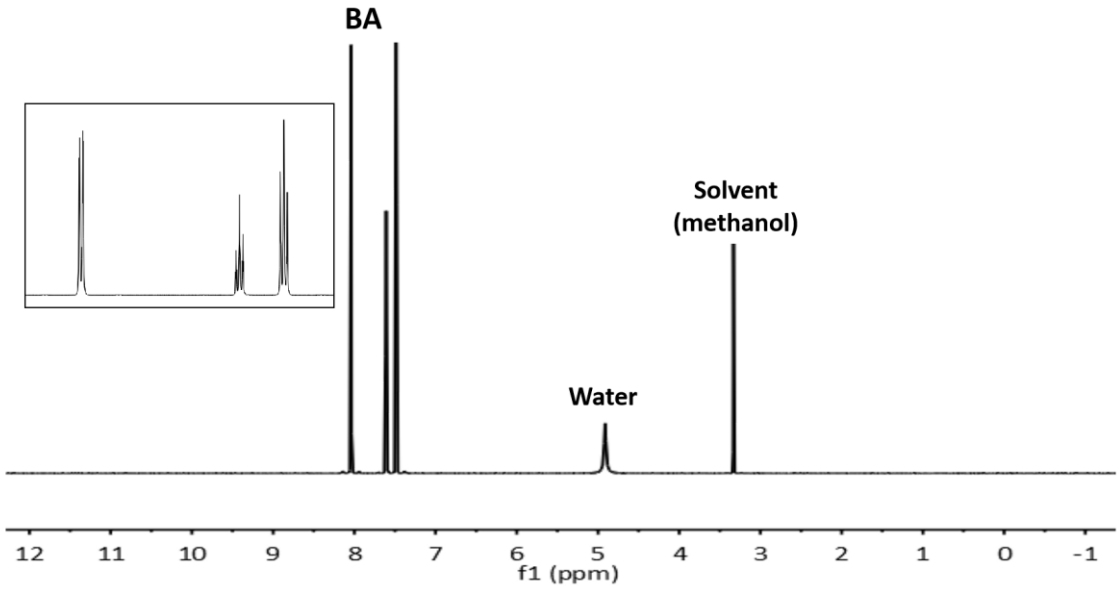

Figure 1 - ^{1}H NMR spectrum of BA in CD_3OD.

5.1.2 BA in acetone-d_6

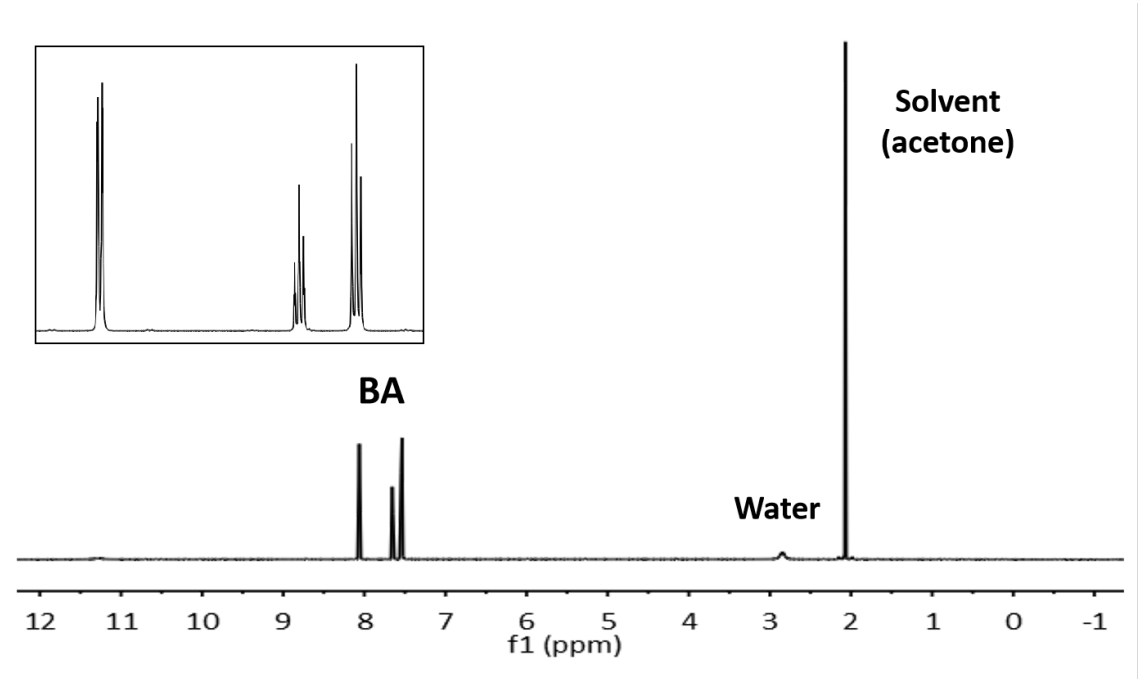

Figure 2 - ^{1}H NMR spectrum of BA in acetone-d_6.

5.2 qNMR using BA as an internal standard

Two examples are provided of the value assignment by qNMR of the mass fraction content of organic compounds using BA as the ISRM and the associated measurement uncertainty budgets. In the first example BA was used in a solution in CD_3OD with 1,4-BTMSB-d_4 as analyte. In the second example acetone-d_6 was the solvent with maleic acid (MA) as the analyte.

These are intended as "best case" illustrations and should not be regarded as representative of the uncertainty budget achievable when quantifying more structurally complex compounds. The signals for quantification in these examples are clearly separated from each other, have narrow, well-resolved signal shape and there is no significant interference from impurities or solvent signals.

A thorough shimming procedure was used to maximize the homogeneity of the instrument field. Gravimetric determinations were carried out using a microbalance with a readability of 0.1 µg and a measurement uncertainty for individual mass determinations of less than 100 mg net of 1.3 µg.

The BA was provided by NIST as a high-purity CRM (NIST PS1).[20] The mass fraction content of BA in the material certified by NIST was 999.92 [+0.04, −0.06] $mg.g^{-1}$. The BTMSB-d_4 and maleic acid used as analytes were donated by WAKO Chemicals. Deuterated solvents were purchased from commercial suppliers and were used without further treatment or purification. Borosilicate glass NMR tubes with 5 mm internal diameter rated for use in 800 MHz spectrometers and purchased from a commercial supplier were used for all measurements.

5.2.1 **BA (IS) & BTMSB-d_4 (Analyte) in CD_3OD**

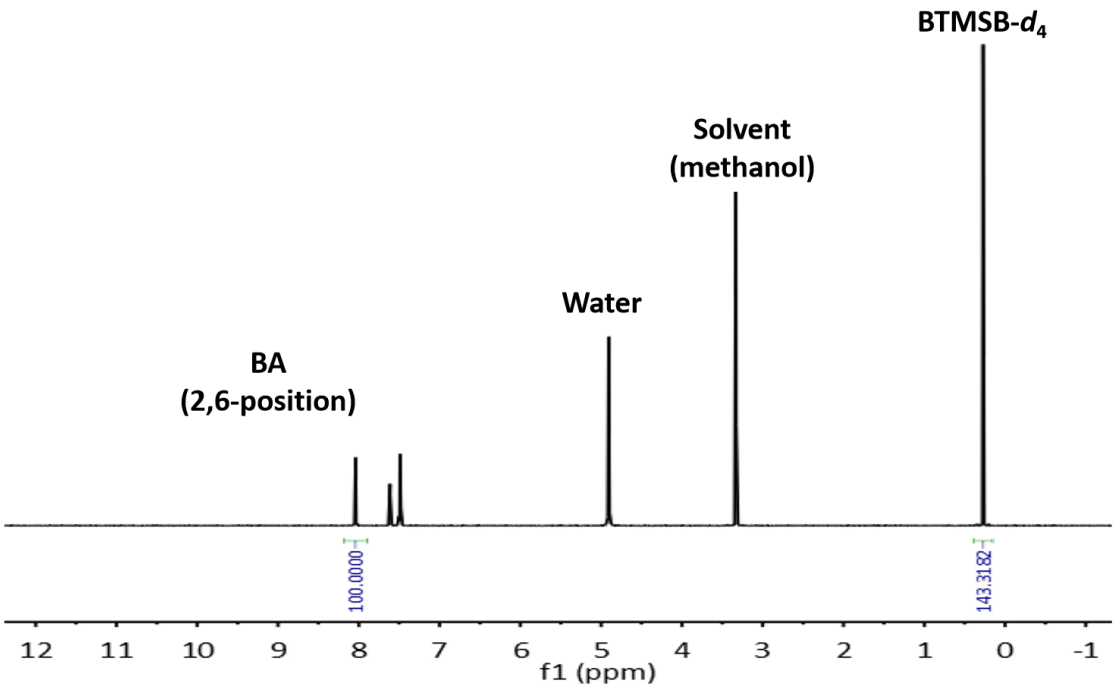

Figure 3 - ^{1}H NMR spectrum of BA + BTMSB-d_4 in CD_3OD.

The optimized gravimetric and NMR parameters for the qNMR assignment using a Bruker

Ascend NMR spectrometer with 5 mm CPQCI probe are given in Table 1. The sample was made up in solution in approximately 2 mL of CD_3OD with 10 min of ultrasonication. Then 800 µL was transferred into the NMR tube for analysis.

Table 1 – NMR parameters for BTMSB-d_4 purity assignment using BA in CD_3OD.

Parameter	*Value*
BA Sample size (mg)	2.9 – 7.5
BTMSB-d_4 Sample size (mg)	1.9 – 2.2
Number of Transients	16
Receiver gain	8
Acquisition time (s)	2.5
Relaxation delay (s)	54.6
Pulse offset (ppm)	4.2
Spectral width (ppm)	20
Data points	639652
Temperature (K)	296
Spinning	Off
Integral ratio (BTMSB-d_4:BA)	1.4 - 3.3

A baseline correction window of one hundred times the FWHM was applied to each integrated signal. The integration range covered eighty times the FWHM. Four independent sample mixtures were prepared and each sample was measured six times. The measurement uncertainty budget for one of the samples is reproduced in Table 2. The integral ratio is the mean of the six replicate values obtained for this sample. The standard uncertainty of the ratio is the standard deviation of the mean. The other uncertainty components are Type B estimations. The relative contribution of each component to the uncertainty of the combined result for this sample is displayed in Figure 4. The mass fraction content of BTMSB-d_4 assigned for this sample was 998.8 ± 0.7 $mg.g^{-1}$.

Table 2 – Uncertainty budget for BTMSB-d_4 purity by qNMR using BA in CD_3OD.

Uncertainty sources	Value	Type	Standard Uncertainty	Sensitivity coefficient	Uncertainty Component
I_S/I_A (repeatability)	1.4313	A	0.00032	0.69785800	2.24E-04
Analyte signal 1H Nuclei	17.9964	B	0.0003	-0.05550112	1.67E-05
IS signal 1H Nuclei	1.9996	B	0.0003	0.49951006	1.50E-04
Analyte Molar Mass (g/mol)	226.502	B	0.0130	0.00440976	5.73E-05
IS Molar Mass (g/mol)	122.123	B	0.0060	-0.00817881	4.91E-05
Analyte mass (mg)	2.2271	B	0.00029	-0.44849143	1.29E-04
IS mass (mg)	7.5422	B	0.00029	0.13243148	3.28E-05
IS purity ($g.g^{-1}$)	0.99992	B	0.000025	0.99890023	2.50E-05
				Combined Uncertainty	0.00031
Purity of BTMSB-d_4	**998.8**		**± 0.7**	**$mg.g^{-1}$**	

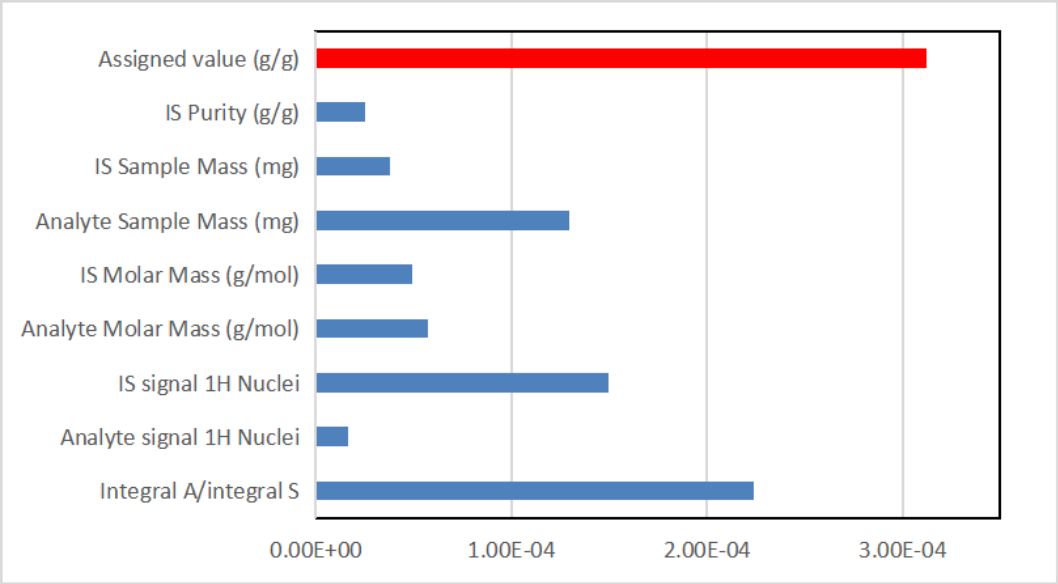

Figure 4 - Relative uncertainty components: BTMSB-d_4 assignment using BA in CD_3OD.

5.2.2 **BA (IS) & MA (Analyte) in acetone-d_6**

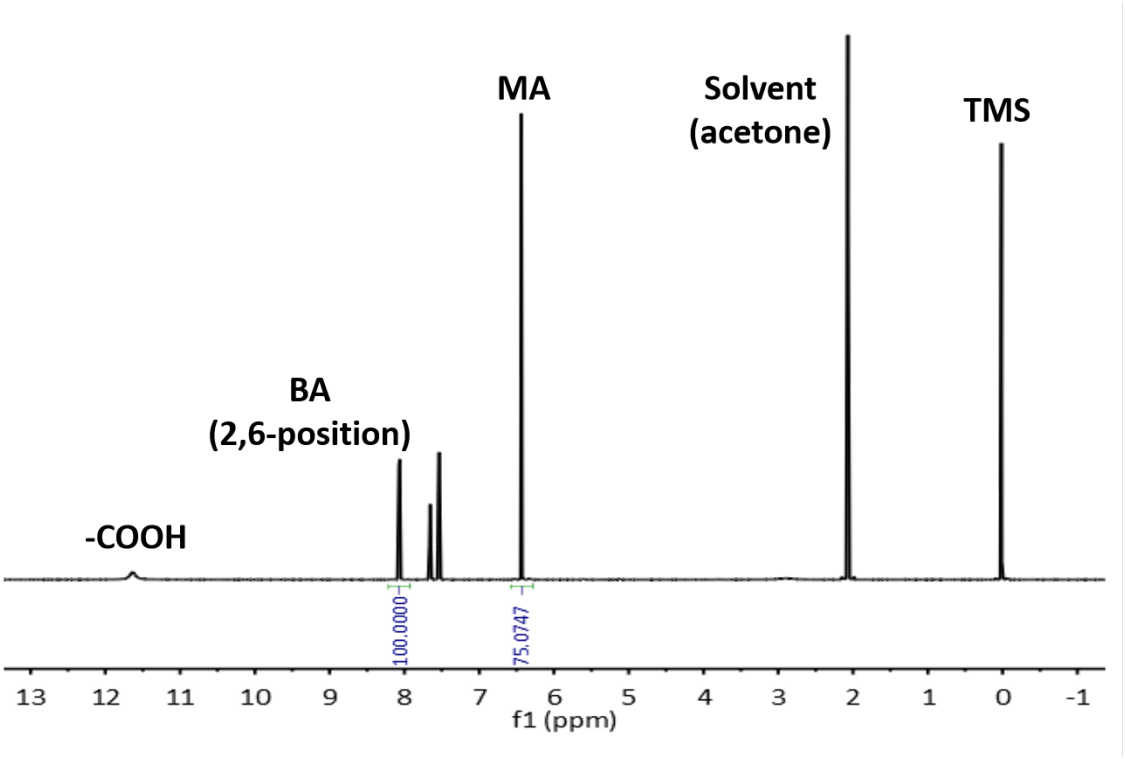

Figure 5 - ^{1}H NMR of BA + MA in acetone-d_6

The optimized gravimetric and NMR parameters for the qNMR assignment using a Bruker Ascend NMR spectrometer with 5 mm CPQCI probe are given in Table 3. The sample was made up in solution in approximately 2 mL of acetone-d6 with 30 s vortex agitation. Then 800 µL was transferred into the NMR tube for analysis. The experimental NMR parameters used for the measurement are given in Table 3.

The integration range start and end points were placed 20 Hz beyond the ^{13}C satellite signals. Results from six independent sample mixtures each measured six times were obtained. The measurement uncertainty budget from the combined results for the thirty-six replicate determinations is reproduced below in Table 4. The relative contribution of each component to the uncertainty of the result obtained for this sample is displayed in Figure 6. The mass fraction content of maleic acid assigned by qNMR using BA as ISRM in this solvent was 997.4 ± 0.9 mg.g^{-1}.

Table 3 - NMR experiment parameters for MA assignment using BA in acetone-d_6.

Parameter	*Value*
BA Sample size (mg)	2.1 – 3.9
MA Sample size (mg)	2.3 – 6.5
Number of Transients	16
Receiver gain	8
Acquisition time (s)	3
Relaxation delay (s)	43.3
Pulse offset (ppm)	7.25
Spectral width (ppm)	20
Data points	639652
Temperature (K)	296
Spinning	Off
Integral ratio (MA:BA)	0.6 – 2.1

Table 4 – Uncertainty budget for MA purity by qNMR using BA in acetone-d_6.

Uncertainty sources	Value	Type	Standard Uncertainty	Sensitivity coefficient	Uncertainty Component
I_A/I_S (repeatability)	1.1842	A	0.00039	0.84225477	3.26E-04
Analyte signal ^{1}H Nuclei	1.9996	B	0.0003	-0.49879806	1.50E-05
IS signal ^{1}H Nuclei	1.9996	B	0.0003	0.49879806	1.50E-05
Analyte Molar Mass (g/mol)	116.072	B	0.0040	0.00859291	3.44E-05
IS Molar Mass (g/mol)	122.123	B	0.0060	-0.00816715	4.90E-05
Analyte mass (mg)	3.0115	B	0.00029	-0.33119656	9.56E-05
IS mass (mg)	2.9642	B	0.00029	0.33648592	9.71E-05
IS purity (g.g^{-1})	0.99992	B	0.000025	0.99747639	2.49E-05
				Combined Uncertainty	4.2E-04
Purity of MA	**997.4**	**± 0.9**		**mg.g^{-1}**	

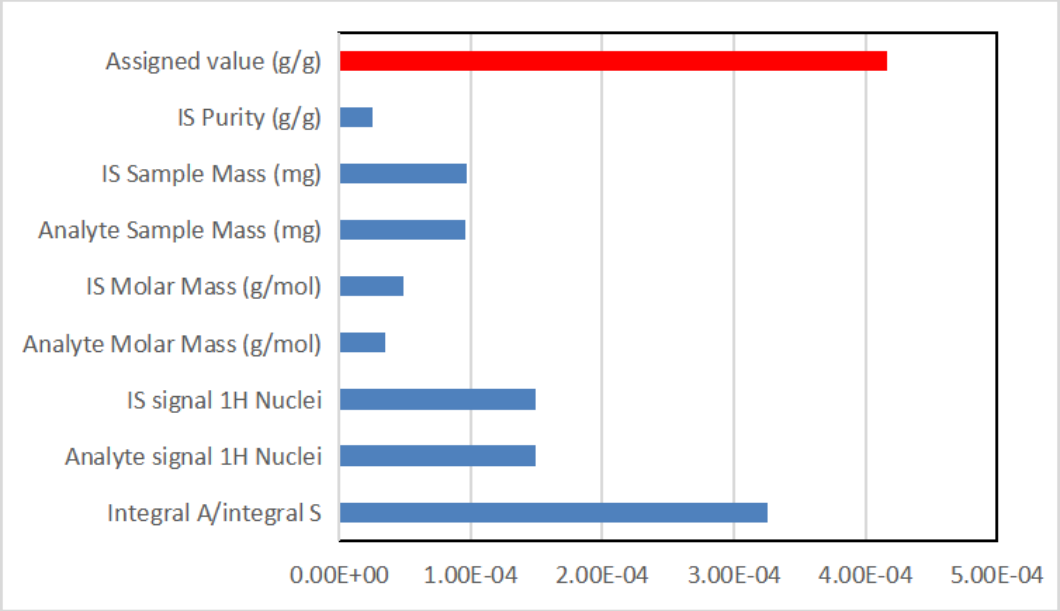

Figure 6 - Relative uncertainty components: MA assignment using BA in acetone-d_6

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