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Internal Standard Reference Data for qNMR: Dimethyl Sulfone [ISRD-02]

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1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is well-established as the pre-eminent method for the qualitative structural analysis of organic molecules. The potential for its application for quantitative organic analysis was also recognized soon after NMR instruments became commercially available. [1] However it has only been recently, as spectrometer capabilities have achieved a level of accuracy and precision comparable to those attainable by chromatographic techniques, that this potential has been widely realized in practice. As a result quantitative NMR (qNMR) methods, particularly for the assignment of the purity of individual organic compounds, are now actively and extensively employed. pauli3, beyer, bharti As evidence of its increasing application in this role, a recent 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 also meets the metrological requirements for a primary ratio measurement procedure. [7] Validated qNMR methods [8], [9] 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 [10] for individual organic analytes. [11], [12], [13], [14] 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 in a calibration hierarchy to a specific pure material. [15]

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.

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

w_{A} is the mass fraction of the analyte in the material subject to assignment, w_{S} the independently established mass fraction content of the internal standard, I_{A} and I_{S} are the integrals of the quantified signals, N_{A} and $\mathrm{N_S}$ 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 samples of the analyte and internal standard used in preparation of the solution subject to the qNMR measurement.

In optimal cases where the data processing is 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 $1\ \mathrm{mg\ g^{-1}}$ on an absolute basis, equivalent to a relative uncertainty of 0.1 %.16,17 Factors including, *inter alia*, the lineshape and multiplicity of the signals integrated, the extent of overlap with the main peak of interfering signals from impurities present, the nature of the internal standard and solvent used, the magnetic field strength, the hardware settings and performance characteristics of the spectrometer as well as the approach taken to transform the free induction decay (FID) signal generated by the NMR experiment and integrate the signals of the resulting frequency domain spectrum all contribute to the overall uncertainty of the final 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). [18] It should be noted that although these principles should apply generally to quantitative measurement involving any NMR-active nuclei the recommendations in this document are only intended for assignments by ^{1}H qNMR.

The second goal of this report is to describe a set of seven 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 constitute a "universal" set of higher-order, SI-traceable internal standards. Other groups have proposed specific compounds or sets of compounds suitable for use as qNMR internal standards. [12], [19], [20], [21] Although there is some commonality between the internal standards recommended in the current literature and our proposal, the focus of the earlier papers is primarily their suitability for application for purity assignments by qNMR rather than their utility as SI-traceable primary measurement standards.

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 seven compounds constituting the "universal" ISRM set, together with an outline of their solubility and suitability for use in four representative deuterated NMR solvents, are described in Figure 1 below.

At least three of the internal standards described in Figure 1 are applicable to each solvent class and provide reference signals distributed across the ^{1}H chemical shift range

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

- Certified Reference Material (CRM) [22] produced and characterized by a National Metrology Institute (NMI) using methods other than qNMR or has been assigned by qNMR using an NMI CRM as the internal standard;
- predominantly one organic component ($w_S > 995$ mg g^{-1});
- value assigned with small associated standard uncertainty ($u(w_s) < 2$ 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 areas to be integrated;
- inert in solution and soluble at a level in excess 2 mg mL^{-1};
- readily handled for accurate mass determinations:
  - non-hygroscopic
  - non-volatile
  - not subject to electrostatic effects
- having a ratio of quantifiable protons to the molar mass of the ISRM sufficient to allow for practical gravimetric operations.

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

The solvents listed in Figure 1 are intended as representative of those with similar capabilities for solubilizing each ISRM rather than as a prescriptive set for use in qNMR. These are however the most readily available deuterated solvents and a majority of reported applications of solution qNMR use one of these solvents.

Compounds recommended as ISRMs for use with $CDCl_3$ as solvent (BTFMBA, DMTP, $DMSO_2$ and BTMSB) should be suitable for use in other chlorinated (CD_2Cl_2, $C_2D_2Cl_4$) or non-polar (benzene-d_6, toluene-d_8, THF-d_8, pyridine-d_5) solvents.

Likewise, compounds recommended as suitable ISRMs for use with DMSO-d_6 (BTFMBA, MA, $DMSO_2$ and DSS-d_6) are anticipated to be suitable for use in other polar organic solvents (acetonitrile-d_3, acetone-d_6, DMF-d_7).

The third goal and the focus of this specific document is to provide guidance regarding the use and limitations of dimethyl sulfone as an ISRM for qNMR analysis.

Dimethyl sulfone ($DMSO_2$) is one of the ISRMs listed in Figure 1. Due to its ready solubility and stability in solution in a range of solvents, it is suitable for potential use as an internal standard for qNMR purity assignments of analytes soluble in most deuterated solvents. The attached annexes describe specific properties and applications of dimethyl sulfone.

It displays the majority of the characteristics of an ideal qNMR ISRM outlined above. One caveat is that as a result of the relatively large number of protons (6) giving rise to the quantification signal combined with the relatively small molar mass of $DMSO_2$ the sample size of $DMSO_2$ will often be relatively small (< 2mg) unless a large amount of an analyte is available and the analyte has a high level of solubility in the selected solvent. As a result of this limitation in sample size the contribution to the overall uncertainty from gravimetric operations with $DMSO_2$ is large relative to that possible with other ISRMs.

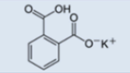
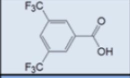
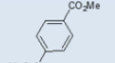
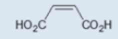
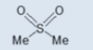
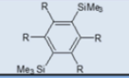
ISRM	KHP	BTFMBA	DMTP	MA	$DMSO_2$	BTMSB	DSS-d_6
Structure							$Me_3\,Si(CD_2)_3SO_3Na$
δ (ppm)	8.3-7.0 (4H)	8.4-8.5 (2H) 8.2-8.4 (1H)	8.1 (4H) 3.9 (6H)	6.3 (2H)	3.0 (6H)	[7.5 (4H, R=H)] 0.2 (18H)	0.1 (9H)
Density ($g.cm^{-3}$)	1.64 ± 0.17	1.72 ± 0.04	1.2 ± 0.24	1.53 ± 0.03	1.4 ± 0.03	1.0 ± 0.02	1.27 ± 0.03
Solvent ⇩	Solubility (mg/mL)						
D_2O	> 10	< 1	< 1	> 5	> 10	< 1	> 5
d_6-DMSO	> 2	> 10	> 2	> 10	> 5	> 2	> 5
CD_3OD	> 2	> 10	*	*	> 5	> 2	> 5
$CDCl_3$	< 1	> 5	> 5	< 1	> 10	> 5	< 1

Figure 1 — qNMR ISRM Suite [23]

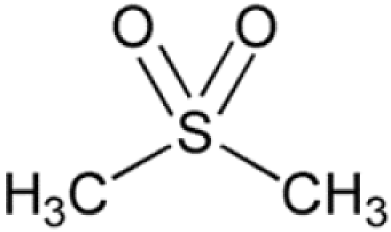
* soluble but unsuitable due to (trans)esterification reaction with CD_3OD

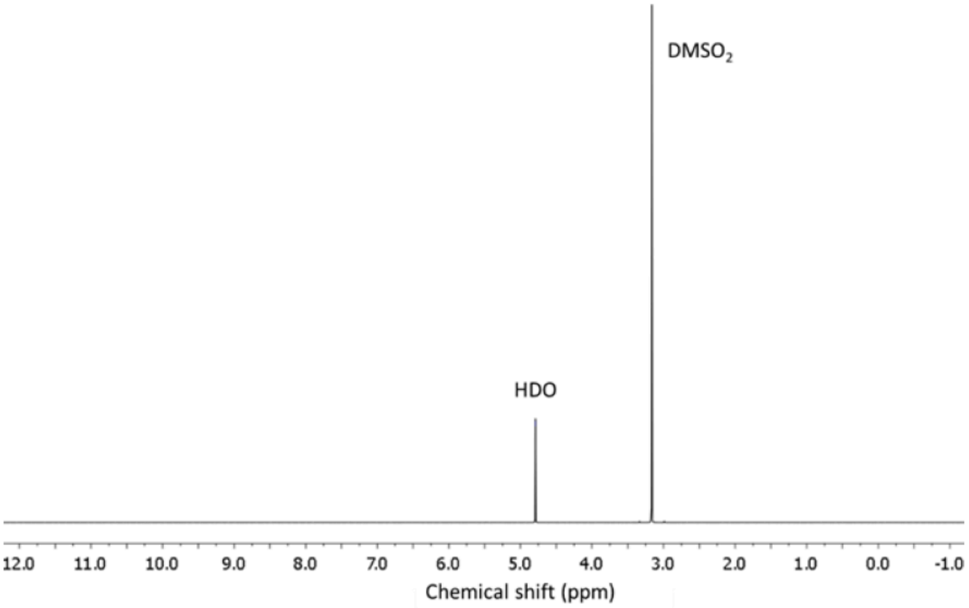
KHP	Potassium hydrogen phthalate
BTFMBA	3,5-*bis*-Trifluromethylbenz acid
DMTP	Dimethyl terephthalate
MA	Maleic acid
$DMSO_2$	Dimethyl sulfone
BTMSB	1,4-*bis*-Trimethylsilylbenze (R=H), BTMSB-d_4 (R = D), BTMSB-F4 (R = F);
DSS-d_6	3-(Trimethylsilyl)-hexadeuteropropane 1-sulfonic acid [also known as 4,4-Dimethyl-4-silapentane-1-sulfonic acid-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 Dimethyl Sulfone

2.1. Physical Properties

Name	**Dimethyl Sulfone**
Structure	+ 
Synonym	Methyl sulfone, Methylsulfonylmethane
CAS Registry Number	67-71-0
Molecular Formula	$C_2H_6O_2S$
Molar Mass [24], [25]	$94.136\mathrm{g}\cdot\mathrm{mol}^{-1}$, $u = 0.005\mathrm{g}\cdot\mathrm{mol}^{-1}$
Melting point [26]	109°C
Density	$1450\mathrm{kg}\cdot\mathrm{m}^{-3}$ [26] $1385 \pm 30\mathrm{kg}\cdot\mathrm{m}^{-3}$ [27]
Appearance	White crystalline powder
^{1}H NMR [28]	δ 3.14 (s, 6H)
^{13}C NMR	δ 42.6



400MHz spectra of DMSO$_2$ in other NMR solvents are provided in Appendix A1.1.

Figure 2 — ^{1}H NMR spectrum of DMSO$_2$ in D_2O: JEOL ECS-400 spectrometer with Royal probe.

2.2. Solvent Compatibility

NMR solvents suitable for use with $DMSO_2$ include D_2O, DMSO-d_6, CD_3OD and $CDCl_3$. $DMSO_2$ is soluble at levels in excess of 10 mg mL^{-1} in DMSO-d_6 and CD_3OD and in excess of 5 mg mL^{-1} in D_2O and $CDCl_3$. [23]

2.3. Quantification signal

The six magnetically equivalent methyl protons of dimethyl sulfone give rise to a singlet absorption at a chemical shift in the range 3.1ppm – 3.2ppm on the δ scale. The position of the resonance is a function of factors including, but not limited to, the solvent, temperature, pH, spectrometer and the concentration of $DMSO_2$ and analyte in the solution. The homogeneity of the spectrometer magnetic field should be optimized such that the full width at half maximum (FWHM) of the $DMSO_2$ resonance signal is less than 1Hz when the base of the resonance retains a suitable Lorentzian peak shape.

2.4. Impurities and artefact signals

In practice the main interferences in a qNMR solution using $DMSO_2$ as internal standard come from the residual non-deuterated solvent. The approximate chemical shifts of these signals are given in Table 1 below. In the case of solutions in D_2O the signal due to residual HDO could be attenuated if desired by the use of a (water) signal suppression pulse sequence, at the cost of introducing additional non-linearity into the signal responses. [29]

2.5. Solvent recommendations and advisories

2.5.1. D_2O

The signal due to residual HDO at 4.8ppm will not interfere with the quantification of the $DMSO_2$ peak resonance. D_2O is a suitable solvent for use with $DMSO_2$ and a water-soluble analyte if the residual water peak does not interfere with the analyte quantification resonance signal.

2.5.2. DMSO-d_6 and related solvent

Signals are observed due to residual DMSO-d_5 at 2.5ppm and, at a varying level depending on the extent to which the DMSO-d_6 has been dried and protected from atmospheric moisture, from the presence of H_2O somewhere in the range 3.3ppm – 4.8ppm. If necessary, baseline correction algorithms can be used to attenuate or eliminate interference from the residual DMSO-d_5 peak in the quantification of the $DMSO_2$ peak resonance at the cost of introducing potential bias into the resultant integral quantifications.

2.5.3. Methanol-d_4 and related solvents

Signals are observed due to residual CD_2HOD at 3.3ppm and, at a varying level depending on the extent to which the CD_3OD has been dried and protected from atmospheric moisture, due to an HOD peak at 4.8ppm. For accurate integration if using this solvent, care must be taken to correct, if necessary, for a contribution from the residual CD_2HOD peak to the quantification of the $DMSO_2$ peak resonance. Baseline correction algorithms can be used to diminish or eliminate interference with the $DMSO_2$ peak at the cost of a potential bias in the resultant integral quantifications.

2.5.4. Chloroform-d and related solvents

Residual $CHCl_3$ peak at 7.25ppm will not interfere with the quantification of the $DMSO_2$ peak resonance. This solvent is recommended for non-polar analytes if the residual solvent peak does not interfere with the analyte quantification resonance signal.

Table 1. Solvent Parameters for $DMSO_2$

Solvent	qNMR signal - Singlet, 6H (ppm) (a)	Integration range (ppm) (a)	T_1 (s) (a)	Residual Solvent (ppm)	Comments:
D_2O	3.1	2.9 – 3.3	4-5	**4.8** (b)	
DMSO-d_6	3.1	2.9 – 3.3	3-4	2.5	H_2O peak at 3.3 – 4.8ppm
CD_3OD	3.1	2.9 – 3.3	3-5	3.3	HOD peak at 4.8ppm
$CDCl_3$	3.1	2.9 – 3.3	4-5	7.25	

(a) Indicative values only. The observed value in a specific qNMR solution will be a function of factors including concentration of $DMSO_2$ and analyte, solution 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), Chapter 1, see p. 6). The purity of the internal standard used for the measurement, the source of traceability to the SI for the value assigned to the analyte, is established independently prior to the qNMR experiment.

The gravimetric procedure used for the preparation of the NMR solution has to be fully validated and fit for purpose, [30], [31] and the spectrometer performance, experimental parameters and the protocol for signal processing and integration must be optimized, [8], [9], [32] in order to produce a result for the ratio of the integral of the analyte and standard signals that accurately reflects the amount of substance fraction of the hydrogen nuclei giving rise to the signals. [33] Only when these conditions are met can the assigned mass fraction purity of the analyte also be regarded as properly traceable to the SI. [11], [12], [34] 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 should 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). [22]

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

1. CRM [22] characterized for mass fraction purity and value assigned by an NMI;
2. CRM produced by a Reference Material Provider accredited to ISO 17034:2016 [35] 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 and Sample Size

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 a recent publication. [30] Achieving an overall relative standard measurement uncertainty for the result of a qNMR assignment of 0.1 % requires the relative uncertainty associated with individual gravimetric operations typically to be less than 0.03 %. If the combined standard uncertainty of a single mass determination is 3μg, a level achievable with modern electronic microanalytical balances, this corresponds to a minimum sample size of 10mg.

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 Appendix A1.2 below, gravimetric operations were undertaken using a balance associated with a measurement uncertainty estimate of 1.3μg for individual mass determinations. In this case a minimum sample size of 4mg achieves a relative uncertainty in individual gravimetric operations below 0.03 %. In addition to the measurement uncertainty of the gravimetric operations, high accuracy measurements require additional correction for sample buoyancy effects [31] and the $^1H/^2H$ isotope composition of the quantified signals. The value and associated uncertainty of the $^1H/^2H$ isotope composition of each quantification signal can be obtained using an on-line calculator application. [25] As noted previously, because of the combination of the large number of hydrogens giving rise to the $DMSO_2$ quantification signal and its relatively small molar mass, when preparing a sample for qNMR in practice it can be problematic to use a sufficient mass of $DMSO_2$ such that the relative uncertainty of the gravimetric operation is below 0.03 %

As sample preparation for qNMR involves mass determinations in the milligram range using sensitive balances, the loss of minute (effectively 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. [36], [37] It is recommended that mass determinations be performed in an area where the humidity is maintained at a relatively stable level in the relative humidity 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, pre-treatment of the sample with an electrostatic charge remover or deioniser is advisable prior to the mass determination. Materials subject to qNMR analysis should be evaluated for their hygroscopicity, for example by measurement of the potential for change in the observed mass of a sample as a function of relative humidity using a dynamic sorption balance. [38] 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.

3.4. NMR spectrometer optimization

There is no specification of minimum NMR spectrometer field strength for purity measurements. Increasing the field strength enhances signal separation and sensitivity, both of which should increase the accuracy and precision of qNMR measurements. Careful optimization of the lineshape (shimming) is critical in order to achieve reliable qNMR results. [39] 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 1Hz. Experience has shown that these lineshape requirements are more easily achieved using an inverse probe than a direct type. For lower field magnets (< 300MHz), 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 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 a range one hundred times the FWHM on each 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 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 attenuated because of the low (1.1 %) natural abundance of the ^{13}C isotopomer even though the decoupling efficiency for individual ^{13}C satellite signals is variable. The potential for the introduction of additional bias due to ^{13}C-decoupling is negligibly small in most cases.

The 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 and 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 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 total **signal** response. However this potential 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 of the results should not be impacted by the use of different pulse lengths but the acquisition time to achieve equivalent levels of precision 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 2.5s 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 $0.4\text{Hz} \cdot \text{pt}^{-1}$ in order to have accurately defined signals that will give accurate area measurements and suitable precision 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 dimethyl sulfone 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, which is 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° pulse, which should also be determined for each mixture under investigation. The 90° pulse is 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 RD is a multiple T_1. After a 90° pulse, if available instrument time permits, a repetition time equivalent to 10 times T_1 of the signal with the longest relaxation time will lead to the recovery of $>$ 99.99 % 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 may be sufficient for equivalent (even if incomplete) magnetization re-equilibration.

Thus the recommended pulse RT for high accuracy quantification is given by:

$$\mathrm{RT} = \mathrm{RD} + \mathrm{AQ} = n * T_1 \tag{2}$$

$$(n = 10\text{–}15)$$

The number of transients (scans) should be determined according to the concentration of the sample, 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 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) <1Hz	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
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	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	> 20ppm	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	> 2.5s	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 2.5s is a useful starting point and 4s has been found to be suitable for many applications.
Digital resolution	$<0.4\text{Hz}\cdot\text{pt}^{-1}$	The digital resolution is the reciprocal of the acquisition time. Suitable signal shape sensitivity requires not less than $0.4\text{Hz}\cdot\text{pt}^{-1}$.
Signal Integral Ratio	1:1	The preference are sample sizes such that the integral ratio for the quantification signals is close to equivalent. However in practice this ratio can vary within the range 10:1 to 1:10 provided the S/N ratio of the lower intensity peak is > 1000.

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 while 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 analyte/IS mixture should be measured at least three times in the NMR system. To correct for potential instrument drift, independent measurements for a particular sample mixture should be non-continuous. The sample tube should be ejected from the spectrometer probe and the measurement process (tuning, locking, shimming) repeated for each replicate for each sample. To avoid potential unwanted contributions due to spinning sidebands, it is recommended to undertake the measurement using sample spinning disabled. This presumes a high degree of field homogeneity has been achieved.

3.6. NMR signal integration

In order to integrate in excess of 99.9 % of each quantified signal the integration range should extend from the centre of the signal at least seventy six times the FWHM on either side of the signal being measured. The limits of the integration range should be based on the outermost signals if a multiplet is subject to integration. An alternative rule-of-thumb that generally produces acceptable results is to use a range extending 30Hz beyond the furthest ^{13}C satellites as the start and end points for the integration ranges. A consistent approach should be employed for all signals subject to integration. It is also important to apply a suitable procedure for the baseline correction and check its validity by analysing standard samples. Practical experience has shown that manual baseline assignment currently works best when very high accuracy qNMR results are required. [32], [39] A window function can be applied as a final data treatment parameter to enhance the S/N ratio. [9] To avoid line broadening effects, an exponential multiplication factor not greater than 0.3Hz should be used. The window function in use at the BIPM with the JEOL-ECS 400 was typically no greater than 0.05Hz—0.10Hz and in some cases it was not used at all.

3.7. Measurement uncertainty

Evaluation of the measurement equation previously presented (**Equation (1)**) allows for identification of individual factors potentially influencing the input quantities for the measurement uncertainty as shown in the diagram in **Figure 3**.

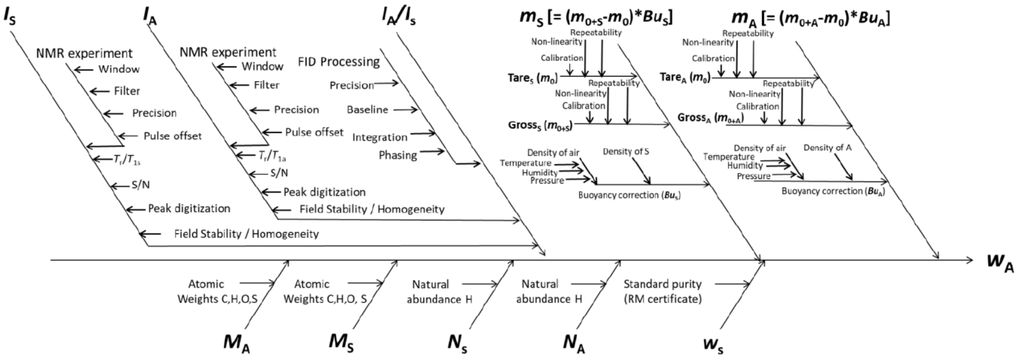

I_S
I_A
I_A/I_S
m_S [= (m_0+S-m_0)*Bu_S]
m_A [= (m_0+A-m_0)*Bu_A]
NMR experiment
Window
Filter
Precision
Pulse offset
T_r/T_1s
S/N
Peak digitization
Field Stability / Homogeneity
NMR experiment
Window
Filter
Precision
Pulse offset
T_r/T_1a
S/N
Peak digitization
Field Stability / Homogeneity
FID Processing
Precision
Baseline
Integration
Phasing
Repeatability
Non-linearity
Calibration
Tare_S (m_0)
Repeatability
Non-linearity
Calibration
Gross_S (m_0+S)
Density of air
Temperature
Humidity
Pressure
Density of S
Buoyancy correction (Bu_S)
Repeatability
Non-linearity
Calibration
Tare_A (m_0)
Repeatability
Non-linearity
Calibration
Gross_A (m_0+A)
Density of air
Temperature
Humidity
Pressure
Density of A
Buoyancy correction (Bu_A)
w_A
Atomic Weights C,H,O,S
Atomic Weights C,H,O, S
Natural abundance H
Natural abundance H
Standard purity (RM certificate)
M_A
M_S
N_S
N_A
w_S

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. [12], [32], [40] Since these measurements should 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 these 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 contribute significantly to the observed precision in the value assignment in addition to that arising from the method repeatability.

The final assigned value will be similar regardless of the approach used, although the contributions of the factors 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 the $^1H/^2H$ isotope distribution of the quantification signals, with their associated uncertainties, were calculated based on the values for atomic weights and hydrogen isotope distribution in the 2016 revision of the IUPAC Technical report of the Atomic weights of the elements, [24], [25] the uncertainties of individual gravimetric operations are based on balance performance characteristics corrected for buoyancy effects [31] 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 [8], [11], [12], [33] including recently a Bayesian approach using a Monte Carlo calculation of the results of replicate sample analysis. [41] Examples of measurement uncertainty budgets for qNMR analysis using $DMSO_2$ as the ISRM are provided in Appendix A1.2.

4. Acknowledgements

The work described in this report was made possible by a collaborative research agreement between the NMIJ/AIST (Japan) and the BIPM and the donation by JEOL France of an ECS-400 NMR spectrometer to the BIPM. The provision of chemical standards by WAKO Pure Chemicals is also acknowledged.

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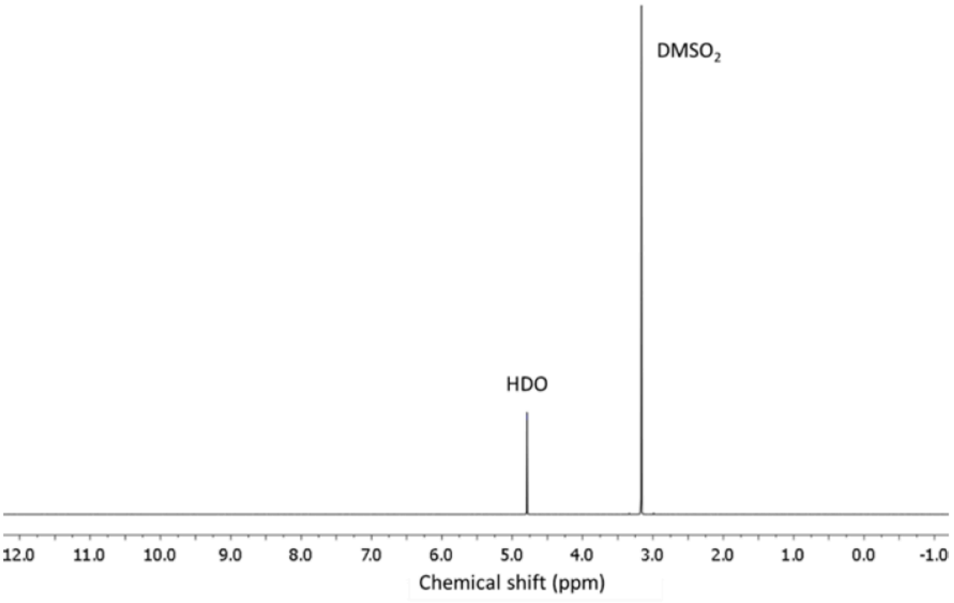
DISCLAIMER: Commercial NMR instruments, software and materials are identified in this document in order to describe some procedures. This does not imply a recommendation or endorsement by the BIPM nor does it imply than any of the instruments, equipment and materials identified are necessarily the best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

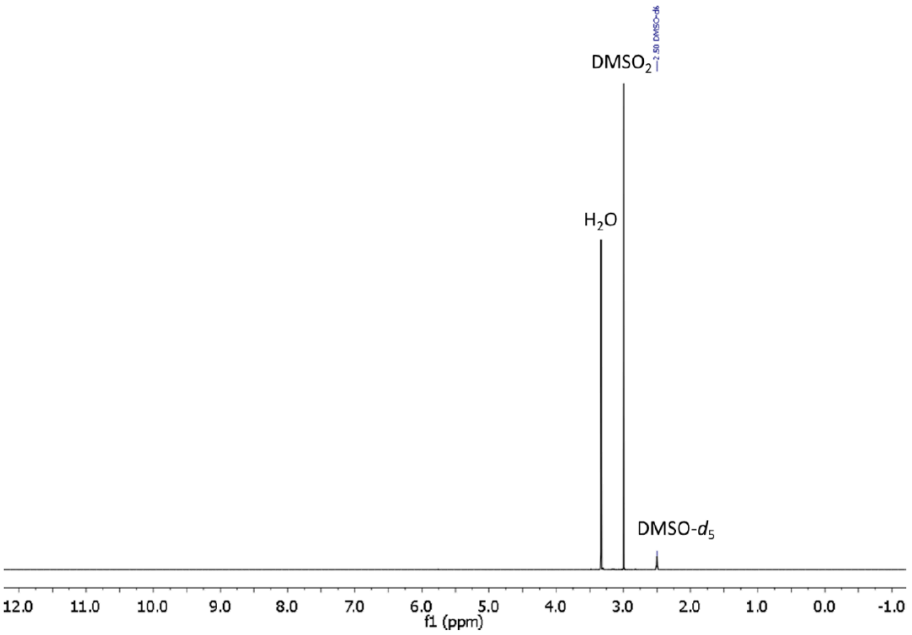
Appendix 1. Annexes

A1.1. Solution NMR Spectra of Dimethyl sulfone

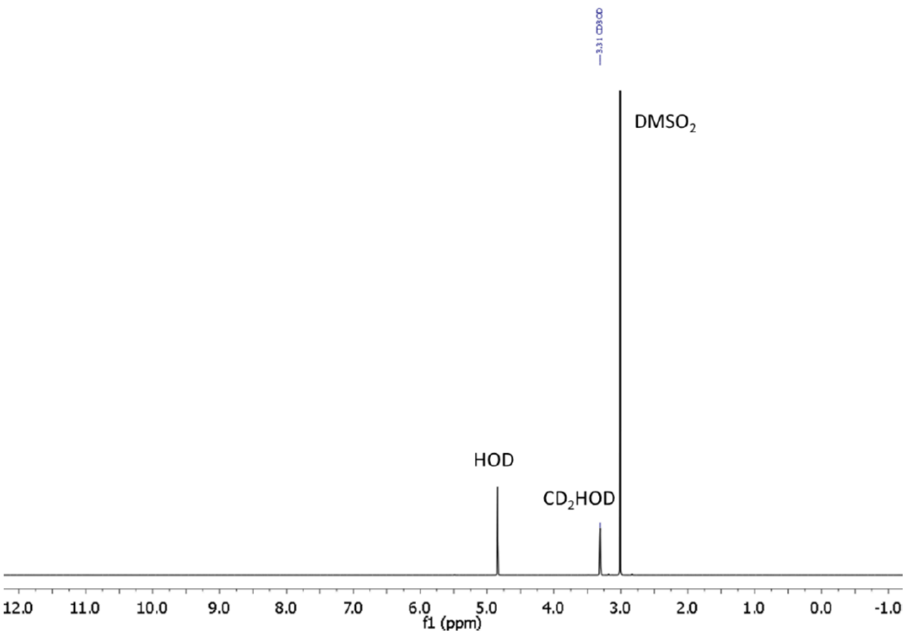
A1.1.1. $DMSO_2$ in D_2O



A1.1.2. $DMSO_2$ in DMSO-d_6



A1.1.3. DMSO$_2$ in CD$_3$OD



A1.2. qNMR using DMSO$_2$ as internal standard

Two examples are provided of the value assignment by qNMR of the mass fraction content of organic compounds using DMSO$_2$ as the ISRM. In the first example DMSO$_2$ was used in a solution in D$_2$O with MA as analyte. In the second example CDCl$_3$ was the solvent with DMTP 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 complex resonance signals or with 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. As a result the uncertainty contribution due to the repeatability of the signal integration is smaller and the relative uncertainty contribution due to the uncertainty associated with gravimetry and the purity of the internal standard correspondingly greater than would be anticipated for more typical applications.

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

The DMSO$_2$ was obtained from a commercial provider and used as supplied without additional treatment. The purity of the DMSO$_2$ was assigned in separate qNMR experiments in solution in D$_2$O using a high-purity CRM for KHP (either NIST SRM 84L or NMIJ CRM 3001b) as the internal standard. By our internal assignment the mass fraction content of DMSO$_2$ in the material used as the ISRM for the qNMR applications described in this sheet was 996.5 ± 0.8 mg g^{-1}, consistent with the purity value reported by the material provider.

The MA and DMTP used as analytes and deuterated solvents were purchased from commercial suppliers and used without further treatment or purification. Commercial borosilicate glass NMR tubes with 5mm internal diameter rated for use in 500MHz spectrometers were used for all measurements.

A1.2.1. $DMSO_2$ (IS) and MA (Analyte) in D_2O

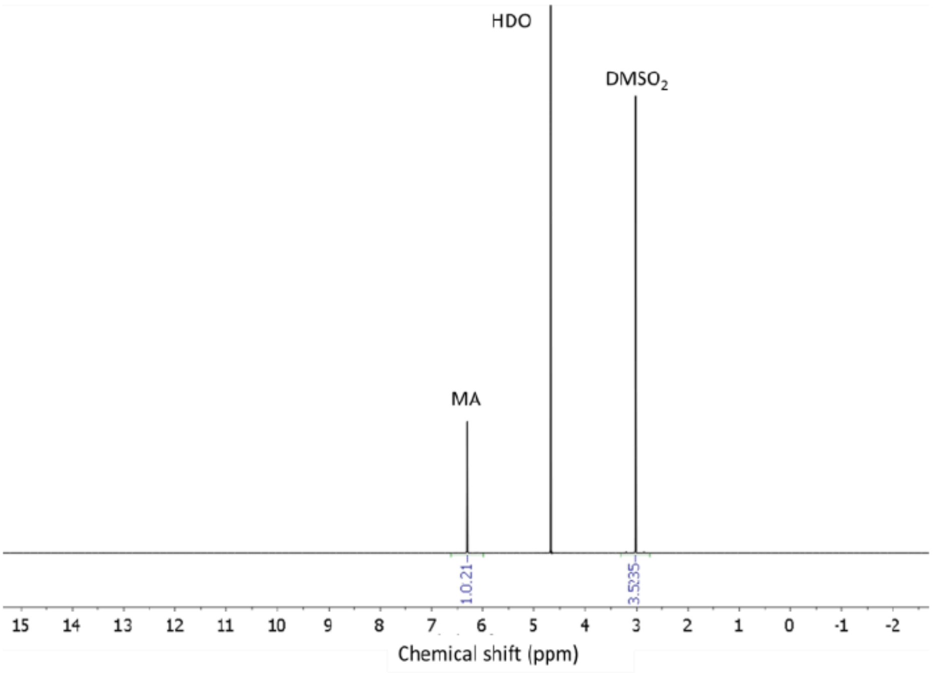


Figure 1.1 — ^{1}H NMR spectrum of MA + $DMSO_2$ in D_2O.

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 1.1. The sample was made up in solution in approximately 1mL of D_2O and 800µL were transferred into the NMR tube for analysis.

Table 1.1. Parameters for purity assignment of a sample of MA using $DMSO_2$ in D_2O

Parameter	Value
MA Sample size (mg)	2.3 – 3.7
$DMSO_2$ Sample size (mg)	3.5 – 4.9
Number of Transients	32
Receiver gain	Automatic
Acquisition time (s)	4
Relaxation delay (s)	65
Pulse offset (ppm)	4.8
Spectral width (ppm)	400

Data points	639652
Temperature (K)	298
Spinning	Off
Integral ratio (MA:DMSO$_2$)	0.13 – 0.29 [(a)]

(a) integral ratio reported for information only—not necessarily the “optimal” value

A baseline correction window of one hundred times the FWHM was used for 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 measurement uncertainty budget for one of the samples is reproduced in Table 1.1. The integral ratio is the mean of the four 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 1.2. The mass fraction content of MA in this material assigned by qNMR using DMSO$_2$ as ISRM in solution in D$_2$O was 999.7 ± 1.6 mg g^{-1}.

Table 1.2. Uncertainty budget for MA purity by qNMR using DMSO$_2$ as ISRM in D$_2$O.

Uncertainty source	Value	Uncertainty Evaluation Type	Standard Uncertainty	Sensitivity coefficient	Relative Uncertainty
I_A	17834	-	-	-	-
I_S	97943	-	-	-	-
Integral A/Integral S	0.1821	A	0.00003	5.490168296	1.75E-04
Analyte signal ^{1}H Nuclei	1.9996	B	0.0003	-0.499928572	1.50E-04
IS signal ^{1}H Nuclei	5.9988	B	0.0003	0.166646102	5.00E-05
Analyte Molar Mass (g · mol^{-1})	116.080	B	0.004	0.008611795	3.44E-05
IS Molar Mass (g · mol^{-1})	94.1284	B	0.0062	-0.010620144	6.61E-05
Analyte Sample Mass (mg)	2.3545	B	0.00124	-0.424573019	5.28E-04
IS Sample Mass (mg)	3.5063	B	0.00124	0.285103149	3.54E-04
IS Purity (g · g^{-1})	0.9965	B	0.0004	1.003168262	4.01E-04
Assigned value (g · g^{-1})	0.9997		0.00079		7.92E-04
				Combined Uncertainty	0.000791

Uncertainty source	Value	Uncertainty Evaluation Type	Standard Uncertainty	Sensitivity coefficient	Relative Uncertainty
				ν_{eff}	1655
Analyte mass function ($g \cdot g^{-1}$):	0.9997	± 0.0016		k	2
Analyte purity (% mass):	99.97	± 0.2		Expanded Uncertainty	0.00158

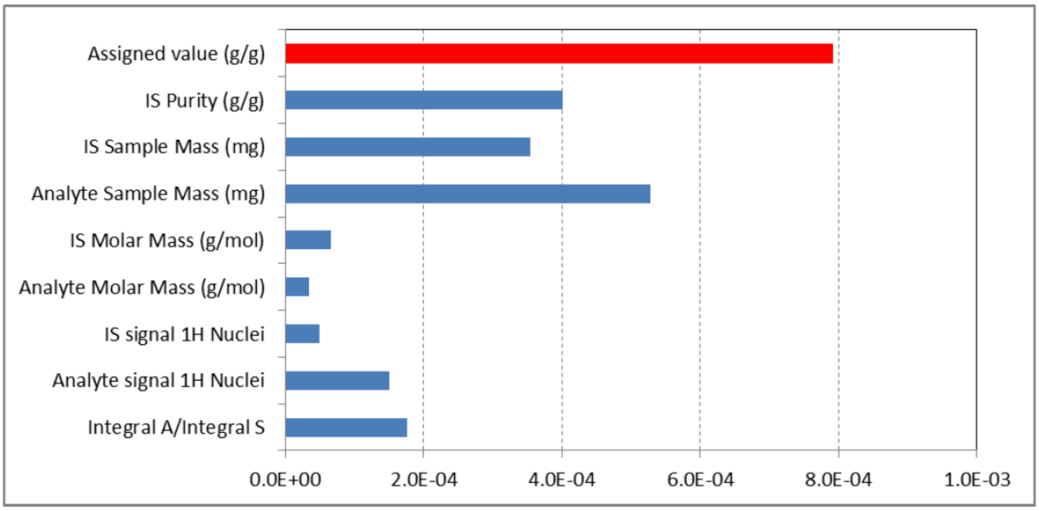


Figure 1.2 — Relative uncertainty components (in blue) for the uncertainty in the assigned purity value (in red) for MA using $DMSO_2$ as ISRM in D_2O.

A1.2.2. $DMSO_2$ (IS) and DMTP (Analyte) in $CDCl_3$

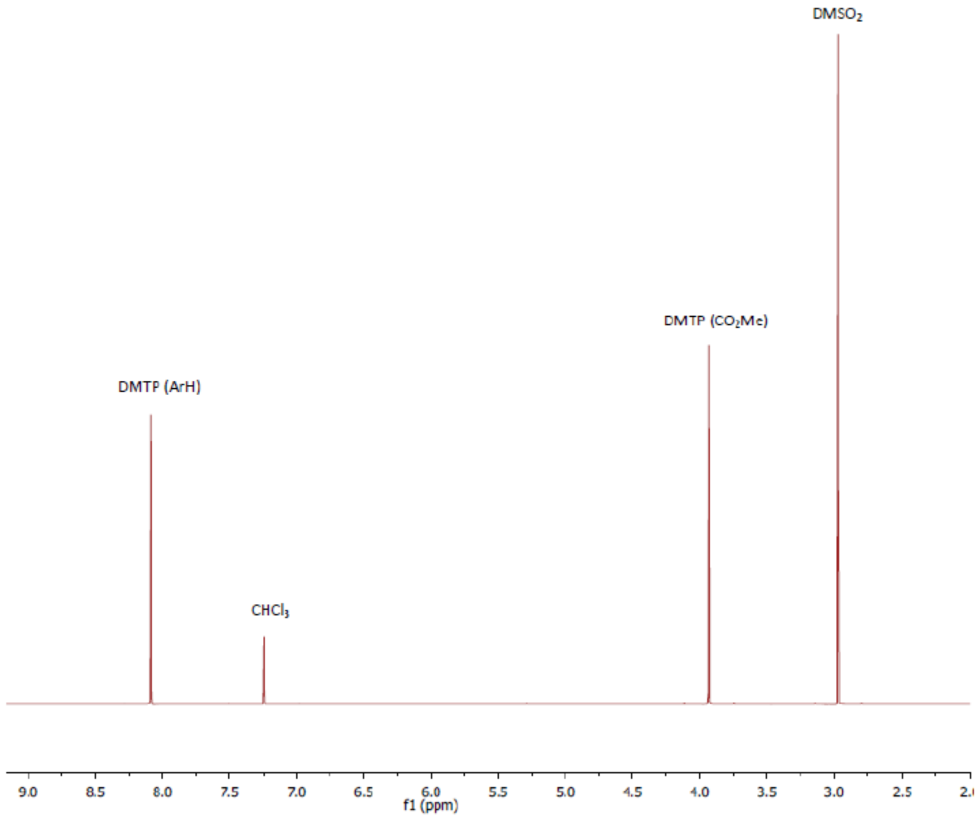


Figure 1.3 — ^{1}H NMR of $DMSO_2$ + DMTP in $CDCl_3$.

D_2O was not a suitable solvent since the non-polar DMTP is insufficiently water soluble. DMTP is however readily soluble in non-polar organic solvents such as $CDCl_3$. In this case the signal at 3.9ppm corresponding to 6 hydrogens due to the two equivalent methyl ester groups in DMTP was used as the signal for quantification.

Representative NMR parameters used for the measurement are given in Table 1.3.

Table 1.3. qNMR parameters for DMTP assignment using $DMSO_2$ as ISRM in $CDCl_3$.

Parameter	Value
$DMSO_2$ Sample size (mg)	6—10
DMTP Sample size (mg)	8 – 22
Number of Transients	32
Receiver gain	34
Acquisition time (s)	4

Relaxation delay (s)	50
Pulse offset (ppm)	7.0
Spectral width (ppm)	400
Data points	39979
Temperature (K)	298
Spinning	Off
Integral ratio (MA:$DMSO_2$)	0.7 – 1.0

Baseline correction was performed over the whole spectral width using a multipoint baseline correction algorithm. The integration range start and end points were placed 30Hz beyond the ^{13}C satellite signals, with the signal corresponding to the four equivalent aromatic protons of DMTP used for quantification. Results from four independent sample mixtures each measured five times were obtained. The measurement uncertainty budget is reproduced below in Table 1.4. The relative contribution of each component to the uncertainty of the combined result is displayed in Figure 1.4. The mass fraction content of DMTP in the material in $CDCl_3$ using $DMSO_2$ as the ISRM was assigned as $999.0 \pm 1.0 mg \cdot g^{-1}$.

Table 1.4. Uncertainty budget for DMTP purity by qNMR using $DMSO_2$ as ISRM in $CDCl_3$.

Uncertainty source	Value	Uncertainty Evaluation Type	Standard Uncertainty	Sensitivity coefficient	Relative Uncertainty
I_A	31	-	-	-	-
I_S	43	-	-	-	-
Integral A/ Integral S	0.7154	A	0.00009	1.396509056	1.28E-04
Analyte signal ^1H Nuclei	5.9988	B	0.0003	-0.16653249	5.00E-05
IS signal ^1H Nuclei	5.9988	B	0.0003	0.166537919	5.00E-05
Analyte Molar Mass ($g \cdot mol^{-1}$)	194.194	B	0.008	0.005144315	4.37E-05
IS Molar Mass ($g \cdot mol^{-1}$)	94.1284	B	0.0062	-0.01061311	6.61E-05
Analyte Sample Mass (mg)	8.5689	B	0.00124	-0.11658382	1.45E-04

Uncertainty source	Value	Uncertainty Evaluation Type	Standard Uncertainty	Sensitivity coefficient	Relative Uncertainty
IS Sample Mass (mg)	5.8213	B	0.00124	0.17161031	2.13E-04
IS Purity ($g \cdot g^{-1}$)	0.9964	B	0.0004	1.002604474	4.01E-04
	Assigned value ($g \cdot g^{-1}$)	0.9990		0.00050	
5.05E-04					Combined Uncertainty
0.000505					ν_{eff}
967	Analyte mass function ($g \cdot g^{-1}$):	0.9990	± 0.0010		k
2	Analyte purity (% mass):	99.90	± 0.1		Expanded Uncertainty

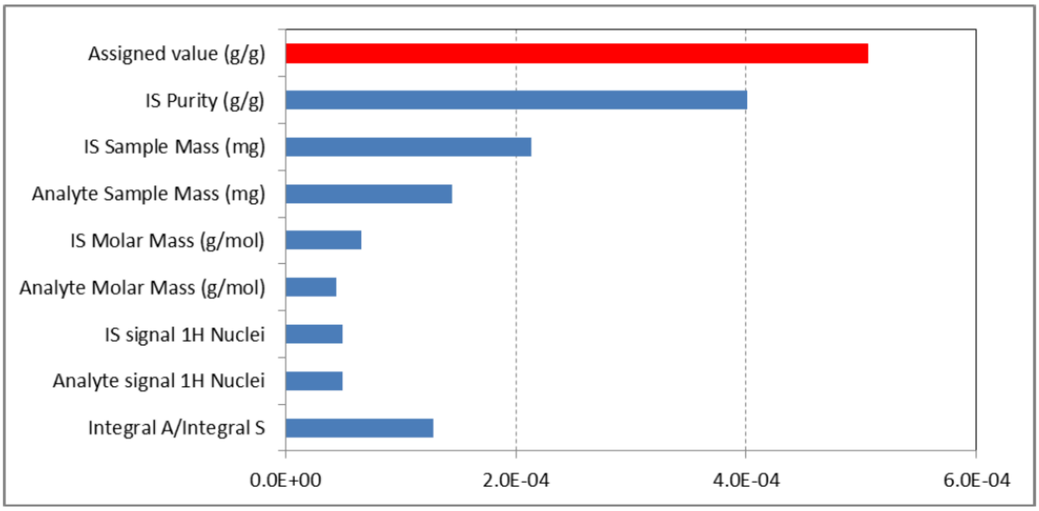


Figure 1.4 — Relative uncertainty components (in blue) for the uncertainty in the assigned purity value (in red) for DMTP using $DMSO_2$ as ISRM in $CDCl_3$.

The result obtained for the purity assignment of DMTP agreed within its associated uncertainty with values obtained by qNMR analyses using other IS/solvent combinations.

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