Bureau International des Poids et Mesures

# Internal Standard Reference Data for qNMR: 4,4-Dimethyl-4-silapentane-1-sulfonic acid-

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# Internal Standard Reference Data for qNMR: 4,4-Dimethyl-4-silapentane-1-sulfonic acid- [ISRD-07] Contents


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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 commercially available. [1] However it has only been more recently, as instrumental capabilities have achieved a level of accuracy and precision comparable to those attainable by chromatographic techniques, that this potential has begun to be widely realized. As a result quantitative methods based on NMR spectroscopy, particularly for the assignment of the purity of individual organic compounds, are now actively and extensively implemented. [2], [3], [4] As evidence, a recent editorial in the *Journal of Medicinal Chemistry* [5] 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 also potentially meets the metrological requirements for a primary ratio measurement procedure. [6] Validated qNMR methods [7]are now being used, generally in combination with data obtained by orthogonal chromatographic techniques, to assign the purity of organic materials intended for use as Primary Reference Materials ISO 17511:2003 for individual organic analytes. [9], [10], [11] The availability of properly characterized Primary Reference Materials is in turn an essential initial step in establishing the metrological traceability for all measurement results for an organic analyte linked in a calibration hierarchy to a specific pure material. [12]

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

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

$w_{\mathrm{A}}$ is the mass fraction of the analyte in the material subject to assignment, $w_{\mathrm{S}}$ the independently established mass fraction content of the internal standard, $I_{\mathrm{A}}$ and $I_{\mathrm{S}}$ are the integrals of the quantified signals, $N_{\mathrm{A}}$ and $N_{\mathrm{S}}$ the number of $^{1}$H nuclei contributing to each quantified signal, $M_{\mathrm{A}}$ and $M_{\mathrm{S}}$ the molar masses of the analyte and internal standard and $m_{\mathrm{A}}$ and $m_{\mathrm{S}}$ the masses 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 have been reported to reach the level of 1 mgg$^{-1}$ on an absolute basis, equivalent to 0.1 % relative. [13] However this level of uncertainty is difficult to achieve on a routine basis and in addition is limited on a case by case basis. 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). [14] It should be noted that although these principles should apply to quantitative measurement involving any NMR-active nuclei the recommendations in this document are intended for assignments by $^{1}$H qNMR.

The second is to describe a set of seven internal standard reference materials (ISRMs) listed in Figure 1 below 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, potentially SI-traceable internal standards for use to underpin $^{1}$H qNMR measurements. Different groups have previously proposed sets of compounds for use as qNMR internal standards. [13], [15], [16], [17] Although there is some commonality with our proposal the focus of the earlier papers is the application of the materials for subsequent purity assignments rather than of their suitability as SI-traceable primary measurement standards for qNMR. At least one ISRM compound from the set we propose should be suitable for use for the assignment of a given organic compound soluble in a specified NMR solvent.

The third goal and the focus of this specific document is to provide guidance on the use and limitations of the sodium salt of 3-(trimethylsilyl)-hexadeuteropropane-1-sulfonic acid (DSS-$d_6$) as an ISRM for qNMR analysis. In general, 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 one organic component ($w_S > 995\ \mathrm{mg\,g^{-1}}$);
- value assigned with small standard uncertainty ($u(w_S) < 1\ \mathrm{mg\,g^{-1}}$);
- providing unique NMR signals, either as singlet or simple multiplet resonances, having Lorentzian lineshape and narrow signal width;
- free of significant impurities interfering with the areas to be integrated;
- inert in solution in NMR solvent;
- soluble in the chosen NMR solvent at a level in excess of $2\ \mathrm{mg\,mL^{-1}}$;
- 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%.[(1)]

[(1)] 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.

It is recognized that these characteristics constitute a "wishlist" rather than prescriptive requirements and that not all the materials constituting the proposed ISRM suite meet all these specifications.

The solubility estimates of the ISRMs in the individual solvents listed in Figure 1 are intended as also being indicative of those for solvents having similar capabilities. The four shown were selected as being 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 internal standards are applicable to each solvent class and provide quantification signals distributed across the standard $^{1}$H chemical shift range.

DSS-$d_6$ does not meet all the requirements listed above due to its significant associated water content as a result of which the mass fraction content of organic component in a sample of the material is lower than the suggested minimum level. Despite this DSS-$d_6$ can be used as an effective internal standard for qNMR purity assignments of analytes soluble in $D_2O$, DMSO-$d_6$, $CD_3OD$ when for other reasons other ISRMs would not be suitable.

The following section of this reference document and the attached annexes describes specific properties and applications of DSS-$d_6$ for use as an ISRM for qNMR.

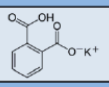
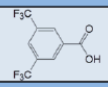
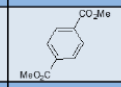
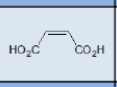
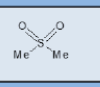
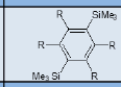
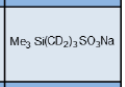
| ISRM | KHP | BTFMBA | DMTP | MA | $DMSO_2$ | BTMSB-$d_4$ | DSS-$d_6$ |
|---|---|---|---|---|---|---|---|
| Structure |  |  |  |  |  |  |  |
| δ (ppm) | 8.3-7.0 (4H) | 8.4-8.5 (2H)<br>8.2-8.4 (1H) | 8.1 (4H)<br>3.9 (6H) | 6.3 (2H) | 3.0 (6H) | 0.2 (18H) | 0.1 (9H) |
| Density ($g.cm^{-3}$) | 1.64 ± 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 |
| H content ($mg.g^{-1}$) | 19.6 | 11.6 | 20.6 (4H)<br>30.9 (6H) | 17.2 | 63.8 | 79.5 | 44.5 |
| Solvent ⇩ | Solubility (mg/mL) | | | | | | |
| $D_2O$ | > 10 | < 1 | < 1 | > 5 | > 10 | < 1 | > 5 |
| $d_6$-DMSO | > 2 | > 10 | > 5 | > 10 | > 5 | > 2 | > 5 |
| $CD_3OD$ | > 2 | > 10 | > 2 * | * | > 5 | > 2 | > 5 |
| $CDCl_3$ | < 1 | > 5 | > 10 | < 1 | > 10 | > 5 | < 1 |

**Figure 1 — qNMR ISRM Suite [18]**

* 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 $CD_3OD$

KHP Potassium hydrogen phthalate

BTFMBA 3,5-Bis-(trifluromethyl)benz acid

DMTP Dimethyl terephthalate

MA Maleic acid

$DMSO_2$ Dimethyl sulfone

BTMSB 1,4-*bis*-Trimethylsilylbenze (R=H); BTMSB-$d_4$ (R = D)

DSS-$d_6$ Sodium - 1,1,2,2,3,3-hexadeutero-3-(trimethylsilyl)propa 1-sulfonate [Sodium 4,4-dimethyl-4-silapentane-1-sulfonate -$d_6$ ]

$D_2O$ Deuterium oxide

DMSO-$d_6$ Dimethyl sulfoxide-$d_6$ / Hexadeuterodimethy sulfoxide

$CD_3OD$ Methanol-$d_4$ / Tetradeuteromethan

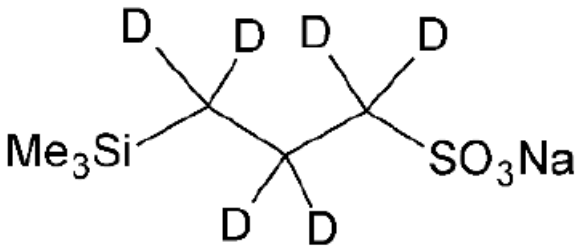
$CDCl_3$ Chloroform-*d* / Deuterochloroform

# 2. Properties of DSS-$d_6$

## 2.1. Physical Properties

IUPAC Name Sodium 1,1,2,2,3,3-hexadeutero-3-trimethylsilylpropane-1-sulfonate

Structure +




| | |
|---|---|
| Synonym | Sodium 4,4-Dimethyl-4-silapentane-1-sulfonate-$d_6$ |
| CAS Registry Number | 284664-85-3 |
| Molecular Formula | $C_6H_9D_6NaO_3SSi$ |
| Molar Mass [19] | 224.354 $gmol^{-1}$, $u = 0.009$ $gmol^{-1}$ |
| Density | 1270 $kgm^{-3}$ [20] |
| Appearance | White powder |
| $^1$H NMR [21] | $\delta$0.1 (s, 9H) |

4400 MHz spectrum of DSS-$d_6$ in DMSO-$d_6$ is given in Annex A1.1.

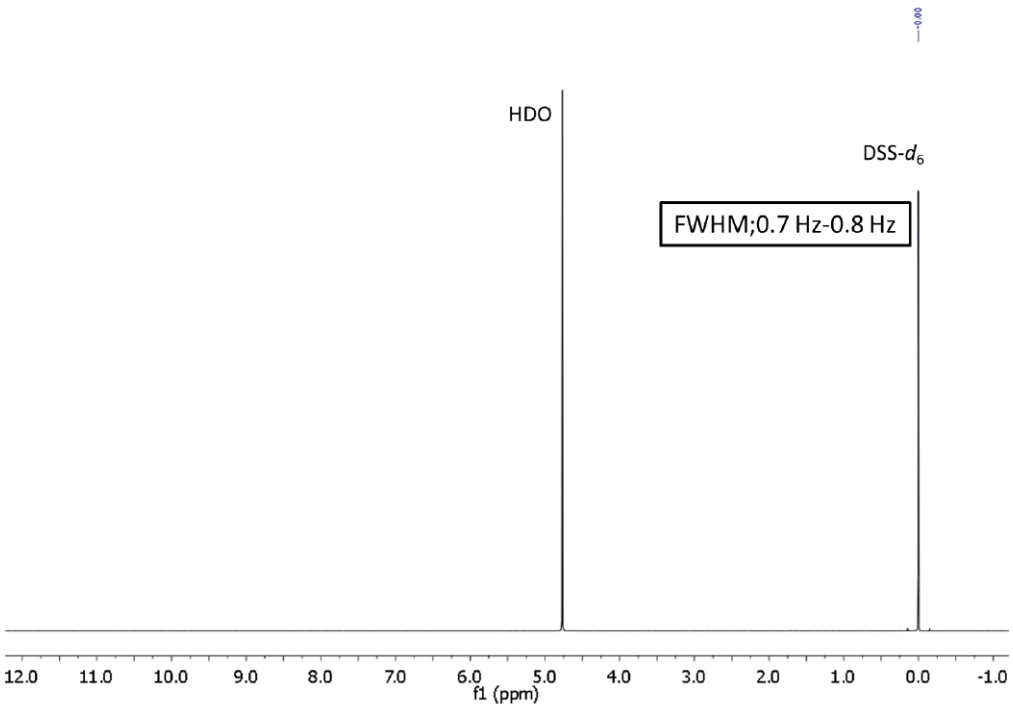



**Figure 2 — $^1$H NMR spectrum of DSS-$d_6$ in $D_2O$: JEOL ECS-400 spectrometer with Royal probe.**

## 2.2. NMR Solvent Compatibility

NMR solvents suitable for use with DSS-$d_6$ are $D_2O$, DMSO-$d_6$ and $CD_3OD$. DSS-$d_6$ is soluble at levels in excess of 5 $mgmL^{-1}$ in each solvent.

## 2.3. NMR quantification signals

The nine magnetically equivalent protons of the trimethylsilyl substituent of DSS-$d_6$ are observed as a singlet occurring at a chemical shift in the range (0.0 ppm) – (0.1 ppm). The exact position of the resonance is a function of other factors including but not limited to the solvent, temperature and the concentration of DSS-$d_6$ and other analytes in the solution. For quantification results the homogeneity of the spectrometer magnetic field should be optimized such that the full width at half maximum (FWHM) of this residual solvent signal is less than 1 Hz while the base of the DSS-$d_6$ trimethylsilyl resonance retains a suitable Lorentzian peak shape.

## 2.4. Impurities and artefact signals

Samples of DSS-$d_6$ analysed in our laboratory have typically not indicvated the presence of significant levels (> 0.1 %) of related structure impurities. However the material is hygroscopic which can make high accuracy gravimetric operations difficult if the humidity level in the measuring environment is either relatively high or low.

The main interferences in a solution containing DSS-$d_6$ come from signals due to residual non-deuterated solvent. Typical chemical shifts are given in Table 1 below.

## 2.5. Solvent recommendations & advisories

### 2.5.1. $D_2O$

$D_2O$ is a suitable choice as solvent for use with DSS-$d_6$.

### 2.5.2. DMSO-$d_6$ and related solvents

DSS-$d_6$ is sufficiently soluble in this solvent and other non-polar solvents such as acetone-$d_6$ and acetonitrile-$d_3$ for solution qNMR measurements.

### 2.5.3. Methanol-$d_4$ and related solvents

$CD_3OD$ is a suitable choice as solvent for use with DSS-$d_6$.

### 2.5.4. Chloroform-$d$ and related solvents

DSS-$d_6$ is not soluble in this solvent. It is not suitable for use with non-polar deuterated solvents in general.

**Table 1. Solvent and qNMR parameters for DSS-$d_6$**

| Solvent | qNMR signal - Singlet, 9H (ppm) [(a)] | Integration range (ppm) [(a)] | $T_1$ (s) [(a)] | Residual Solvent (ppm) | Comments: |
|---|---|---|---|---|---|
| $D_2O$ | 0.1 | -0.1 – 0.2 | 6-7 | 4.8 [(b)] | |
| DMSO-$d_6$ | 0.1 | -0.1 – 0.2 | < 6 | 2.5, 3.2 [(b)] | |
| $CD_3OD$ | 0.1 | -0.1 – 0.2 | < 6 | 3.2, 4.8 [(b)] | |
| $CDCl_3$ | | **Not Suitable** | | | Insufficiently soluble |

(*a*) Indicative values only. The observed value in a specific qNMR solution will be a function of factors including concentration of DSS-$d_6$ and analyte, temperature, instrument, etc.

(*b*) Chemical shift of residual HDO signal is strongly pH dependent

# 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 (Equation (1), see p. 6). The mass fraction purity of the internal standard used for the measurement, 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, [22], [23] and the spectrometer performance, experimental parameters and the protocol for signal processing and integration must be optimized, [4], [7], [24] 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. [25] Only when these conditions are met can the assigned mass fraction purity of the analyte also be regarded as properly traceable to the SI. [9], [26], [27] 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 qNMR must comply as far as possible with the criteria described in the Introduction regarding composition, physical characteristics, inertness, solubility, impurity profile and suitability for accurate gravimetry. 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). [28]

To maintain SI-traceability the sources of the internal standard should be either a:

1. Certified Reference Material (CRM) characterized for its mass fraction purity and value assigned by a National Metrology Institute;
2. CRM produced by a Reference Material Provider accredited to ISO 17034:2016 ISO 17034:2016 requirements;
3. high purity material subject to a validated measurement procedure for purity assignment by qNMR using as an internal standard a CRM of type 1) or 2).

## 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. A recent publication describes recommended practice in this area in the context of qNMR sample preparation. [23] Achieving an overall relative standard measurement uncertainty for a qNMR assignment of 0.1 % requires the relative uncertainty associated with individual gravimetric operations 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.

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 A1.1 below, gravimetric operations were undertaken using a balance associated with a measurement uncertainty estimate of 1.3 μg for individual mass determinations. In this case a minimum sample size of 4 mg achieves a relative uncertainty in individual gravimetric operations below 0.03 %. In addition to the measurement uncertainty of the balance reading, for high accuracy measurements correction for sample buoyancy effects and the contribution to the overall measurement uncertainty associated with this correction should also be taken into consideration. [24]

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.30 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 another potential source of bias for mass determinations, particularly for high polarity, hygroscopic compounds. In these cases, treatment of the sample with an electrostatic charge remover or deioniser 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. [31] 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 requisite might not be attainable which impacts on the level of measurement uncertainty associated with the assigned value. In no case 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 minimise differences in the integrated signal intensities due to differential rates of relaxation. For highest accuracy assignments use of broadband heteronuclear decoupling should in general be avoided 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 digitisation 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 $\mathrm{Hzpt^{-1}}$ 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 maleic acid 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 10 $T_1$ should be used as a minimum where highest accuracy results are sought.

Thus the pulse RT is given by:

$$\mathrm{RT} = \mathrm{RD} + \mathrm{AQ} = n * T_1 \qquad (2)$$

$$(n = 10\text{–}15)$$

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 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 for can still lead to acceptable results, but the reported measurement uncertainties increase as the S/N ratio decreases.

**Table 2. 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 | 90 ° | Should not change the quality of the results, but the use of a 90 ° 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 | $10-15 \times T_1$ | After 90 ° 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 $Hzpt^{-1}$ | The digital resolution is the reciprocal of the acquisition time. Suitable signal shape sensitivity requires not less than 0.4 $Hzpt^{-1}$. |

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 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) repeated each time for each sample.

## 3.6. NMR signal integration

The integration range should extend on each side 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. An alternative acceptable method is to use a range extending 30 Hz beyond the furthest $^{13}C$ satellites as the start/end points for the integration ranges as this generally exceeds the above described width.

It is important to apply a suitable algorithm for the baseline correction and check its validity by analysing standard samples. Practical experience has shown that manual baseline assignment generally works best when high accuracy qNMR results are required. ISO 17034:2016 A final data treatment parameter that can be applied is an adequate window function. For $^{1}H$ NMR, an exponential multiplication a factor not greater than 0.3 Hz should be used. The exponential multiplication factor in use at the BIPM with the JEOL-ECS 400 is typically no greater than 0.05 Hz − 0.10 Hz and in some cases is not used at all.

## 3.7. Measurement uncertainty

Evaluation of the measurement equation previously presented (Equation (1), see p. 6) identifies the factors influencing the input quantities for the measurement uncertainty as shown in the diagram in Figure 3.

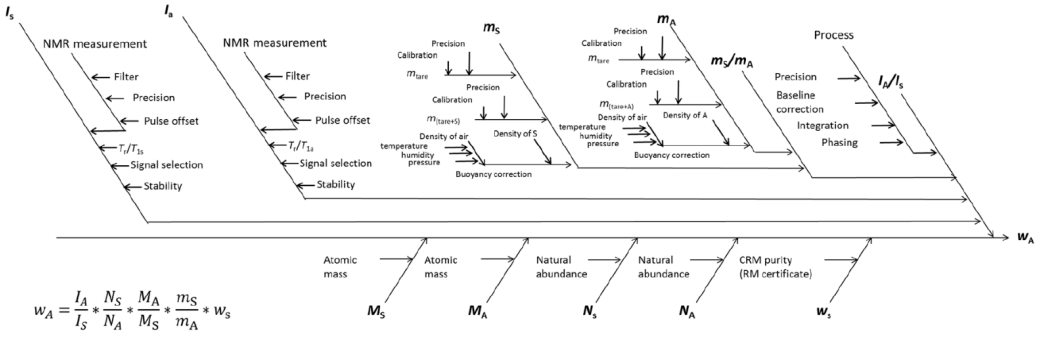



**Figure 3 — Ishikawa diagram for input quantities considered for the measurement uncertainty estimation by qNMR**

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. [22], [32] Since these measurements come from at least two independent solutions each containing different sample masses, the 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 3 of the 2016 revision of the IUPAC Technical report of the Atomic weights of the elements, [19] the uncertainties of mass determinations are based on balance performance characteristics and are corrected for buoyancy effects [24] 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. [26], [33] Examples of “best case” measurement uncertainty budgets for qNMR analysis are provided in the examples given in Annex A1.1.

# 4. Acknowledgements

The work described in this report was made possible by the munificent donation by JEOL France of an ECS-400 NMR spectrometer to the BIPM and was generously supported by the contribution of chemical standards by WAKO Pure Chemicals.

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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.

# Annex 1. Annexes

## A1.1. qNMR using DSS-$d_6$ as internal standard

Examples are provided of the value assignment by qNMR of the mass fraction content of organic compounds using DSS-$d_6$ as the ISRM and the associated measurement uncertainty budgets. In the examples DSS-$d_6$ was used in a solution in $D_2O$ with $DMSO_2$ and MA as analyte.

These are intended as "best case" illustrations and should not be regarded as representative of the uncertainty budget achievable when quantifying against more convoluted resonances or with more structurally complex compounds. The signals for quantification are clearly separated from each other, have narrow, well-resolved signal shape and there is no significant interference from impurities or solvent signals. As a result the uncertainty contribution due to the reproducibility of the signal integration is smaller (and the relative uncertainty contribution due to gravimetry and the purity of the internal standard correspondingly greater) than should be anticipated for more general applications.

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 DSS-$d_6$ was donated by WAKO and used without additional treatment. The purity was assigned in a separate qNMR experiment in solution in $D_2O$ using a high-purity CRM for KHP (NMIJ CRM 3001b) as the internal standard. The mass fraction content of the DSS-$d_6$ assigned by our internal qNMR measurement was $922.7 \pm 0.9$ mgg$^{-1}$.

The $DMSO_2$ and MA used as analytes for purity assignment and deuterated solvents were purchased from commercial suppliers and used without further treatment or purification. Borosilicate glass NMR tubes with 5 mm internal diameter rated for use in 500 MHz spectrometers and purchased from a commercial supplier were used for all measurements.

### A1.1.1. DSS-$d_6$ (IS) & $DMSO_2$ (Analyte) in $D_2O$

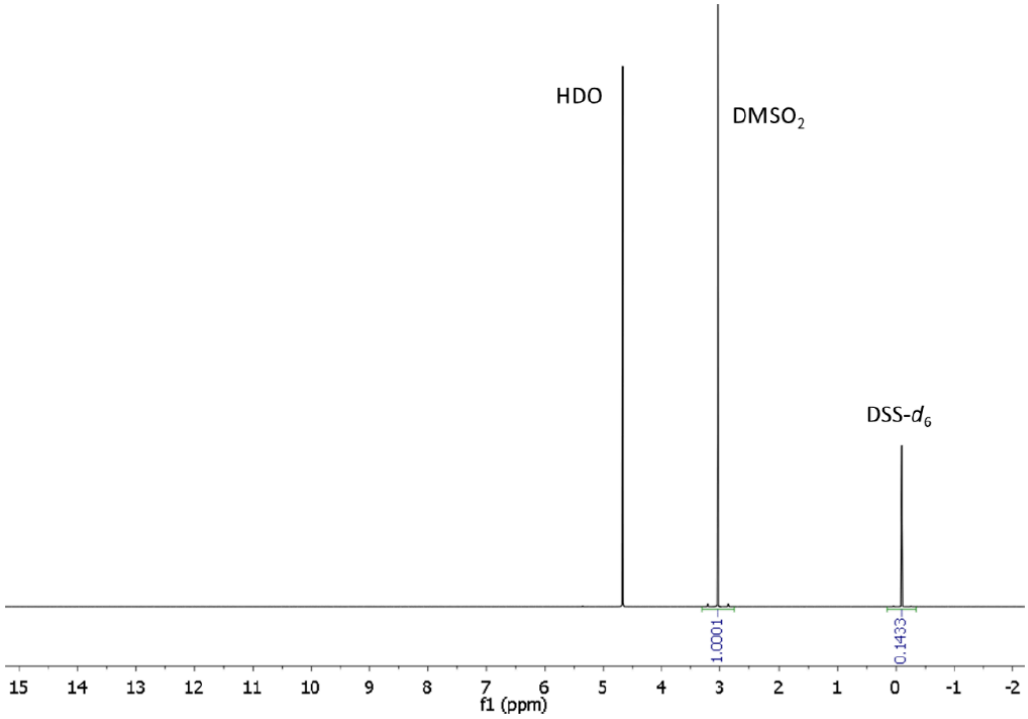


**Figure A1.1 — $^1$H NMR spectrum of DSS-$d_6$ and $DMSO_2$ in $D_2O$.**

The optimized gravimetric and NMR parameters for the qNMR assignment using a JEOL ECS-400 spectrometer equipped with a Royal probe are given in Table A1.1. The sample was made up in solution in approximately 1 mL of $D_2O$ and 800 µL was transferred into the NMR tube for analysis.

**Table A1.1. NMR experiment parameters for $DMSO_2$ purity assignment using DSS-$d_6$ in $D_2O$.**

| Parameter | Value |
|---|---|
| $DMSO_2$ Sample size (mg) | 3.2 – 5.4 |
| DSS-$d_6$ Sample size (mg) | 1.2 – 1.7 |
| Number of Transients | 32 |
| Receiver gain | Automatic |
| Acquisition time (s) | 4 |
| $T_1$ (longest signal except for solvent) (s) | < 6.5 |
| Relaxation delay (s) | 65 |

| | |
|---|---|
| Pulse offset (ppm) | 1.6 |
| Spectral width (ppm) | 400 |
| Data points | 639652 |
| Temperature (K) | 298 |
| Spinning | Off |
| Integral ratio ($DMSO_2$:DSS-$d_6$) | 3.2 – 7.1 |

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 four times. The integral ratio is the overall mean of the four replicate values obtained for each of the four samples, normalized for the different sample sizes used in their preparation. The standard uncertainty of the normalized 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 A1.2. The mass fraction content of $DMSO_2$ assigned for this sample was $996.2 \pm 2.4$ mgg$^{-1}$.

**Table A1.2. Uncertainty budget for $DMSO_2$ purity by qNMR using DSS-$d_6$ in $D_2O$.**

| Uncertainty sources | Value | Type | Standard Uncertainty | Sensitivity coefficient | Uncertainty Component |
|---|---|---|---|---|---|
| $I_A/I_S$ (repeatability) | 4.5173 | A | 0.00057 | 0.220540149 | 1.47E-04 |
| Analyte signal $^1$H Nuclei | 5.9988 | B | 0.0003 | -0.1660718 | 4.98E-05 |
| IS signal $^1$H Nuclei | 8.9982 | B | 0.0003 | 0.110716064 | 3.32E-05 |
| Analyte Molar Mass | 94.128 | B | 0.0069 | 0.010583796 | 7.33E-05 |
| IS Molar Mass | 224.3544 | B | 0.0081 | -0.004440437 | 3.62E-05 |
| Analyte Mass (mg) | 3.2520 | B | 0.00124 | -0.306344253 | 3.81E-04 |
| IS Mass (mg) | 1.2351 | B | 0.00124 | 0.80659988 | 1.00E-03 |
| IS Purity (g/g) | 0.9227 | B | 0.00045 | 1.079691679 | 4.86E-04 |
| | | | Combined Uncertainty | | 1.19E-03 |
| Purity of $DMSO_2$ | $996.2 \pm 2.4$ mgg$^{-1}$ | | | | |

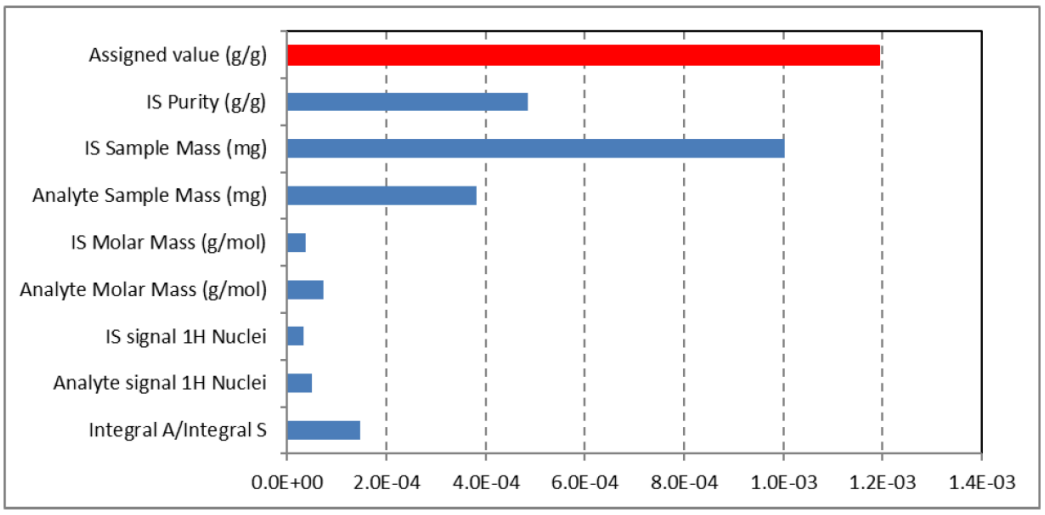


**Figure A1.2 — Relative uncertainty components: $DMSO_2$ assignment using DSS-$d_6$ in $D_2O$.**

### A1.1.2. DSS-$d_6$ (IS) & MA (Analyte) in $D_2O$

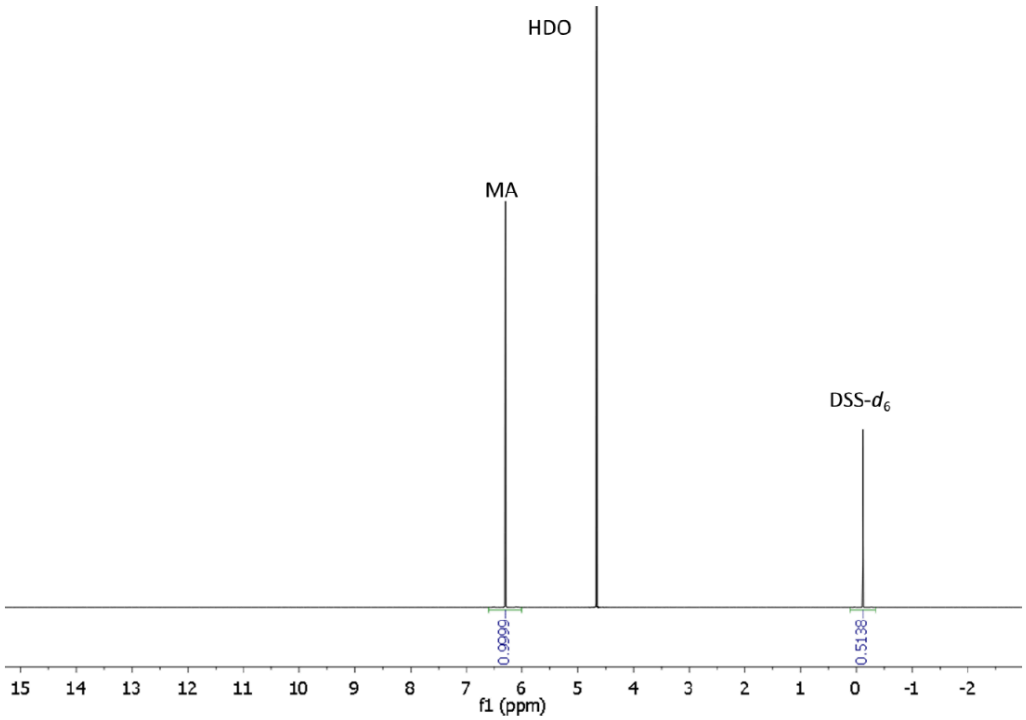


**Figure A1.3 — $^1H$ NMR of DSS-$d_6$ and MA in $D_2O$.**

The experimental NMR parameters used for the measurement are given in Table A1.3.

**Table A1.3. NMR experiment parameters for MA assignment using DSS-$d_6$ in $D_2O$.**

| Parameter | Value |
|---|---|
| DSS-$d_6$ Sample size (mg) | 1.3 – 1.8 |

| | |
|---|---|
| MA Sample size (mg) | 5.1 – 6.2 |
| Number of Transients | 32 |
| Receiver gain | Automatic |
| Acquisition time (s) | 4 |
| $T_1$ (longest signal except for solvent) (s) | < 6 |
| Relaxation delay (s) | 60 |
| Pulse offset (ppm) | 3.1 |
| Spectral width (ppm) | 400 |
| Data points | 639652 |
| Temperature (K) | 298 |
| Spinning | Off |
| Integral ratio (MA: DSS-$d_6$) | 1.33 – 1.95 |

The integration range start and end points were placed 30 Hz beyond the $^{13}C$ satellite signals. Results from four independent sample mixtures each measured four times were obtained. The measurement uncertainty budget is reproduced below in Table A1.4. The integral ratio is the overall mean of the four replicate values obtained for each of the four samples, normalized for the different sample sizes used in their preparation. The standard uncertainty of the normalized ratio is the standard deviation of the mean. The relative contribution of each component to the uncertainty of the result for this material is displayed in Figure A1.4. The mass fraction content of MA from the results for this sample was $998.7 \pm 2.2$ mgg$^{-1}$.

**Table A1.4. Uncertainty budget for MA purity by qNMR using DSS-$d_6$ in $D_2O$.**

| Uncertainty sources | Value | Type | Standard Uncertainty | Sensitivity coefficient | Uncertainty Component |
|---|---|---|---|---|---|
| $I_A/I_S$ (repeatability) | 1.7512 | A | 0.00031 | 0.570282686 | 1.99E-04 |
| Analyte signal $^1H$ Nuclei | 1.9996 | B | 0.0003 | -0.499444643 | 1.50E-04 |
| IS signal $^1H$ Nuclei | 8.9982 | B | 0.0003 | 0.110987321 | 3.33E-05 |
| Analyte Molar Mass (g/mol) | 116.072 | B | 0.0040 | 0.008604052 | 3.44E-05 |

| Uncertainty sources | Value | Type | Standard Uncertainty | Sensitivity coefficient | Uncertainty Component |
|---|---|---|---|---|---|
| IS Molar Mass (g/mol) | 224.354 | B | 0.0081 | -0.004451393 | 3.63E-05 |
| Analyte Sample Mass (mg) | 5.1383 | B | 0.00124 | -0.194360718 | 2.41E-04 |
| IS Sample Mass (mg) | 1.3641 | B | 0.00124 | 0.732123383 | 9.10E-04 |
| IS Purity (g/g) | 0.9227 | B | 0.00045 | 1.082355595 | 4.87E-04 |
| | | | Combined Uncertainty | | 1.08E-03 |

Purity of MA998.7 ± 2.2 $mgg^{-1}$

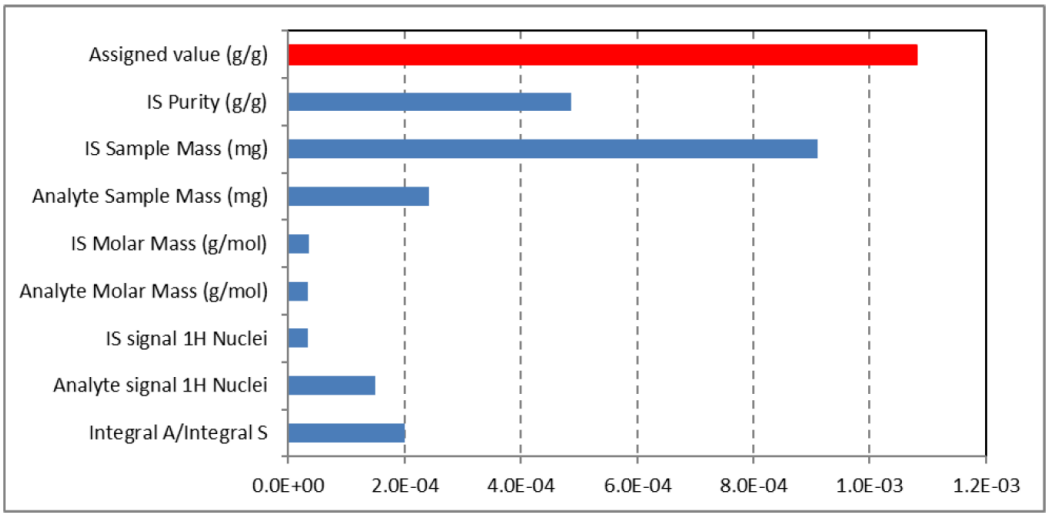


**Figure A1.4 — Relative uncertainty components: MA assignment using DSS-$d_6$ in $D_2O$**

# References


[1] Jungnickel, J.; Forbes, J.; *Anal. Chem.* 1963, **35**, 938–942

[2] Pauli, G.; Jaki, B.; Lankin, D.; *J. Nat. Prod.* 2005, **68**, 133–149

[3] Holzgrabe, U. (ed); *NMR Spectroscopy in Pharmaceutical Analysis*, Elsevier, 2008

[4] Bharti, S.; Roy, R.; *Trends Anal. Chem.*, 2012, **35**, 5-26

[5] Cushman, M.; Georg, G.; Holzgrabe, U.; Wang, S.; *J. Med. Chem.* 2014, **57**, 9219−9219

[6] Milton, M.; Quinn, T.; *Metrologia* 2001, **38**, 289–296

[7] Malz, F.; Jancke, H.; *Pharm. Biomed.* 2005, **38**, 813–823

[8] ISO 17511:2003 *In vitro diagnostic medical devices—Measurement of quantities in biological samples—Metrological traceability of values assigned to calibrators and control materials*

[9] Saito, T.; Ihara, T. et al ; *Accredit. Qual. Assur.* 2009, **14**, 79–89

[10] Huang, T. *et al* ; *Talanta* 2014, **125**, 94–101

[11] Davies, S. *et al* ; *Anal. Bioanal. Chem.*, 2015, **407**, 3103-3113

[12] De Bièvre, P., Dybkaer, R., Fajgelj, A. and Hibbert, D.; *Pure Appl. Chem.*, 2011, **83**, 1873–1935.

[13] Weber M., Hellriegel C., Rueck A., Sauermoser R., Wuethrich J.; *Accredit. Qual. Assur.* 2013, **18**, 91–98

[14] See information on the SI at: https://physics.nist.gov/cuu/Units/

[15] Wells, R.; Cheung J.; Hook, J.; *Accredit. Qual. Assur.* 2004, **9**, 450–456

[16] Rundlöf, T.; *et al*; *J. Pharm. Biomed. Anal.*; 2010, **52**, 645-651

[17] Miura, T.; Sugimoto, N., Suematsu, T. and Yamada, Y; Poster, SMASH Conference 2015

[18] Solubility data for individual ISRMs was determined by Dr Taichi Yamazaki (NMIJ), unpublished data obtained on secondment at the BIPM (2017)

[19] Meija, J., et al (2016): *Atomic weights of the elements 2013, Pure Appl. Chem*, 2016, **88**, 265-293

[20] Density data by pyncnometry provided by WAKO Chem (August 2017)

[21] Dr Taichi Yamazaki (NMIJ), unpublished data obtained on secondment at the BIPM (2017)

[22] Yamazaki, T. ; Nakamura, S. ; Saito, T.; *Metrologia*, 2017, **54**, 224-228

[23] Reichmuth, A.; Wunderli, S.; Weber, M.; Meier, V.R.; *Microchim. Acta* 2004, **148**, 133-141

[24] Saito, T. et al ; *Metrologia*, 2004, **41**, 213-218

[25] Le Gresley, A.; Fardus, F.; Warren, J.; *Crit. Rev. Anal. Chem.* 2015, **45**, 300-310

[26] Saito, T.; Ihara, T.; Miura, T.; Yamada, Y.; Chiba, K.; *Accredit. Qual. Assur.* 2011, **16**, 421-428

[27] Eurolab Technical Report 01/2014; *Guide to NMR Method Development and Validation – Part 1: Identification and Quantification*

[28] JCGM Guide 200:2012 *International Vocabulary of Metrology*

[29] ISO 17034:2016 *General requirements for the competence of reference material producers*

[30] Scorer, T.; Perkin, M.; Buckley, M. ; *NPL Measurement Good Practice Guide No. 70* (2004)

[31] Final Report for CCQM Pilot study CCQM-P150.a: Data acquisition and process in a qNMR method

[32] Saed Al-Deen, T.; Hibbert, D. B.; Hook, J. M.; Wells, R. J.; *Accredit. Qual. Assur.* 2004, **9**, 55–63

[33] Toman, B.; Nelson, M.; Lippa, K.; *Metrologia*, 2016, **53**, 1193-1203

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