Bureau International des Poids et Mesures

Internal Standard Reference Data for qNMR: Potassium Hydrogen Phthalate [ISRD-03]

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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. [2], [3], [4], [5] 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 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 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 interferences 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 assigned value. Evidently, regardless of the precision of a qNMR measurement result, 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 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:

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 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 of 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 representative of those with similar properties rather than as a prescriptive set for use in qNMR. However these are the most readily available deuterated solvents and the 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).

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

The third goal and the focus of the remainder of this report is to provide guidance on the use and limitations of potassium hydrogen phthalate as an ISRM for qNMR analysis.

Potassium hydrogen phthalate (KHP) is one of the ISRMs listed in Figure 1. The use of KHP as a primary standard for acidometric titration and pH measurements is well established. [23] More recently material certified for use as a calibrator for titrimetric analysis has been shown to be suitable for use as an internal standard for qNMR assignments of analytes soluble in D_2O. [24] KHP can in principle also be used in solution in DMSO-d_6 or CD_3OD and related solvents if D_2O is not a suitable solvent for the target analyte. In DMSO-d_6 however there exists the potential for interference due to the signal associated with the acidic hydrogen in KHP. The following sections of this reference document and the attached annexes focus on specific properties and applications of potassium hydrogen phthalate for use as an ISRM for qNMR.

Because it is an important primary standard for acid-base titration measurements KHP has the advantage of being available as a CRM certified by an NMI. [25] These CRMs possess all the characteristics of a qNMR ISRM outlined above and in particular are produced at high levels of purity assigned with small associated measurement uncertainty. One caveat is that the NMR signals of KHP used for quantification are relatively disperse and complex compared with the reference signals of the other materials listed in Figure 1.

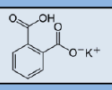
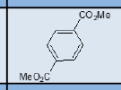
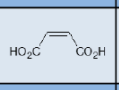
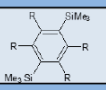
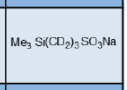
ISRM	KHP	BTFMBA	DMTP	MA	$DMSO_2$	BTMSB	DSS-d_6
Structure							
δ (ppm)	8.3-7.0 (4H)	8.4-8.5 (2H) 8.2-8.4 (1H)	8.1 (4H) 3.9 (6H)	6.3 (2H)	3.0 (6H)	[7.5 (4H, R=H)] 0.2 (18H)	0.1 (9H)
Density ($g.cm^{-3}$)	1.64 ± 0.17	1.72 ± 0.04	1.2 ± 0.24	1.53 ± 0.03	1.4 ± 0.03	1.0 ± 0.02	1.27 ± 0.03
Solvent ⇩	Solubility (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 [26]

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

KHP Potassium hydrogen phthalate

BTFMBA 3,5-*bis*-Trifluromethylbenzoic acid

DMTP Dimethyl terephthalate

MA Maleic acid

$DMSO_2$ Dimethyl sulfone

BTMSB 1,4-*bis*-Trimethylsilylbenzene (R=H), BTMSB-d_4 (R = D), BTMSB-F_4 (R = F);

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

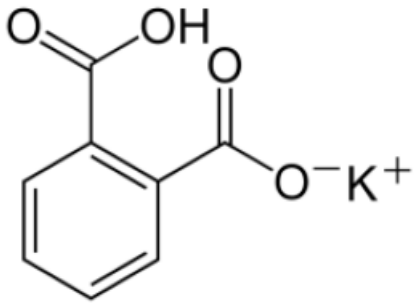
D_2O Deuterium oxide

DMSO-d_6	Dimethyl sulfoxide-d_6 / Hexadeuterodimethyl sulfoxide
CD_3OD	Methanol-d_4 / Tetradeuteromethanol
$CDCl_3$	Chloroform-d / Deuterochloroform

2. Properties of Potassium Hydrogen Phthalate

2.1. Physical Properties

Name	**Potassium Hydrogen Phthalate**
Structure	+



Synonym	Potassium biphthalate
CAS Registry Number	877-24-7
Molecular Formula	$C_8H_5KO_4$
Molar Mass [27], [28]	204.223 g/mol, $u = 0.004$ g/mol
Melting point [29]	295 °C (decomposes)
Density	1 640 ± 20 kg/m^3 [29]
Appearance	White crystalline powder
^{1}H NMR [30]	δ 7.7—8.2 (m, 2H); 7.5 – 7.6 (m, 2H)
^{13}C NMR	δ 168.3; 134.9; 132.6; 130.4

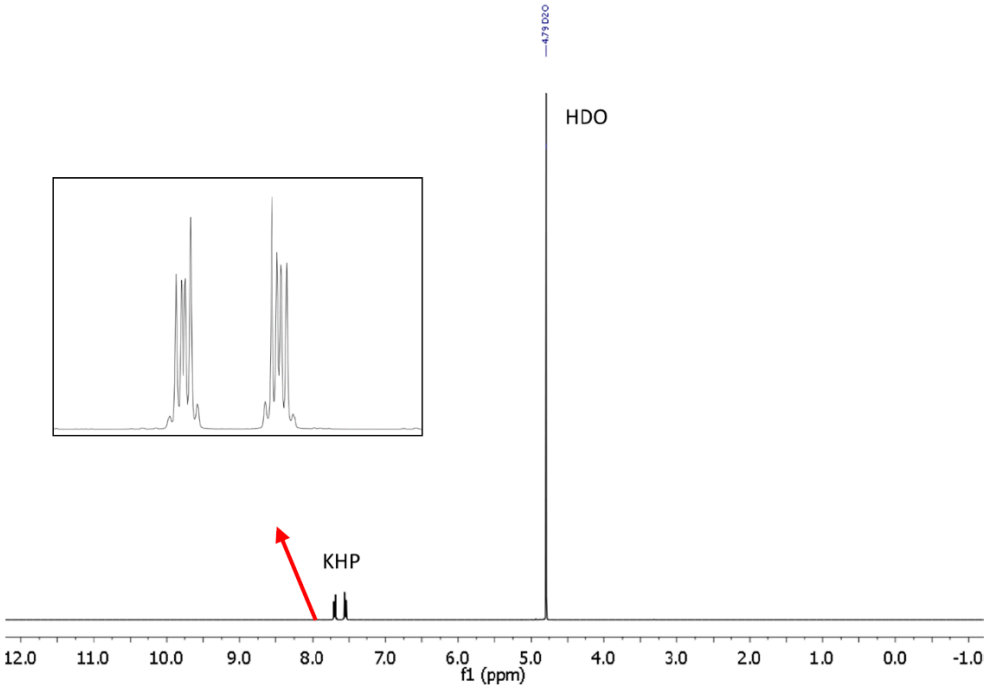


Figure 2 — ^{1}H NMR spectrum of KHP in D_2O: JEOL ECS-400 spectrometer with Royal probe.

2.2. Solvent Compatibility

Where suitable, D_2O is the first choice solvent for use with KHP. KHP is soluble in D_2O at levels in excess of 10 mg mL^{-1}. If necessary it may be used in DMSO-d_6 or CD_3OD but its solubility is limited in each case (less than 2.5 mg mL^{-1}) [26] and the effectiveness of the desired analyte/solvent combination should be verified.

2.3. Quantification signal

Two distinct pairs of magnetically equivalent aromatic protons are present in potassium hydrogen phthalate. These give rise to two multiplets, each corresponding to two hydrogens, occurring over a chemical shift in the range of 8.3 ppm – 7.0 ppm on the δ scale. The exact position of the resonance is a function of other factors including, but not limited to, the solvent, temperature, pH and the concentration of KHP and the analyte in the solution. The proximity of the multiplets generally precludes their separate integration and the combined signals of the four aromatic protons of KHP are normally used for quantification purposes. For optimal results the homogeneity of the spectrometer magnetic field should be optimized such that the full width at half maximum (FWHM) of the residual HDO signal is less than 2 Hz when D_2O is the solvent with the base of the residual water resonance retaining a suitable Lorentzian peak shape.

2.4. Impurities and artefact signals

The main interferences in a solution containing KHP will come from the signals due to residual non-deuterated solvent. The chemical shifts of these signals are given in Table 1 below. Note that in the case of solutions in D_2O the signal due to residual HDO could potentially be attenuated if desired by the use of a (water) signal suppression pulse sequence, at the cost of potentially introducing additional non-linearity into the signal responses. [31]

2.5. Solvent recommendations and advisories

2.5.1. D_2O

D_2O is the recommended choice as NMR solvent for use with KHP. Rapid exchange of the carboxyl proton with deuteron removes interference due to the acidic hydrogen in KHP. D_2O is suitable for 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

In addition to the relatively low solubility of KHP in DMSO-d_6, the use of this solvent can be problematic due to potential interference from the signal due to the acidic hydrogen of KHP. This interference can be attenuated by the addition of D_2O as a co-solvent, at the cost of an additional or increased signal due to HDO. DMSO-d_6 should only be chosen in a case where the analyte lacked sufficient solubility in water and the other ISRMs recommended for use in DMSO-d_6 (see Figure 1, see p. 8) were not suitable for the chosen analyte.

2.5.3. Methanol-d_4 and related solvents

As in the case of DMSO-d_6, CD_3OD can be considered as solvent in a case where the analyte lacked sufficient solubility in water and the other ISRMs recommended for use in CD_3OD (see Figure 1, see p. 8) were not suitable for the analyte. The presence of an exchangeable deuteron in CD_3OD attenuates the potential for interference from the acidic KHP hydrogen.

2.5.4. Chloroform-d and related solvents

KHP is not sufficiently soluble for use as an ISRM in chlorinated or non-polar solvents.

Table 1. Solvent Parameters for KHP

Solvent	qNMR signal - Multiplet, 4H (ppm) [a]	Integration range (ppm) [a]	T_1 (s) [a]	Residual Solvent (ppm)	Comments:
D_2O	7.8, 7.6	7.2 – 8.0	5-6	**4.8** [b]	
DMSO-d_6	8.2, 7.5	7.2 – 8.5	4-6	2.5	Potential for baseline interference from acidic proton of

CD_3OD	7.6	7.2 – 8.0	5-6	3.3 (*b*)	KHP. H_2O peak at 3.3 – 4.8 ppm (*b*) HOD peak at 4.8 ppm (*b*)
$CDCl_3$	Not suitable				Insufficient solubility

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

(*b*) Chemical shift of residual H_2O or HDO signal is strongly pH dependent and can shift in the range 3.3 ppm – 4.9 ppm.

3. Good Practice Guidance for Achieving 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, [32], [33] and the spectrometer performance, experimental parameters and the protocol for signal processing and integration must be optimized, [8], [9], [34] 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. [35] 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], [36] 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 [37] 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. [32] 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 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 Appendix A1.2 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 gravimetric operations, high accuracy qNMR assignments require additional correction for sample buoyancy effects [33] and the $^{1}H/^{2}H$ isotope composition of the quantified signals. The value and associated uncertainty of the $^{1}H/^{2}H$ isotope composition of each quantification signal can be obtained using an on-line calculator application. [28]

As sample preparation for qNMR involves mass determinations in the milligram range using sensitive balances, the loss of even minute (almost invisible) quantities of powder during the gravimetric procedure will have a measurable influence on the balance reading and hence on the input quantities for the qNMR assignment. Environmental conditions for gravimetry and qNMR sample preparation should be controlled throughout the process, subject to minimum change and kept within the operating range recommended by the manufacturer. [38], [39] 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. 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.

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. [40] 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 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 2.5 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 Hz/pt 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 potassium hydrogen phthalate 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:

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

$$(n = 10 - 15)$$

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) < 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
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 Hz/pt	The digital resolution is the reciprocal of the acquisition time. Suitable signal shape sensitivity requires not less than 0.4 Hz/pt.
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 30 Hz 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. [34], [40] 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 0.3 Hz should be used. The window function in use at the BIPM with the JEOL-ECS 400 was typically no greater than 0.05 Hz—0.10 Hz 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**.

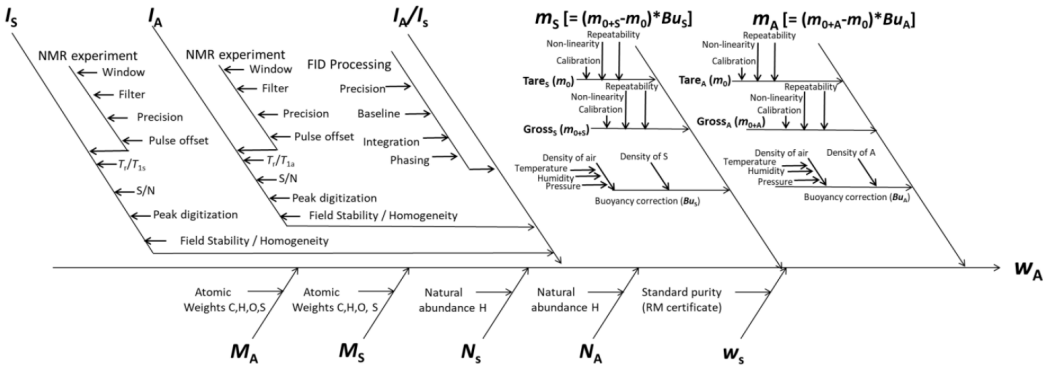


Figure 3 — Ishikawa diagram for input quantities considered for estimation of the measurement uncertainty of a purity assignment by qNMR

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], [34], [41] 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 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 $^{1}H/^{2}H$ 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, [27], [28] the uncertainties of individual gravimetric operations are based on balance performance characteristics corrected for buoyancy effects [33] 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 including qNMR for purity evaluation have been reported [8], [11], [12], [35] including recently a Bayesian approach using a Monte Carlo calculation of the results of replicate sample analysis. [42] An example measurement uncertainty budget for a qNMR using KHP as the internal standard analysis is provided in Appendix A1.2.

4. Acknowledgements

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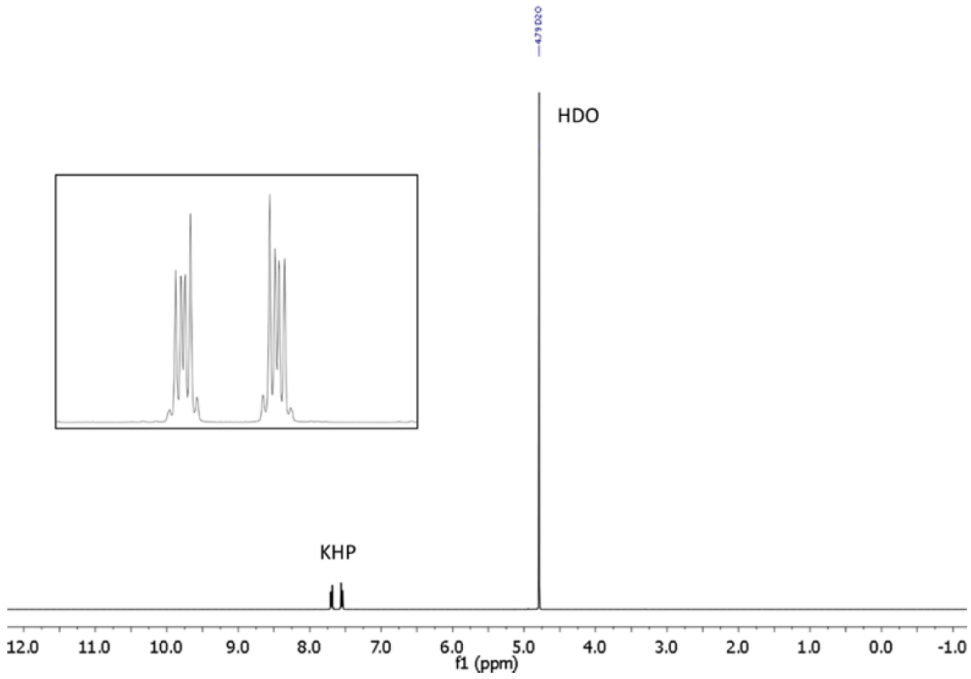
DISCLAIMER: Commercial NMR instruments, software and materials are identified in this document in order to describe some procedures. This does not imply a recommendation or endorsement by the BIPM nor does it imply than any of the instruments, equipment and materials identified are necessarily the best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

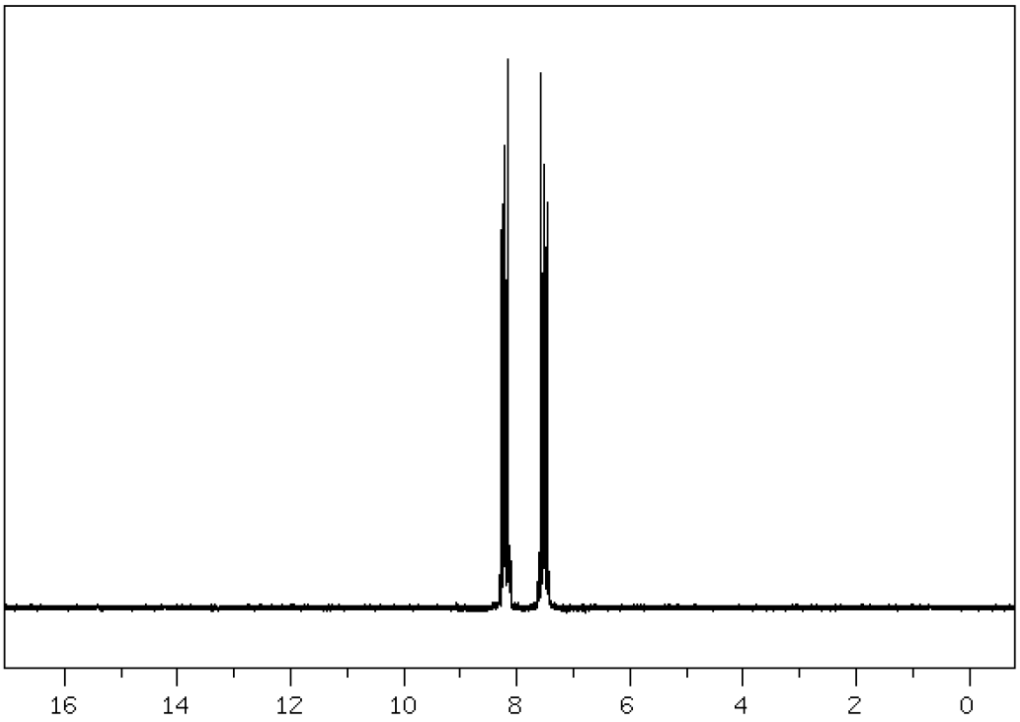
Appendix 1. Annexes

A1.1. Solution NMR Spectra of KHP

A1.1.1. KHP in D_2O ($400\ \mathrm{MHz}$)



A1.1.2. KHP in DMSO-d_6 (90 MHz)



A1.2. qNMR using KHP as internal standard

One example is provided of the value assignment by qNMR of the mass fraction content of organic compounds using KHP as the ISRM. KHP was used in a solution in D_2O with maleic acid (MA) as analyte.

This is intended as "best case" illustration 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 and fully resolved 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 reproducibility 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 routine applications.

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

Two sources of a CRM for high purity KHP, value assigned by an NMI—either NIST SRM 84L or NMIJ CRM 3001b [25]—were obtained and used in compliance with the provider's instructions and without additional treatment. The certified mass fraction of the KHP was $999.93 \pm 0.08\ \text{mg g}^{-1}$ for NIST SRM 84L and $999.91 \pm 0.14\ \text{mg g}^{-1}$ for NMIJ CRM 3001c

The MA used as analyte and D_2O solvent were obtained from commercial suppliers and were used without further treatment or purification. Commercial borosilicate glass NMR

tubes with 5 mm internal diameter rated for use in 500 MHz spectrometers were used for all measurements.

A1.2.1. KHP (IS) and MA (Analyte) in D_2O

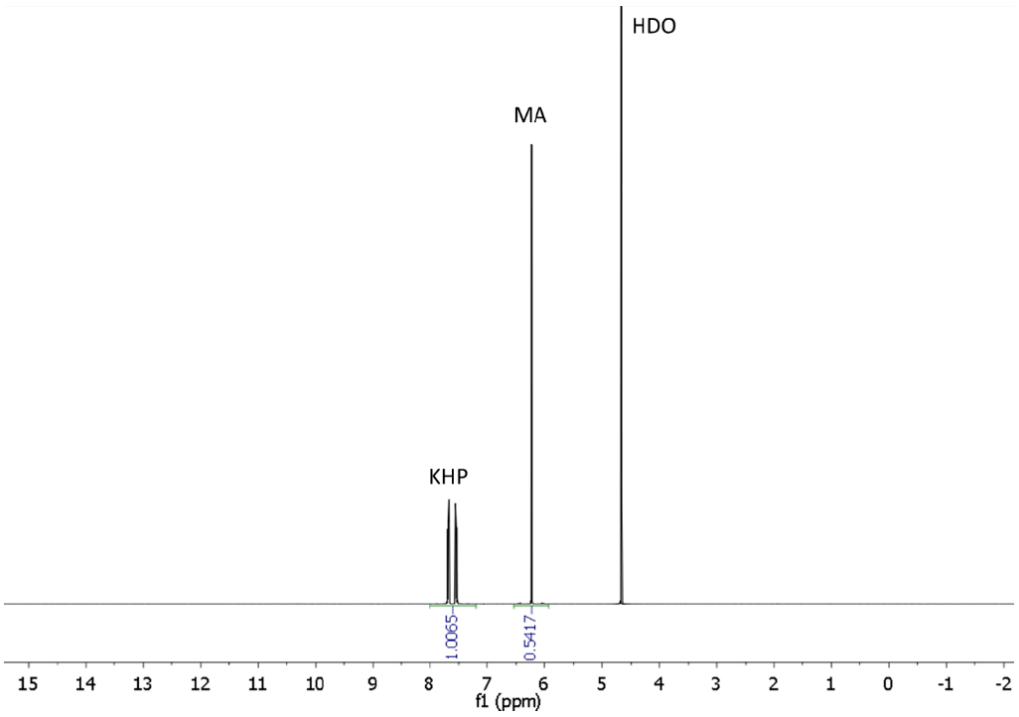


Figure 1.1 — ^{1}H NMR spectrum of MA + KHP in D_2O at 400 MHz**.**

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

Table 1.1. NMR experiment parameters for MA purity assignment using KHP in D_2O

Parameter	Value
MA Sample size (mg)	5.5 – 6.2
KHP Sample size (mg)	9.3 – 10.1
Number of Transients	32
Receiver gain	Automatic
Acquisition time (s)	4
Relaxation delay (s)	80
Pulse offset (ppm)	7.0

Spectral width (ppm)	400
Data points	639652
Temperature (K)	298
Spinning	Off
Integral ratio (MA:KHP)	0.48 – 0.58 (a)

(*a*) integral ratio reported for information only — not necessarily "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 using NMIJ CRM 3001 as the source of KHP, is reproduced in Table 1.2. The integral ratio is the mean of all replicates obtained for the four samples, normalized to take into account the different masses of analyte and standard used in the preparation of each 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 is displayed in Figure 1.2. The mass fraction content of the MA material assigned by qNMR using this set of samples was $999.5 \pm 1.1\ \mathrm{mg\ g^{-1}}$.

Table 1.2. Uncertainty budget for MA purity by qNMR using KHP as ISRM in D_2O.

Uncertainty source	Value	Uncertainty Evaluation Type	Standard Uncertainty	Sensitivity coefficient	Relative Uncertainty
I_A	11679	-	-	-	-
I_S	48569	-	-	-	-
Integral A/Integral S	0.2405	A	0.00004	4.156567996	1.80E-04
Analyte signal ^{1}H Nuclei	1.9996	B	0.0003	-0.499871958	1.50E-04
IS signal ^{1}H Nuclei	3.9992	B	0.0003	0.249933509	7.50E-05
Analyte Molar Mass (g/mol)	116.072	B	0.004	0.008611413	3.44E-05
IS Molar Mass (g/mol)	204.222	B	0.0059	-0.004894392	2.90E-05
Analyte Sample Mass (mg)	2.5469	B	0.00124	-0.392455128	4.88E-04
IS Sample Mass (mg)	9.3139	B	0.00124	0.107317447	1.33E-04
IS Purity (g/g)	0.99991	B	0.00007	0.999633934	7.00E-05

Uncertainty source	Value	Uncertainty Evaluation Type	Standard Uncertainty	Sensitivity coefficient	Relative Uncertainty
Assigned value (g/g)	0.9995		0.00057		5.69E-04
				Combined Uncertainty	0.000568
				ν_{eff}	394
Analyte mass function (g/g):	0.9995	± 0.0011		k	2
Analyte purity (% mass):	99.95	± 0.1		Expanded Uncertainty	0.00158

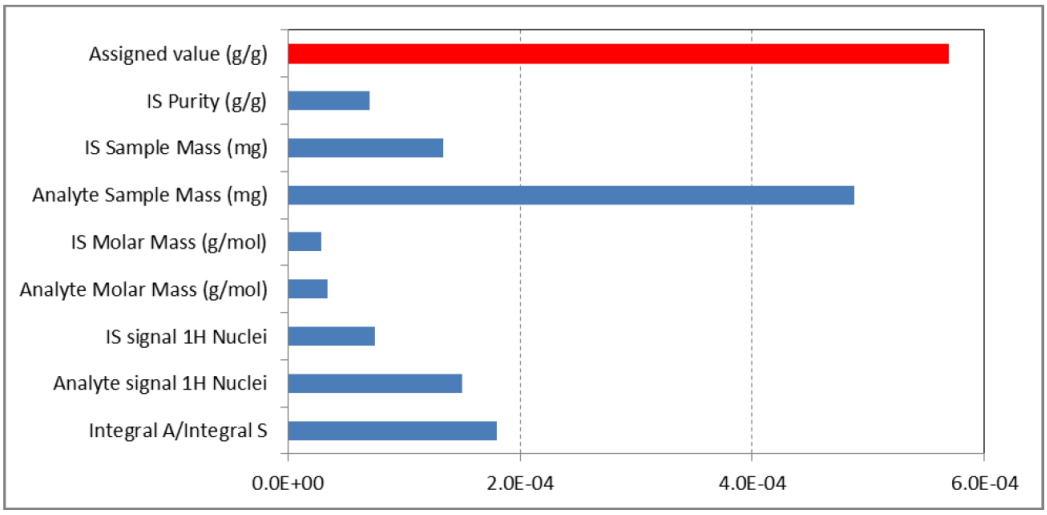


Figure 1.2 — Relative uncertainty components (in blue) for the uncertainty in the assigned purity value (in red) for MA using KHP as ISRM in D_2O.

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