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Quantitative nuclear magnetic resonance spectroscopy — Purity determination of organic compounds used for foods and food products — General requirements for ^{1}H NMR internal standard method

Spectroscopie par résonance magnétique nucléaire quantitative — Détermination de la pureté des composés organiques utilisés dans les aliments et les produits alimentaires — Exigences générales pour la méthode de l'étalon interne par RMN ^{1}H

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Foreword

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Reliable quantification of food components is important for food safety and can be used as a measurement tool for food authenticity. Presently, chromatography such as gas chromatography (GC) and liquid chromatography (LC) is used in the majority of regulatory work associated with foods and food products. To obtain reliable quantification results with these methods, the use of certified reference materials (CRMs) is required, for which metrological traceability of the certified value, as measurement standards, is essential. However, obtaining such CRMs to fulfil these requirements is almost impossible in many cases as conventional methods that can establish metrological traceability, such as the mass balance, have limited applications. Therefore, the establishment of a simple, rapid, widely applicable and reliable purity quantification method, with a focus on the establishment of metrological traceability, for the characterization of measurement standards for food analyses is an essential. Quantitative nuclear magnetic resonance (qNMR) spectroscopy has been recognized as a quick and simple characterization method. The method is also recognized as metrologically traceable, and uses the purity from a CRM to determine the purity of other analytes. When a certified value of a CRM, whose value is stated as metrological traceable to the International System of Units (SI), is used as a measurement standard for qNMR, the determined purity value of the sample by qNMR can also be traceable to the SI through the CRM. qNMR, therefore, has the potential to provide the SI traceability to measurement standards relevant to food components.[10][17][36]

Quantitative nuclear magnetic resonance spectroscopy — Purity determination of organic compounds used for foods and food products — General requirements for ^{1}H NMR internal standard method

1 Scope

This document specifies general requirements and performance criteria for the determination of purity of organic compounds through the application of solution state proton (^{1}H) quantitative nuclear magnetic resonance (qNMR) spectroscopy using an internal standard method.

This document is applicable to bioactive compounds in functional foods, natural toxins, food additives and pesticides.

This document is applicable to the users pursuing metrological traceability of the measurement results.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
quantitative NMR
qNMR
quantitative analysis using NMR spectroscopy

3.2
proton quantitative NMR
^{1}H quantitative NMR
^{1}H qNMR
quantitative NMR (3.1) spectroscopy using proton (^{1}H) as the observed nucleus

3.3
qNMR procedure
predetermined workflow of quantitative analysis using *qNMR* (3.1) including sample solution preparation, data acquisition and data processing parameters that have been optimized and validated for a specific analyte

3.4
equilibrium magnetization
magnitude of the nuclear magnetization vector that is polarized in the sample after it has be placed into a static magnetic field

3.5
spin-lattice relaxation time
T_1
time needed for a set of spins of magnetically equivalent nuclei to attain macroscopic z-magnetisation, M_z, equilibrium in a magnetic field or to return to this equilibrium after excitation (by a radio frequency (RF) pulse)

Note 1 to entry: The recovery of M_z magnetisation is an exponential saturation process described by the Bloch-equation for M_z:

$$M_z(t) = M_z(t_{eq}) - [M_z(t_{eq}) - M_z(0)] \times \exp(-t/T_1)$$

where

$M_z(t)$	is the time function of M_z;
0	is the time zero;
t_{eq}	is the time equilibrium has been achieved;
t	can be any time between 0 and t_{eq}.

Note 2 to entry: Following a 90° pulse, 63 % of an ensemble of magnetically equivalent spins have relaxed after $1 \times T_1$, over 99 % of spins have relaxed after $5 \times T_1$.

3.6
free induction decay
FID
time-domain NMR signal that results from the precession of the nuclear magnetization vector inside the probe coil after application of an excitation RF pulse to a sample in a static magnetic field

3.7
shimming
process that is carried out to correct any inhomogeneities in the applied magnetic field during an NMR experiment

3.8
spectral width
SW
width of the spectrum after Fourier transformation

Note 1 to entry: It is given in Hz or ppm[1)].

Note 2 to entry: The axis for SW (x-axis) of an NMR spectrum is usually expressed as chemical shift in ppm scale. Resonance frequency of an NMR signal depends on the external magnetic field of the NMR instrument. Relationship between the resonance frequency in Hz and chemical shift, δ, in ppm is as follows:

$$\delta = \frac{\nu_s - \nu_r}{\nu_r}$$

where

δ	is the NMR "chemical shift" of an individual signal, typically expressed in ppm scale;
ν_s	is the absolute resonance frequency of the ^{1}H NMR signal for a sample measured in an NMR instrument, given in Hz;
ν_r	is the absolute resonance frequency of the ^{1}H NMR signal for a chemical shift's reference compound signal measured in the instrument, given in Hz.

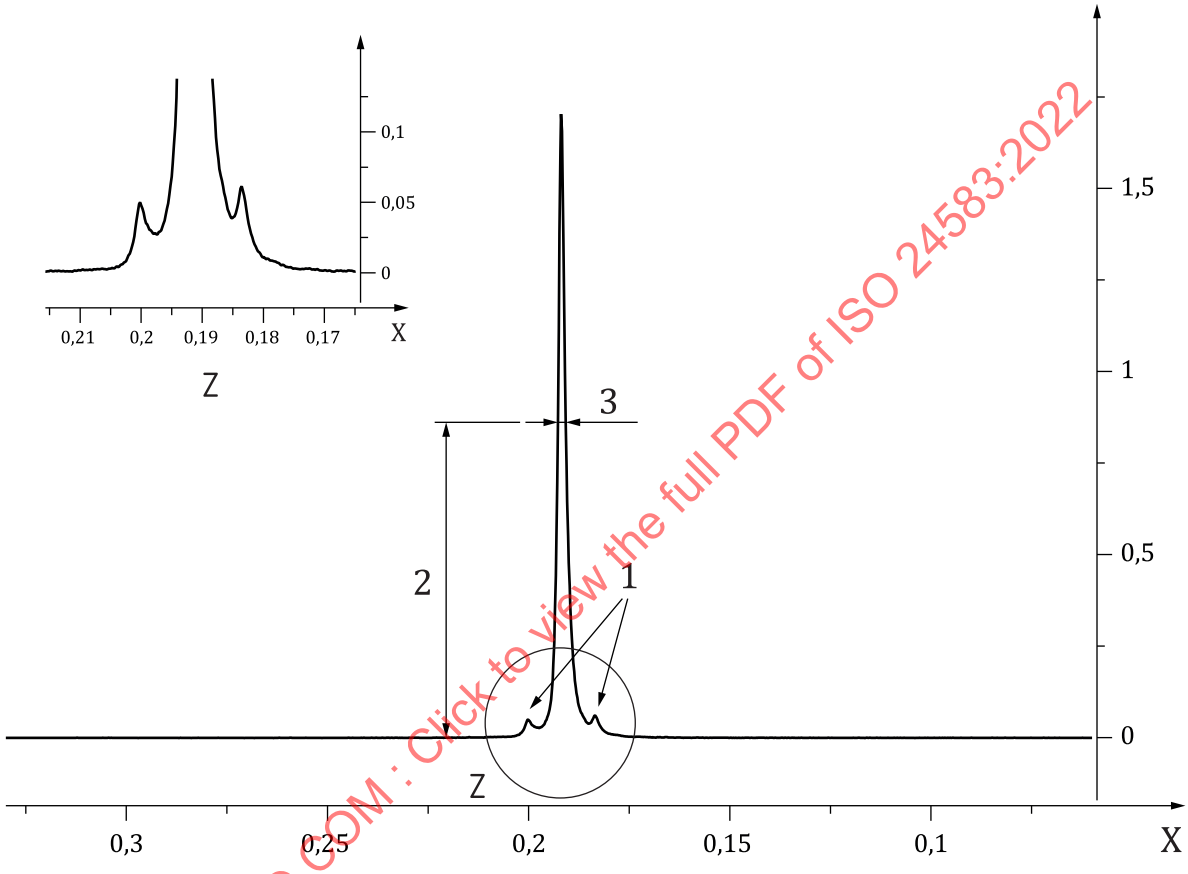
Since the difference in the numerator is usually in Hz and the denominator in MHz, δ is expressed in ppm.

1) ppm = parts per million.

3.9
full width at half maximum
FWHM
width of a line shape at half the maximum signal intensity

Note 1 to entry: It is expressed in Hz.

Note 2 to entry: Figure 1 illustrates FWHM. The example signal is from 1,4-bis(trimethylsilyl)(D_4)benzene (see Table B.1).



Key
X chemical shift (ppm)
Z chemical shift (ppm)
1 ^{29}Si satellite signal
2 half height of signal
3 full signal width at half maximum height

Figure 1 — Illustration of FWHM

3.10
flip angle
pulse angle
pulse flip angle
tilt angle of the bulk nuclear magnetization vector, relative to the static magnetic field after applying an RF pulse of specific duration and amplitude in a static magnetic field at thermal equilibrium

Note 1 to entry: This non-equilibrium magnetization can be induced by applying an RF pulse of sufficient excitation bandwidth and carrier frequency near the Larmor frequency of the nuclear spins.

3.11
repetition time
T_r
time period from the application of the first RF pulse of a pulse sequence until the same pulse is applied again in the subsequent transient

3.12
number of transients
NT
number of scans
number of times that a Fourier transformation-NMR experiment is repeated with the resulting *FIDs* (3.6) accumulated/summed to improve the signal-to-noise ratio of the NMR spectrum

3.13
decoupling
NMR experimental technique to eliminate spin-spin coupling

Note 1 to entry: In this document, only heteronuclear decoupling, e.g. decoupling of ^{1}H-^{13}C spin-spin coupling, is considered.

3.14
dummy scan
steady state scan
transient that is performed to establish a steady state of the magnetization, all parts of the NMR experiment are carried out (e.g. RF pulses, delays, pulsed field gradients), but no data is recorded

3.15
satellite signal
signals arising from fraction of sample containing another NMR active nucleus showing the coupling to this nucleus

3.16
zero filling
insertion of zero values at the end of an *FID* (3.6) signal prior to Fourier transformation, a means to increase the frequency domain resolution of an NMR spectrum

3.17
phase correction
mathematical procedure used to restore a pure absorption lineshape over the whole NMR spectrum

3.18
baseline correction
mathematical procedure used to correct distortions in the baseline of an NMR spectrum

3.19
spectral resolution
degree of distinction and separation of signals used in quantitative analysis

3.20
integrated area
signal area
peak area
integration value of the signal interval between the baseline of the signal and the resonance signal

3.21
minimum weight
smallest sample quantity required for a weighment to just achieve a specified relative accuracy of weighing

[SOURCE: EURAMET Calibration Guide No.18 Version 4.0[9]]

3.22
repeatability
measurement precision under a set of repeatability conditions of measurement

[SOURCE: ISO/IEC Guide 99:2007[5], 2.21]

3.23
calibration
operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication

[SOURCE: ISO/IEC Guide 99:2007[5], 2.39, modified — The notes to entry have been deleted.]

3.24
internal standard
material used as a measurement standard for the purity evaluation for a *qNMR procedure* (3.3) in solution together with a sample

Note 1 to entry: A schematic illustration is given in Figure 2.

3.25
qNMR standard
component of an *internal standard* (3.24) used as the measurement standard for a *qNMR procedure* (3.3)

Note 1 to entry: A schematic illustration is given in Figure 2.

3.26
receiver gain
RG
amplification ratio of the signal by the receiver

3.27
line-broadening
mathematical processing technique by which the *FID* (3.6) is manipulated by exponential function in order to improve the signal-to-noise ratio at the expense of resolution

4 Principles

4.1 General

NMR is one of the most useful techniques for the structure elucidation of organic compounds due to three important features:

a) chemical shifts of resonance signals;

b) spin-spin couplings by neighbouring non-equivalent NMR active nuclei;

c) the proportionality between the integrated area and the number of corresponding ^{1}H nuclei.

The first and third features play an important role in comparing different integrated areas quantitatively. The integrated area is directly proportional to the size of the population of ^{1}H nuclei causing this resonance signal, if the experimental conditions are optimized correctly. Since the integrated areas of two distinct resonances are usually well separated due to their respective chemical shifts it becomes possible to determine the molar ratios of chemical substances or structural moieties giving rise to the signals. In other words, all signals in the spectrum can be assigned to chemical (sub-)structures of the analyte and the qNMR standard. Therefore, if an internal standard, with a certified purity value of identified structure, is added to the sample solution that contains an analyte of known structure,

the purity of the analyte in sample can be determined from the relationship derived in Formulae (3) and (4).

For the i^{th} signal in the ^1H NMR spectrum of a single analyte compound in a sample, a integrated area I_i can be expressed as Formula (1):

$$I_i \propto N_i \frac{m}{VM} P \sin\beta \frac{1-e^{-T_r/T_{1i}}}{1-e^{-T_r/T_{1i}}(\cos\beta)} M_z(t_{eq}) \quad (1)$$

where

I_i is the integrated area of the i^{th} signal of the compound;

N_i is the number of resonating protons for the integrated area of the i^{th} signal (I_i);

V is the volume of the sample solution;

m is the mass of the sample;

M is the molar mass of the compound;

P is the purity (mass fraction) of the compound;

β is the excitation flip angle;

T_{1i} is the spin-lattice relaxation time of the i^{th} ^1H signal of the compound;

T_r is the repetition time;

$M_z(t_{eq})$ is the equilibrium magnetization.

The relaxation times T_{1i} can be different for the ^1H nuclei of different signals.

This formula suggests that N_i and T_{1i} are the only terms that depend on different signals. When $T_r >> T_{1i}$ is met, 1-exp($-T_r/T_{1i}$) becomes unity. Therefore, when T_r for an experimental parameter is set to sufficiently longer than the longest T_1 among signals of interest, the ratio of integrated areas can be proportional to N_i. When this relationship is applied to one compound, all parameters except I and N are common factors. Therefore, the I and N should only be considered. This is the basic principle for the proportionality of integrated areas for one compound that can be summarized in the Formula (2):

$$\frac{I_1}{I_2} = \frac{N_1}{N_2} \quad (2)$$

where

I_1 is the first integrated area of the compound;

I_2 is the second integrated area of the compound;

N_1 is the number of resonating protons for the integrated area of the first signal (I_1);

N_2 is the number of resonating protons for the integrated area of the second signal (I_2).

4.2 Conventions on the sample solutions for the qNMR procedure

In this document, the following conventions are used:

— A sample solution for the qNMR procedure is a mixture of a sample and an internal standard dissolved in a solvent or mixture of solvents.

— The sample (S) consists of a main component, which is the target component for the purity determination, and other components; in this context these are impurities. Hereafter, analyte (A) is referred to as the main component of the sample.

— The internal standard (IS) is a material used as a standard of the qNMR method. The internal standard also consists of a main component for the qNMR method, and other components (impurities). Hereafter, qNMR standard (Q) is referred to as the main component of the internal standard.

Figure 2 is a schematic illustration of the conventions.

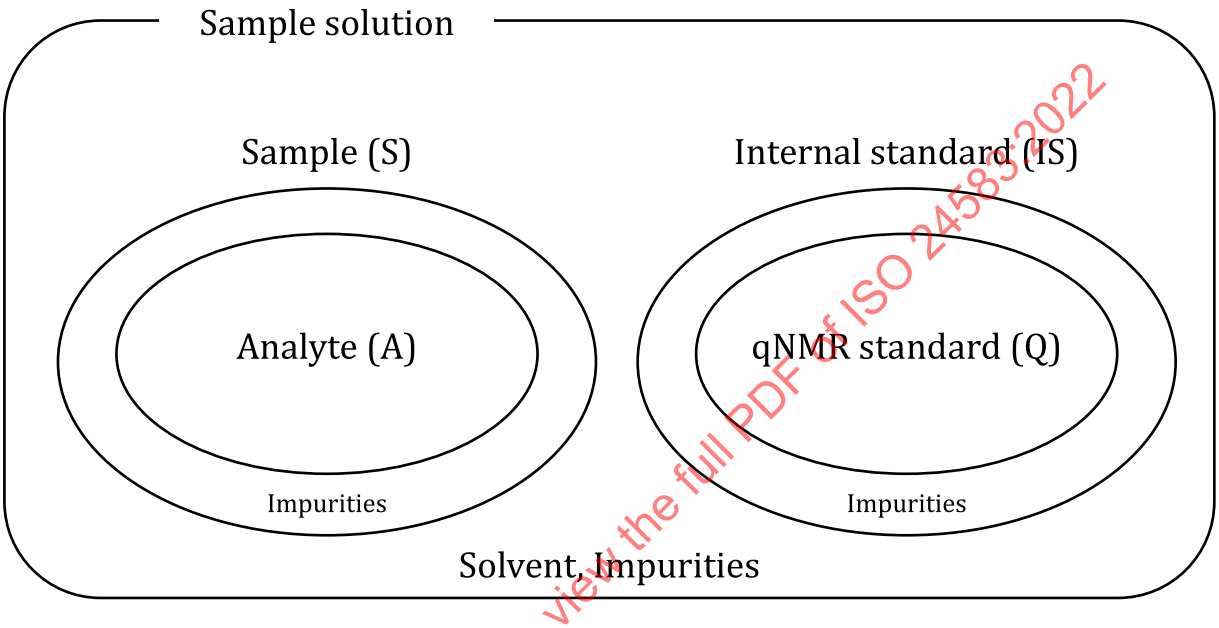


Figure 2 — Schematic illustration of the sample solution

4.3 ^{1}H quantitative NMR (^{1}H qNMR)

Formula (2) can also be applied to signals derived from two compounds in a sample solution. When the purity is determined by qNMR, the two compounds in the sample solution correspond to an analyte in the sample and the qNMR standard in an internal standard. Figure 2 is a schematic illustration of the sample solution for such a case. The sample (S) and the internal standard (IS) both consist of main components and impurities. Since the analyte and the qNMR standard (Q) are the main components, the purities can be expressed by percentages of mass fraction (kg/kg) of the sample and the internal standard.

When the longest T_{1i} satisfies with $T_r >> T_{1i}$ for the two compounds, Formula (3) can be derived from Formula (1):

$$\frac{I_{Q_j}}{I_{A_i}} = \frac{N_{Q_j}}{N_{A_i}} \times \frac{m_{IS}}{m_S} \times \frac{M_A}{M_Q} \times \frac{P_{IS}}{P_S} \tag{3}$$

where

A is the analyte in the sample;

Q is the qNMR standard in the internal standard;

S is the sample;

IS is the internal standard;

I_A is the integrated area of the analyte in the sample (A);

I_Q is the integrated area of the qNMR standard in the internal standard (Q);

N_A is the number of resonating protons for signals originating the analyte in the sample (A);

N_Q is the number of resonating protons for signals originating the qNMR standard in the internal standard (Q);

m_S is the mass of the sample (S);

m_{IS} is the mass of the internal standard (IS);

M_A is the molar mass of the analyte in the sample (A);

M_Q is the molar mass of the qNMR standard in the internal standard (Q);

P_S is the purity (mass fraction) of the sample (S);

P_{IS} is the purity (mass fraction) of the internal standard (IS);

subscript i is the i^{th} signal from the analyte in the sample (A);

subscript j is the j^{th} signals from the qNMR standard in the internal standard (Q).

Formula (3) theoretically indicates that ^{1}H qNMR has the ability to determine the purity of the sample (P_S) through the purity of the internal standard (P_{IS}).

See References [21] and [26] for further information.

5 Technical requirements for the qNMR measurements

5.1 General requirements

When metrological traceability is to be established for the purity values of the qNMR procedures, even if the qNMR results are only for internal use, the laboratory operating the qNMR procedure shall be a competent laboratory.

NOTE 1 Laboratories operating in accordance with ISO/IEC 17025[3] are considered to be competent.

NOTE 2 The qNMR procedure can be used as part of the production process of reference materials, including stability studies and characterization of property values. Reference material producers operating in accordance with ISO 17034[1] are considered to be competent.

5.2 qNMR study design

With the internal standard method, Formula (4) can be derived from Formula (3), which can be used for purity determination:

$$P_{S_{ijkl}} = \frac{I_{A_{ikl}}}{I_{Q_{jkl}}} \times \frac{N_{Q_j}}{N_{A_i}} \times \frac{M_A}{M_Q} \times \frac{m_{IS_k}}{m_{S_k}} \times P_{IS} \quad (4)$$

where subscripts

i and j are the selected analyte (A) signal and the qNMR standard (Q) signal numbers, respectively, from one qNMR acquired data set;

k and l are the numbers of the independent qNMR sample solution preparations and the independent qNMR data acquisitions for each sample, respectively.

The internal standard method requires at least each one isolated signal derived from the analyte (A) (i = 1) and the qNMR standard (Q) (j = 1). If possible, selection and evaluation of two or more signals from each substance can improve the reliability of the purity value. When two or more independent qNMR data sets ($l \geq 2$) for each of two or more independent qNMR sample solutions prepared ($k \geq 2$) are acquired, the measurement quality can be determined using statistical uncertainty evaluation.

For the establishment of metrological traceability of the purity value, uncertainty associated with qNMR procedure shall be evaluated. To provide a reasonable estimate of uncertainty in the purity value evaluated by this method, three or more preparations of sample solutions ($k \geq 3$) shall be prepared and at least three independent qNMR measurements ($l \geq 3$) for each sample shall be conducted for the evaluation of qNMR measurement uncertainty.

NOTE 1 Repeating spectroscopic analysis of a single sample over a short period of time is not necessarily going to provide that number of independent observations.

The following parameters should be considered for obtaining an accurate purity value P_S of the sample:

— determination of accurate mass values (m_S and m_{IS});

NOTE 2 The mass or volume of solvent added does not need to be precisely known to calculate purity via Formula (4).

— determination of accurate integrated areas (I_A and I_Q);

NOTE 3 Signals generated by ^{1}H involved in exchange reactions or molecular dynamics in the NMR timescale (resulting in the broadening of the signal) cannot be used for the purity determination.

— identification of chemical structures to obtain molar mass (M_A and M_Q), and the number of resonating ^{1}Hs for target signals (N_{A_i} and N_{Q_j});

— the uncertainty and metrological traceability of the purity value of the internal standard (P_{IS}), as well as the methods used to determine this value.

NOTE 4 Measured purity values are metrologically traceable to a reference through the purity value of the internal standard (P_{IS}). The metrological traceability of the purity value of the internal standard (P_{IS}) is an important consideration for determining whether it is suitable for the purposes of the measurement.

NOTE 5 Certified purity values of CRMs fulfilling the requirements of ISO 17034[1] are considered to be metrologically traceable to a higher reference.

5.3 Analytical target profile

To obtain purity values appropriate for the application, the following attributes and target values shall be determined and documented prior to starting the qNMR procedure:

— intended use of the purity value;

— intended level of target measurement uncertainty (u_t) of the purity value;

— necessity for the establishment of metrological traceability of the purity value.

NOTE Intended level of u_t can be different for different intended use.

Metrological traceability of the purity value shall be established when the intended use includes calibration purposes.

Based on the predetermined target values, it shall be specified which of the following steps the analyst shall comply with and what level of uncertainty, if applicable, can be accepted. To meet the predetermined targets, the following points should be followed;

— feasibility studies for setting up the measurement procedures for each sample (see 5.4);

— mass evaluation of an internal standard and the sample (see 5.5);

— consideration of qNMR data acquisition (see 5.6);

— consideration of qNMR data processing (see 5.7);

— where applicable, evaluation of measurement uncertainties (see 5.8);

— where applicable, establishment of metrological traceability (see 5.9).

5.4 Feasibility study

A feasibility study shall be conducted during the early stages of the qNMR procedure development. One or more signals from the analyte, and one or more signals from the qNMR standard should be selected. These signals should not interfere with any other signals such as those from solvent or impurities. In order to achieve the intended results, the following points should be checked:

— measurement uncertainty of the balance (available from the balance calibration certificate);

NOTE 1 The balance used is appropriate for the amount of sample and internal standard weighed, e.g. use of a microbalance rather than a balance with coarser resolution.

— measurement uncertainty in use when weighing the sample and the internal standard;

NOTE 2 This uncertainty is usually derived from the uncertainty of the balance at calibration by adding additional terms that characterize the actual usage of the instrument during routine operation,[9][14] and is compared with the target weighing uncertainty requirement.

NOTE 3 Minimum weight which is calculated from the uncertainty in use[9][14] is a good measure for the choice of an appropriate amount of sample and internal standard to fulfil the target weighing uncertainty requirement.

— solubility that ensures complete dissolution of all components;

NOTE 4 Purity results obtained through use of different solvents can be used for the consideration of the solubility.

NOTE 5 See A.2.1 for the explanations.

— existence of one or more isolated signals of the analyte and the qNMR standard;

NOTE 6 Impurities can interfere with the purity determined by qNMR when their signals overlap with those originating from the analyte and/or qNMR standard.

— stability of the solution for the duration of time required for qNMR measurements;

— optimization of the acquisition and processing parameters;

— the signal-to-noise ratio (SNR) of the target signals is high enough to achieve adequate accuracy.

NOTE 7 The relationship between SNR and uncertainty is discussed in detail in References [17], [34] and [37].

The uncertainty associated with the qNMR measurement repeatability should be considered to fulfil u_t.

The ratios of the integrated areas between the analyte and the internal standard should be within the validated conditions.

5.5 qNMR sample solution preparation

Based on the results of the feasibility study, a detailed procedure for the sample preparation should be defined and documented. A calibrated balance shall be used for the gravimetric preparation of the sample and the internal standard. All the weighing information and amount of solvent added for the qNMR sample solution preparation shall be recorded. For each of the qNMR sample solutions prepared, independent observations of qNMR measurements shall be conducted.

When establishing metrological traceability, the internal standard used shall be a CRM with a certified value for chemical purity. A minimum of triplicate qNMR sample solutions shall be prepared.

Based on the desired target uncertainty of the purity, a desired target uncertainty for the mass of the weighed sample and the internal standard shall be determined and documented. To achieve this desired target uncertainty, the mass of the sample and the internal standard should exceed the respective minimum weight. The minimum weight is derived from the measurement uncertainty of the balance.[9][14][22]

NOTE 1 When the mass of the weighed material corresponds to the minimum weight, its uncertainty equals the desired target uncertainty for that mass. See A.2.2 for further information.

Special care should be taken when preparing samples or standards of a hygroscopic or volatile nature to ensure that potential mass changes are negligible with regards to the uncertainty of the mass determination.

The sample and the internal standard shall be dissolved completely into an appropriate amount of NMR solvent/solvents.

It is not necessary to measure the accurate mass or volume of solvent added.

NOTE 2 Adding more solvent does not change the mole ratio of the sample and the internal standard.

NOTE 3 If the concentration of the sample solution is too high, NMR signal broadening, RF receiver saturation, radiation damping or other effects can occur. Adding more solvent to lower the concentration can resolve these problems. However, if the concentration is too low, it can lead to inadequate accuracy in determining purities caused by too low SNR.

NOTE 4 To ensure an adequate fill height of the sample solution in the tube, refer to the manufacturer's recommendations.

5.6 qNMR data acquisitions

5.6.1 Acquisition parameters

All replicates of a qNMR sample shall be measured using the same set of acquisition parameters.

Based on the desired target measurement uncertainty of the purity of the sample, u_t, an allowance for a possible bias shall be determined and documented. T_r, a key parameter to dictate bias of the qNMR signal intensity which directly affects the integrated area, can be determined based on T_1^{max}, the longest T_1 among the target signals. The T_r for the sample solution shall be determined with the consideration for achieving the u_t.

T_r is a time period from the application of the first pulse of a pulse sequence until the next pulse is applied. The relationship between the T_r/T_1 ratio and the flip angle of the excitation pulse is shown in Table 1. For example, if the excitation pulse is set to induce a 90° flip angle and T_r is five or seven times T_1^{max}, then the maximum theoretical bias will be 0,67 % or 0,091 %, respectively.

NOTE 1 The actual bias depends on the T_r/T_1 value of the corresponding NMR signal intensity used for the purity calculation.

Table 1 — Bias of NMR signal intensity (%) due to incomplete recovery of the equilibrium magnetization — Relationship between T_r/T_1 and flip angle of excitation pulse

T_r/T_1	Flip angle of excitation pulse		
	90°	60°	30°
1	36,79	22,54	7,23
2	13,53	7,26	2,05
3	4,98	2,55	0,70
4	1,83	0,92	0,25
5	0,67	0,34	0,091
6	0,25	0,12	0,033
7	0,091	0,046	0,012
8	0,034	0,017	0,004 5
9	0,012	0,006 2	0,001 7
10	0,004 5	0,002 3	0,000 6

The following parameters shall be optimized accordingly and shall be documented:

— Measurement temperature: It is recommended that the stability of the sample temperature should be governed by a precision temperature controller that is part of the NMR spectrometer. If the spectrometer is not equipped with such a controller, the temperature of the room where the NMR instrument is installed should be controlled to be constant during the measurements.

— Spectral width (SW) and pulse offset: The SW shall be at least twice the chemical shift difference of the two most extreme signals of the entire spectrum. It is recommended to set the pulse offset, or the centre position of the SW, near the centre between the two target signals. If more than two signals need to be evaluated a possible bias due to a non-uniform excitation shall be investigated during method development.

Additionally, at least the following parameters should be optimized and documented:

— Flip angle.

NOTE 2 A 90° excitation pulse is often used. The 90° flip angle offers maximum signal intensity but needs five to seven times T_1^{max} for > 99 % relaxation. A 30° excitation pulse gives 50 % of the maximum intensity but takes only three to four times T_1^{max} to > 99 % relaxation. Thus, although fewer scans can be accumulated over time using the 90° pulse, a better SNR will be obtained in a fixed amount of experiment time.

— Use of sample spinning (if necessary).

Sample spinning should be excluded from qNMR measurements. When spinning is used, it shall be carefully checked if the spinning sidebands interfere with any real signals. The spinning sidebands are part of the main signal. Hence, they should be considered for the choice of the integral regions.

When strong spinning sidebands (i.e. at the level of ^{13}C satellite signals or larger) are visible in the spectrum, optimization of the off-axis shims can reduce the intensities of the sidebands. The benefits should be weighed against the possible artefacts when deciding about the usage of sample spinning.

— Use of ^{13}C heteronuclear decoupling during acquisition time.

The ^{13}C satellite signals, which are part of the main signal, can be overlapped with other real signals. Using ^{13}C heteronuclear decoupling can help to resolve such an overlap. On the other hand, ^{13}C heteronuclear decoupling can induce decoupling sideband artefacts or can degrade the spectral resolution by heating the sample. The benefits should be weighed against the possible artefacts when deciding about the usage of ^{13}C heteronuclear decoupling.

When ^{13}C decoupling is executed, the acquisition time (AT) should be long enough to achieve a sufficient number of data points and using a decoupler power level that is low enough to prevent

damage of the probe, e.g. this is readily achieved through bilevel adiabatic composite pulse heteronuclear decoupling.

— Number of transients (NT).

NOTE 3 When the SNR of the target signals is not sufficient, there are two options for improving the SNR. Doubling the SNR (2 × SNR) can be achieved by increasing the NT by a factor of four (4 × NT). The SNR can be improved by preparing more concentrated sample solutions.

— Number of dummy scans.

NOTE 4 One or more dummy scans are usually applied prior to actual data acquisition.

— Real part data points of the complex acquisition (AP).

NOTE 5 AP is determined by AT in seconds and SW in Hz as AP = AT × SW. Reference [26] indicates that samples need an AT of 2 s to 4 s. In cases of samples with very narrow signal linewidths, a further increase of the AT can be useful.

— Receiver gain (RG):

NOTE 6 If the RG is set too high, the incoming signal will be clipped in the analogue digital converter (ADC). This will result in spectral artefacts and render the spectrum unfit for quantitative evaluation. If the RG is set too low, the dynamic range of the NMR receiver is not fully utilized. To obtain proper spectral information, consider optimizing the RG prior to every qNMR experiment. See References [17], [21], [26] and [31] for further information.

5.6.2 qNMR data acquisition

All the acquired data shall be recorded as free induction decay (FID) data. Replicate qNMR data should be acquired independently, which involves the following steps:

— insert the sample solution into the NMR instrument;

— wait enough time for the temperature equilibration of qNMR sample solution in the NMR instrument if the temperature needs to be static;

— tune and match the probe;

— lock and shimming the NMR instrument for the sample solution;

— acquire qNMR data with the parameters shown in 5.6.1;

— eject the sample solution from the NMR instrument.

NOTE When several data acquisitions are repeatedly conducted without ejecting the sample, they are not considered as independent qNMR measurements since shimming conditions cannot be independently set.

5.7 Data processing

5.7.1 Data processing parameters

The spectral processing parameters shall be consistent for each NMR analysis, except for the parameters used for phase correction.

The following steps should be followed for the data processing of qNMR data:

— perform zero-filling at least once to double the FID data points;

— perform a Fourier transformation of the FID data;

— perform a phase correction for the resulting spectrum;

— reference the chemical shift with an appropriate reference signal, e.g. solvent signal;

— determine the integration interval for each target signal;

— record the integration interval for each target signal and corresponding resonating ^{1}Hs;

— determine the baseline for each target signal integration interval;

— perform a baseline correction;

— record the integrated area of each target signal for all qNMR data.

Where possible, two or more signals, especially for the analyte signals, should be determined when the integration interval for each target signal is determined.

To improve the spectral shape or resolution, apodization can be applied to FID data prior to Fourier transformation. Depending on the function used, the analytical framework remains quantitative or not in relation to how this function affects not only the signal shape but also the integrated area. Therefore, the resort to apodization in a quantitation perspective shall be substantiated and/or validated beforehand. Unless validated, an apodization function shall not be applied for the qNMR data set.

NOTE A line-broadening of up to 0,3 Hz by apodization (exponential multiplication) can enhance the SNR without a relevant loss of signal narrowness.[31][32][33][34]

NMR signals present a Lorentzian shape. In theory, to capture over 99,9 % of the whole signal requires an integration range of at least 76 times full width at half maximum (FWHM) for the selected signal. If this is impossible due to neighbouring signals, a sensible (narrower) integration range around the peak of interest should be selected. In any case, to avoid bias, the integration ranges for all the different integrals shall be set in a consistent way. The ratio of the integral range width to the signal's FWHM should be the same for all signals, and not less than ×25 for 99 % recovery or eventually ×10 for 97 % recovery.[30]

It is important to take into consideration the existence of ^{13}C-satellite signals, which provide approximately 1,1 % of an integrated area.[26] ^{13}C satellite signals shall be either included or excluded for all signals. Another solution can be the implementation of ^{13}C decoupling during acquisition.

When a combination of signals is integrated into one interval, care should be taken not to integrate too much unwanted noise. With an appropriate baseline correction, both ends of the integral intervals should be adjusted to the zero level of the spectrum. Baseline correction inside of the integral intervals can cause bias of integrated areas.

See References [17] and [27] for further information.

5.7.2 Purity calculation

Purity shall be calculated according to Formula (4). The mean of purities derived from individual resonance signals is often used as the purity value of the analyte in the sample.

Anomalous purity data found shall be investigated, and the adopted measures for the result of the investigation shall be recorded.

Even when neither metrological traceability nor uncertainty evaluation is necessary, the individual purity values should be checked for any unexpected results (e.g. outliers, high variances or significant deviations from expected values) before calculating the mean of the individual values. If anomalous purity data are observed, the point in question should be investigated. The investigation method and probable explanation should be recorded.

NOTE All the recorded data sets and documents can be helpful to technically and explicitly explain the robustness of results.

5.8 Evaluation of measurement uncertainty

The measurement uncertainty associated with the qNMR measurement result, P_S, should be calculated according to the rules of propagation of uncertainty. An appropriate uncertainty budget sheet should be established and recorded.

When measurement uncertainty is evaluated for the establishment of metrological traceability, the uncertainty sources shall be identified, and their contribution shall be evaluated. At least, the following uncertainty sources shall be evaluated:

— uncertainty associated with repeatability of the purity from one pair of an analyte signal and an qNMR standard signal;

— where possible, uncertainty associated with the purity among different pairs of analyte and qNMR standard signal/signals;

— uncertainty associated with the purity from multiple qNMR sample solution preparations, including measurement uncertainty for weighing the sample and the internal standard;

— uncertainty associated with the purity value of internal standard used.

In addition to these uncertainty sources, the following can be considered:

— uncertainty associated with the molar mass;

— uncertainty associated with the number of resonating nuclei;

— uncertainty associated with other qNMR parameters.

The allowance for a possible degree of purity should be converted to a level of uncertainty. Then it should be evaluated and added as an uncertainty source.

NOTE 1 Examples of the evaluation of uncertainty can be found in Annex C.

NOTE 2 The accuracy profile method can also be considered as an alternative to propagation of uncertainty. [35]

NOTE 3 Triplicate or more qNMR data sets for each sample can provide repeatability to evaluate measurement uncertainty. Triplicate or more sample preparation can provide measurement uncertainty associated with sample preparation.

5.9 Establishment of metrological traceability

With the qNMR procedure, metrological traceability can be established. When metrological traceability is to be established, the records shall be documented taking into account the following items:

— evidence of metrological traceability of the purity value for the internal standard used;

— evidence of calibration for the balance used for qNMR sample solution preparation;

— records of validation data;

— measurement uncertainty budget for the qNMR measurement result.

6 Validation of the qNMR procedure

The qNMR procedure should be validated to fulfil the intended accuracy based on the purpose. All the records for the validation results should be recorded. The validation should be conducted periodically. The intervals should be determined and documented prior to the implementation of qNMR procedures.

An independent and individual validation for all samples would be excessive and inefficient. Instead a representative validation using two CRMs (with certified known chemical purities) which treats them

as a validation sample "S_v" and validation internal standard "IS_v", can be used for the validation of the entire qNMR procedure. The observed absolute difference between the property (purity) value of S_v stated on a reference material document, p_{Sv}, and the measured purity value of S_v by the qNMR procedure using the IS_v, $p_{S_{vqNMR}}$, should be smaller than the combined standard uncertainty of respective uncertainties.

$$\left| p_{S_{vqNMR}} - p_{S_v} \right| \leq k\sqrt{u^2_{S_{vqNMR}} + u^2_{S_v}} \tag{5}$$

where

p_{S_v} and u_{S_v} are the purity and its standard uncertainty of the validation sample to be evaluated obtained from information including the reference material document;

$p_{S_{vqNMR}}$ and $u_{Sv_{qNMR}}$ are the purity and its standard uncertainty for the validation sample evaluated by qNMR using p_{IS_v} and u_{IS_v} of the IS_v as the validation internal standard for the qNMR procedure;

k is the coverage factor at a level of confidence of approximately 95 %.

NOTE 1 The reference material document is a document containing all the information that is essential for using any reference material. The reference material document covers both the product information sheet and the reference material certificate.

If the condition shown in Formula (5) holds, then the measured and property values are consistent with one another within their respective uncertainties (see ISO Guide 33[2]).

NOTE 2 ISO/IEC Guide 98-3[4] provides information to calculate the *k* factor for a defined level of confidence.

The validation shall be conducted at the predetermined and documented intervals. Two materials of the sample (S) and the internal standard (IS) for the validation of the qNMR procedure shall be both CRMs certified for chemical purities, and Formula (5) shall be satisfied.

See References [20], [28] and [29] for further information.

7 NMR instrument qualification

A qualification procedure shall be determined, which ensures stable and suitable conditions for qNMR measurements and which demonstrates that the instrument is fit for purpose.

NOTE 1 The manufacturer's recommended test methods can be useful.

The documentation of the qualification procedure shall include at least:

— the intervals at which the tests are to be performed;

— the test conditions, including the definition of the sample, measurement and processing parameters, and assessment procedure;

— the acceptance criteria to define the minimum requirements the instrument shall fulfil.

At least, a test for the spectral resolution shall be included in the procedure.

If necessary, other factors can be considered.

NOTE 2 Reasonable acceptance criteria can be established by referring to the manufacturer's installation reports.

NOTE 3 Qualification and system suitability tests are given in in several documents, e.g. ASTM E2977-15[15], JIS K 0138[11].

8 Test report

The test report shall include the following information:

— a title (e.g. “Test Report”, “Calibration Certificate” or “Report of Sampling”);

— the name and address of the laboratory;

— the location of performance of the laboratory;

— unique identification that all its components are recognized as a portion of a complete report and a clear identification of the end;

— the name and contact information of the customer;

— identification of the method and/or procedure used;

— a description, unambiguous identification, and, when necessary, the condition of the sample;

— the date(s) of performance of the test;

— the date of issue of the report;

— a statement to the effect that the results relate only to the samples tested;

— the International Standard used;

— the results, including a reference to the clause which explains how the results were calculated, with, where appropriate, the units of measurement;

— additions to, deviations, or exclusions from the method and/or procedure;

— any unusual features observed;

— identification of the person(s) authorizing the report;

— clear identification when results are from external providers.

Annex A
(informative)

Weighing of sample and internal standard

A.1 General

For ^{1}H qNMR measurements, the amounts of the sample and the internal standard to be weighed are usually quite small (milligrams). Since the measurement of mass is one of the factors that affects the measurement uncertainty of the analysis, special attention should be paid to handling procedures and the operation of the balance.

A.2 Handling of the balance

A.2.1 General

Select a balance that is suitable for the intended purpose, taking into consideration the measurement uncertainty requirements, external regulations, productivity, etc. Handling of a balance is also described in References [7], [8], [13] and [16].

A.2.2 Weighing performance

The important parameters of a balance that affect the weighing performance of the balance are the following:[18]

a) maximum capacity;

b) readability (scale interval);

c) repeatability;

d) sensitivity;

e) linearity deviation (nonlinearity);

f) eccentric loading error;

g) sensitivity temperature drift;

h) minimum weight.

The smaller the relative target measurement uncertainty (in per cent), the larger the minimum weight, i.e. the more sample and internal standard have to be weighed to meet the uncertainty requirement. The literature provides guidance to calculate minimum weight from the calibration results.[9][14][22]

NOTE For analytical and micro-balances which are usually used for weighing the sample and the internal standard for qNMR, the dominant contribution factor to measurement uncertainty is repeatability. An approximated calculation of minimum weight can therefore be done using the standard deviation of a series of replicate measurements, see References [12] and [13]. The approximated minimum weight can be derived as:

$$m_{\min} = k \times \frac{S_r}{u_{\mathrm{rt}}}$$

where

k is coverage factor to achieve a 95 % coverage probability;

s_r is standard deviation of not less than 10 replicate measurements with a test weight;

u_{rt} is the relative target measurement uncertainty.

A.2.3 Installation environment

The balance location should be free from vibrations and air currents which strongly influence the stability of the weighing values. To achieve that, the balance should ideally be installed in a room with limited space on the ground floor (or underground). It should be positioned in a place with stable environmental conditions and the weighing table should not be connected to walls. The corner of a room or near the mainstay of the building, where the influence of vibration is assumed to be low, are preferable places to locate the balance. The weighing table (shock-absorbing table) used to support the balance should be sufficiently heavy and robust enough to ensure that there are no vertical distortion or displacement effects, even when an object is placed on the weighing pan. The balance should not be exposed to direct sunlight (or heat from indoor lighting). The balance should also not be exposed to any types of electric or electromagnetic charges. The balance should not be exposed to magnetic fields. Specifically, it should be ensured that the magnetic fields of the NMR apparatus do not interfere with the balance. It is strongly recommended to install the balance in a separate room as per the considerations above. Should this not be possible, the user needs to test that the balance indication is not influenced by the NMR apparatus. Particularly for balances with a readability (scale interval) of 0,1 mg or less, imperceptible tremors can be conveyed to the sample, or affect the weighing sensors, causing instability in the displayed values. This necessitates exercising due caution in installing or moving the balance.

The environment around the balance should also be maintained in order to prevent sudden temperature or humidity changes, and maintenance of the environment will also help to eliminate dropwise condensation, which can cause degradation of components of the balance. Since balances are electronic devices, the installation environment should be maintained at a temperature between 5 °C and 40 °C, and a relative humidity between 20 % and 80 %. Check the manufacturer's manual to verify the installation instructions. If the effects of static electricity are considered, it is recommended to set the relative humidity range between 40 % and 60 %.

If the balance is moved to a different location for use, it can be considered to perform a sensitivity adjustment and recalibration, since gravitational acceleration at the new location is different from the previous location. Unlike a mechanical balance, where measurements are made at the equilibrium position of loads on two weighing pans, an electronic balance uses the relationship between an electromagnetic force and the force of gravity to determine the sample mass.

A.2.4 Operational checks

A.2.4.1 General

Check the items given in A.2.4.2 to A.2.4.4 before using a balance.

A.2.4.2 Warm-up period

After powering on, allow a necessary amount of time before using the balance so that the measurement cell gets into a thermal equilibrium. Typically, for balances with a readability (scale interval) of 10 mg or more, allow a warm-up period of at least 30 min. For balances with a readability of 1 mg or less, allow at least 2 h. For balances with a readability of below 0,01 mg, allow at least a day. Consult the manufacturer's manual for the appropriate warm-up period. It is recommended to have the balance powered on continuously in order to exclude human errors and to expedite the sample preparation.

A.2.4.3 Levelling

It is important to level balances correctly: most balances have a bubble level which shall be brought to the centre by adapting the feet of the balance. Some balances are equipped with an electronic levelling system. The balance needs to be adjusted after levelling. This can be done using built-in weights (if available) or external calibrated weights.

A.2.4.4 Sensitivity adjustment

Balances show a drift of sensitivity as a function of temperature changes and as a function of time. The higher the resolution (number of digital increments) of a balance is, the larger the sensitivity drift can become. Therefore, a periodic sensitivity adjustment is advisable. With the sensitivity adjustment function of a balance (built-in adjustment weights), it is possible to perform an automated sensitivity adjustment at the current ambient temperature between zero point and nominal capacity without interference of a user. For a balance that is not equipped with a built-in adjustment function, a sensitivity adjustment can be performed manually using a calibrated reference weight with a nominal value near the maximum capacity of the balance. In this case, caution and proper handling by a trained user needs to be executed to avoid the balance being de-adjusted.

A.3 Specific considerations

A.3.1 General

Important points to be considered when weighing are given in A.3.2 to A.3.7.

A.3.2 Preparation

Prepare the work environment so that it is easy to perform any task, e.g. by arranging the sample material and handling tools where they can be easily reached. Since the body temperature or exhaled breath of the analyst causes air convection into the windscreen containing the balance or around the weighing pan, the analyst should consider wearing gloves, a coat with long sleeves, and where appropriate, a mask and be attentive to the laboratory clothing when weighing.

A.3.3 Handling volatile, hygroscopic or electrically charged samples

Readings should be taken after the balance is stabilized. For samples that are volatile and/or hygroscopic, phase change can cause instability in measurement results. Electrostatically charged samples can equally cause instability. In that case, use appropriate balance accessories to minimize the instability, while monitoring the environmental conditions in which the balance is used.

A.3.4 Recording the measured value

Readings should be taken after the balance is stabilized. For cases where a balance reading is not stable, a readout time should be established, ensuring that the measured value is read and recorded after a fixed time interval by all analysts.

A.3.5 Environmental conditions

Ambient temperature and humidity should be recorded. It should be ensured that they do not change during the weighing procedure to avoid an influence on the measurement result.

A.3.6 Cleanliness

Regular checks for cleanliness should be made to prevent any unintended materials from being incorporated into the weighing procedure. The balance should be disassembled easily. The weighing pan and weighing chamber should be regularly and thoroughly cleaned using a glass cleaner and lint-free cloth.

A.3.7 Eliminating external influences on the measurement results

A.3.7.1 General

It is recommended that major sources of external influence on the measurements are eliminated as much as possible. If there are special circumstances that make it impossible to completely eliminate an external influence or there seems to be a significant dependence on the measurement results, then

consider making corrections to the measured values. Typical examples of sources of external influence are given in A.3.7.2 to A.3.7.5.

A.3.7.2 Temperature difference between the sample (including the container) and the area around the weighing pan

There can be a difference between the temperatures of a sample and the area around the weighing pan due to factors such as the sample being brought in from another room, or from refrigerated storage, heat processing, the physical properties of the sample, or the conduction of body heat. If the temperature of the sample and the container is higher than the temperature inside the measuring chamber, an upward air flow will be generated around the weighing pan, creating a force that pushes the sample upward, thereby reducing the measured value or causing instability. If the temperature relationship is reversed, the relative force is also reversed, which increases the measured value or causes instability. Since these are physical phenomena occurring in the vicinity of the weighing pan, they cannot be avoided, even when the balance is contained within draft shields. For this reason, if possible, measurements should be performed with the sample at the same temperature as the inside of the draft shield and the container.

A.3.7.3 Drafts (such as those from an air conditioner)

Since air currents caused by heating or cooling equipment, or by the actions of the analyst performing the measurements in the room, can cause instability in the values displayed on the balance if the air flows directly impact upon the weighing pan, due consideration should be given to the airflow direction of the heating or cooling equipment and the weighing procedure. If the balance is equipped with doors which, when opened, will allow direct air current to enter and impact upon the balance directly, do not open these any wider than is necessary.

A.3.7.4 Static electricity

For a sample that is susceptible to being charged due to friction, such as a powder, when the relative humidity is lower than 40 %, static electricity can be generated in the sample container and inside the measuring chamber, causing upward or downward reading fluctuations and errors in the measurement values due to interactions between the generated electrical charge and the balance. Weighing of such samples, therefore, requires special consideration. If it is not possible to maintain an environment with a higher relative humidity, or to use an anti-static container, or if there is not enough time to wait for the accumulated charges to dissipate, then it is recommended that weighing is done after removing as much of the electrostatic charge as possible from the sample and the container with the use of an ionizer or some other tool to promote charge dissipation. Avoid using devices that cause direct air currents on the weighing pan as this causes instability in the values displayed by the balance.

A.3.7.5 Density of sample

Using Archimedes' principle, when the sample being measured has a density that is significantly different from the 8,0 g/cm^3 density of the standard weight used to perform the sensitivity adjustment of the balance, the buoyant force results in a bias in the displayed values and measurement results. If necessary, calculate the air density from the ambient temperature, relative humidity and air pressure, and correct the measurement results to take this buoyancy effect into account.

A.4 Routine operation

Performance checks on the balance are performed periodically in-between calibrations according to an established operating procedure. Usually the risk of the application, i.e. the consequences of inaccurate weighings, and the required weighing accuracy, determine the frequency of performance checks. Control charts can equally be used to determine the frequency of performance checks. The

reference weights used for the performance checks should be metrologically traceable and periodically re-calibrated. The performance checks should assess the systematic and random error of a balance:

a) The systematic error (bias) should be assessed using a reference weight around the nominal capacity of the balance or the maximum weighing range.

b) The random error (repeatability) should be assessed with a reference weight of around 5 % of the nominal capacity. Alternatively, it can be assessed with a weight close to the expected minimum weight, however great care has to be taken by the operator in this case as the handling of very small weights is error prone and can jeopardize the assessment.

In addition to testing weighing instruments with external reference weights, it is accepted practice to adjust the instruments by means of built-in weights. This makes it possible to reduce the frequency of tests with external reference weights which assess the systematic error of the balance.

See References [7], [13], [14] and [19] for further information.

Annex B
(informative)

Internal standards and solvents

B.1 General

For qNMR using the internal standard method that is shown in 5.2 (Formula (4)), the selection of an internal standard and the solvent is one of the important steps as the sample should be dissolved in the solvent along with the internal standard. In this annex, the selection criteria for an internal standard and solvent for an analyte are explained. Since there is no single internal standard that is suitable for use with all target analytes, it is necessary to understand the characteristics of the various internal standards available and to choose the most suitable internal standard when considering the solubility and stability of the sample and the internal standard.

B.2 Selection of internal standards

B.2.1 General

The sample (S) and the internal standard (IS) both consist of main components and impurities. Since the analyte and the qNMR standard are the main components, the contents are represented by percentages of purities (mass fraction) of the sample (P_S) and the internal standard (P_{IS}).

When selecting an internal standard, consider the factors described in B.2.2 to B.2.6.

B.2.2 Purity

The higher the purity of the internal standard, the fewer the potential interferences will be from impurity signals. There can be many impurities present when the purity is low, thus signals originating from the impurities have more chance of overlapping with the analyte signals, making it more challenging to measure the integrated areas (I). Even when weighing was conducted accurately in the sample preparation procedure, a bias in the integrated areas due to overlap, can result in a bias in the resulting purity value.

To check for impurities in the internal standard, several spectra of the internal standard spectra using different solvents with the same parameter set for the qNMR measurements can be acquired.

2D NMR experiments can be helpful to check for any overlapping signals of impurities in some cases.

B.2.3 Properties

Ideally, the internal standard should not be volatile, sublimable or hygroscopic. It is difficult to achieve stable weighing results for these materials.

NOTE Hygroscopic materials can be stabilized in their hydrate formation.

B.2.4 Signal position

The signal originating from the internal standard should not interfere with the analyte signal. At least one signal from the analyte and the internal standard shall be sufficiently separated from any other signal.

NOTE There is often no signal near 0 ppm.

B.2.5 Signal shape

For an internal standard, the ideal signal shape is a sharp singlet signal. If the signal is a multiplet due to spin-spin coupling, it shall be considered that the signal range is wider and the SNR is lower as compared to a singlet signal. The simple FWHM factor rule cannot be applied and the integral range shall be chosen with care.

B.2.6 Solubility and stability in a solvent

Solubility in a solvent and stability in the solvent are important.

For reference, Table B.1 gives examples of available materials and standard solutions that are useful as internal standards for qNMR. The table also contains their properties, suitable solvent types as well as the chemical shifts of signals. Some internal standards for qNMR measurement can also be found on the website of the Bureau International des Poids et Mesures (BIPM)[6].

Table B.1 — Example of internal standards for the qNMR procedure

Name	State of matter	Suitable solvent	Chemical shift[a] (ppm)
Sodium 3-(trimethylsilyl)-1-(D_6)propanesulfonate (DSS-d_6)[e]	Solid	Aqueous/organic solvent (e.g. deuterium oxide, (D_6)dimethyl sulfoxide, (D_4)methanol)	0,0
DSS-d_6 standard solution (deuterium oxide solution)[b,e]	Solution	—	0,0
3-(Trimethylsilyl)(D_4)propionic acid sodium salt (TSP-d_4)[e,g]	Solid	Aqueous solvent (e.g. deuterium oxide)	0,0
1,4-Bis(trimethylsilyl)(D_4)benzene (1,4-BTMSB-d_4)	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_6)acetone, (D_4)methanol, (D_3) acetonitrile, (D_2)dichloromethane)	0,2
1,4-BTMSB-d_4 standard solution ((D_6) dimethyl sulfoxide solution)[b]	Solution	—	0,2[f]
1,4-Bis(trimethylsilyl)-2,3,5,6-tetrafluorobenzene	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_6)acetone, (D_4)methanol, (D_3) acetonitrile, (D_2)dichloromethane, (D_8) toluene, (D_6)benzene, (D_8)tetrahydrofuran)	0,4

[a] Chemical shifts can vary slightly, depending on the measurement conditions, such as the type of deuterated solvent used, the sample concentration and solution temperature during the measurement.

[b] The standard solution containing a fixed amount of the reference material prepared for qNMR measurement is available. When the standard solution is used for the preparation of a qNMR sample solution, it should be ensured that an accurate amount of the qNMR standard is evaluated. For achieving this, preparation can be either volumetric or gravimetric. The gravimetric preparation is more accurate than the volumetric preparation because density depends on the temperature that is an additional source of uncertainty.

[c] This is the value of deuterium oxide solution.

[d] This is the value of (D_6)dimethyl sulfoxide solution.

[e] Great care should be taken when selecting an internal standard from these candidates of internal standards and it should be carefully evaluated that the selected internal standard has no interaction with the sample in the solution.

[f] In order to simplify the qNMR measurement and the interpretation of qNMR spectra, the chemical shift of 1,4-BTMSB-d_4 has been set at 0 ppm in Japanese pharmacopeia 17 (JP17)[10].

[g] This is a deliquescent material.

Table B.1 *(continued)*

Name	State of matter	Suitable solvent	Chemical shift[a] (ppm)
Thymol	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_4)methanol, (D_3)acetonitrile)	1,2 2,2 3,2 6,6 6,7 7,1
Dimethylmalonic acid	Solid	Aqueous/Organic solvent (e.g. deuterium oxide, (D_6)acetone)[c]	1,4
Ethyl 4-dimethylaminobenzoate	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_4)methanol, (D_3)acetonitrile)	1,4 3,0 4,3 6,7 7,9
2,3,5,6-Tetramethyl-*p*-benzoquinone (Duroquinone)	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_4)methanol, (D_3)acetonitrile)	2,0
1,2,4,5-Tetramethylbenzene	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_4)methanol, (D_3)acetonitrile)	2,2 6,9
Dimethyl sulfone	Solid	Aqueous/Organic solvent (e.g. deuterium oxide, (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_6)acetone, (D_4)methanol, (D_3)acetonitrile, (D_2) dichloromethane)	3,0
1,3,5-Trimethoxybenzene	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_6)acetone, (D_4)methanol, (D_3) acetonitrile, (D_2)dichloromethane)	3,9 6,3
Dimethyl terephthalate	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_6)acetone, (D_3)acetonitrile, (D_2)dichloromethane)	4,0 8,1

[a] Chemical shifts can vary slightly, depending on the measurement conditions, such as the type of deuterated solvent used, the sample concentration and solution temperature during the measurement.

[b] The standard solution containing a fixed amount of the reference material prepared for qNMR measurement is available. When the standard solution is used for the preparation of a qNMR sample solution, it should be ensured that an accurate amount of the qNMR standard is evaluated. For achieving this, preparation can be either volumetric or gravimetric. The gravimetric preparation is more accurate than the volumetric preparation because density depends on the temperature that is an additional source of uncertainty.

[c] This is the value of deuterium oxide solution.

[d] This is the value of (D_6)dimethyl sulfoxide solution.

[e] Great care should be taken when selecting an internal standard from these candidates of internal standards and it should be carefully evaluated that the selected internal standard has no interaction with the sample in the solution.

[f] In order to simplify the qNMR measurement and the interpretation of qNMR spectra, the chemical shift of 1,4-BTMSB-d_4 has been set at 0 ppm in Japanese pharmacopeia 17 (JP17)[10].

[g] This is a deliquescent material.

Table B.1 *(continued)*

Name	State of matter	Suitable solvent	Chemical shift[a] (ppm)
Methyl 3,5-dinitrobenzoate	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_3)acetonitrile)	4,0 9,0 9,2
Ethylene carbonate	Solid	Aqueous/organic solvent (e.g. deuterium oxide, (D_6)dimethyl sulfoxide, (D_4)methanol, (D_3)acetonitrile)	4,5
Benzyl benzoate	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_4)methanol, (D_3)acetonitrile)	5,4 7,3 to 7,6 8,1
Maleic acid	Solid	Aqueous/organic solvent (e.g. deuterium oxide, (D_6)dimethyl sulfoxide, (D_6)acetone, (D_4)methanol)	6,3
Benzoic acid	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_6)acetone, (D_4)methanol, (D_3) acetonitrile, (D_2)dichloromethane)	7,5 7,7 8,0
Potassium hydrogen phthalate	Solid	Aqueous solvent (e.g. deuterium oxide)	7,5[c] 7,6[c]
Pentachlorobenzene	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_4)methanol, (D_3)acetonitrile)	7,9 9,2
1,2,4,5-Tetrachloro-3-nitrobenzene	Solid	Organic solvent (e.g. (D_1)chloroform, (D_6)dimethyl sulfoxide, (D_4)methanol, (D_3)acetonitrile)	8,5[d]
3,5-Bis(trifluoromethyl)benzoic acid	Solid	Organic solvent (e.g. (D_6)dimethyl sulfoxide, (D_6)acetone, (D_4)methanol, (D_3)acetonitrile)	8,2 8,5
Calcium formate	Solid	Aqueous solvent (e.g. deuterium oxide)	8,4
3,5-Dinitrobenzoic acid	Solid	Organic solvent (e.g. (D_4)methanol)	8,9 9,0

[a] Chemical shifts can vary slightly, depending on the measurement conditions, such as the type of deuterated solvent used, the sample concentration and solution temperature during the measurement.

[b] The standard solution containing a fixed amount of the reference material prepared for qNMR measurement is available. When the standard solution is used for the preparation of a qNMR sample solution, it should be ensured that an accurate amount of the qNMR standard is evaluated. For achieving this, preparation can be either volumetric or gravimetric. The gravimetric preparation is more accurate than the volumetric preparation because density depends on the temperature that is an additional source of uncertainty.

[c] This is the value of deuterium oxide solution.

[d] This is the value of (D_6)dimethyl sulfoxide solution.

[e] Great care should be taken when selecting an internal standard from these candidates of internal standards and it should be carefully evaluated that the selected internal standard has no interaction with the sample in the solution.

[f] In order to simplify the qNMR measurement and the interpretation of qNMR spectra, the chemical shift of 1,4-BTMSB-d_4 has been set at 0 ppm in Japanese pharmacopeia 17 (JP17)[10].

[g] This is a deliquescent material.

B.3 Examples of internal standards in the official methods

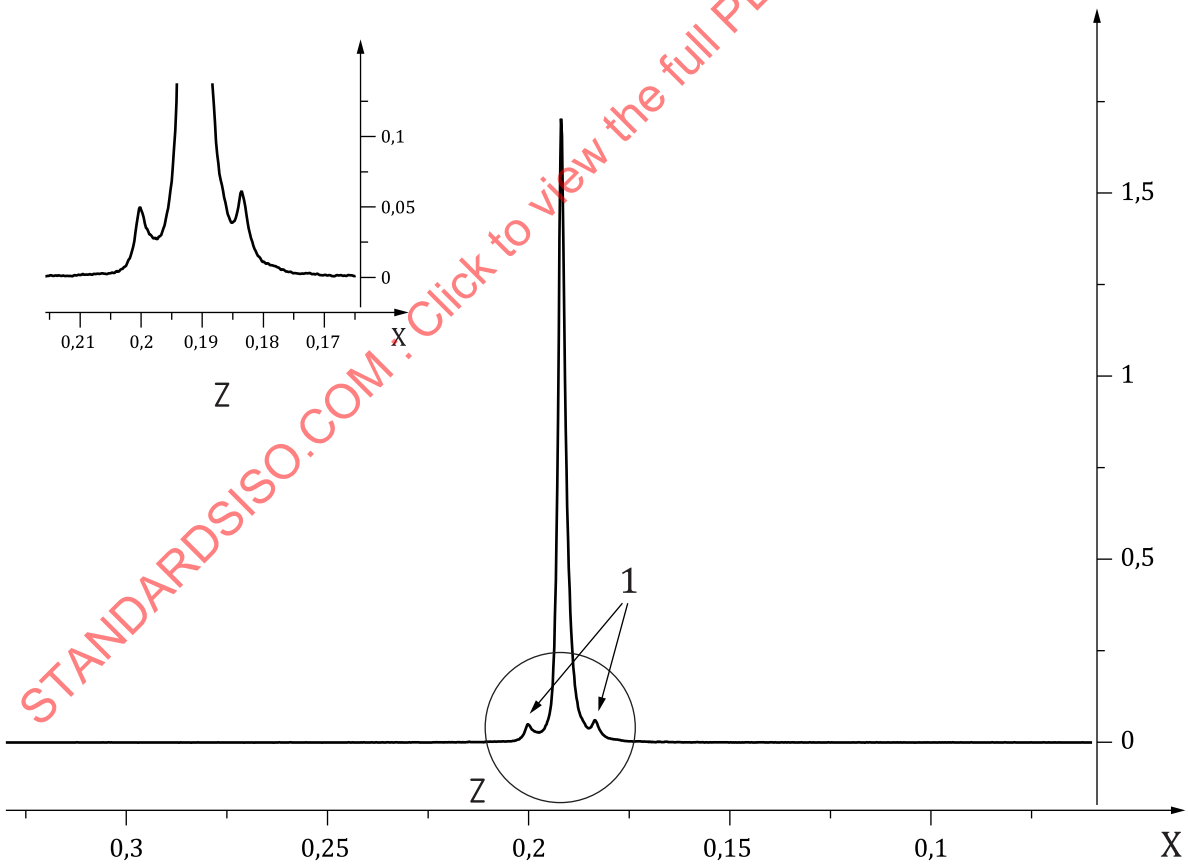
B.3.1 General

This clause gives examples of internal standards adopted in Japanese Pharmacopoeia (JP) and Japan's Specifications and Standards for Food Additives (JSFA).

B.3.2 Example 1 — 1,4-Bis(trimethylsilyl)(D_4)benzene (1,4-BTMSB-d_4)

1,4-BTMSB-d_4 has a singlet signal around 0,2 ppm (shown in Figure B.1) that is used as the reference signal for the quantification. On both sides of this main signal a doublet signal caused by a spin-spin coupling to silicon (^{29}Si) of around 6,7 Hz is found. This doublet signal derives from the 1,4-BTMSB-d_4 and shall be integrated with the reference signal for the quantification. 1,4-BTMSB-d_4 has four deuterium its benzene ring. Since complete deuteration is difficult, tiny signals from the 4 residual protons on the benzene ring can be observed around 7,3 ppm. When performing quantification of an analyte that has signals around this chemical shift, it is necessary to carefully check for the possibility of interferences.

It has also been confirmed that (D_1)chloroform without a stabilizer causes decomposition of 1,4-BTMSB-d_4 over time. Accordingly, when (D_1)chloroform is used as a deuterated solvent for qNMR sample solution preparation, it is necessary to perform the NMR measurements immediately after the sample solution has been prepared.



Key

X chemical shift (ppm)
Z chemical shift (ppm)
1 ^{29}Si satellite signals

Figure B.1 — Signal splitting of 1,4-BTMSB-d_4

B.3.3 Example 2 — Sodium 3-(trimethylsilyl)-1-(D_6)propanesulfonate (DSS-d_6)

DSS-d_6 is completely stable as a monohydrate in a laboratory environment (in temperature range from 15 °C to 25 °C, and a relative humidity of 20 % to 80 %). Therefore, when DSS-d_6 should be used, it is necessary to place the DSS-d_6 standard in a laboratory environment for 30 min or longer before its use. Furthermore, since the moisture content of DSS-d_6 that is stabilized as a monohydrate is about 74 g/kg, when DSS-d_6 is used as an internal standard, the signal derived from the water will also be observed in the ^{1}H qNMR spectrum of the sample solution. This means that it is necessary to think about separating it from the sample signal.

B.4 Selection of solvents

B.4.1 General

Solubility and stability of the sample and the internal standard are two important considerations when selecting a solvent. Additionally, impurities in the solvent can interfere with signals from the sample and the internal standard. When selecting a solvent, consider the items described in B.4.2 to B.4.5.

B.4.2 Consideration of solubility

If the sample and the internal standard are not completely dissolved in a solvent, accurate quantitative results cannot be obtained. As solubility changes under different conditions (e.g. type of a solvent used, temperature, pH), prior information on the solubility of both the sample and the internal standard can be helpful to select the solvent.

The determination of complete dissolution is typically done by visual inspection. To be thorough, it is recommended to use a transparent glass container to prepare the solution, and illuminate the solution with a fluorescent lamp. If there is even a slight cloudiness, or any particles are floating in the solution, the quantitative results can be adversely affected. This check should be performed meticulously. If there is any difficulty determining whether the sample and the internal standard are completely dissolved, it is recommended to use a different solvent.

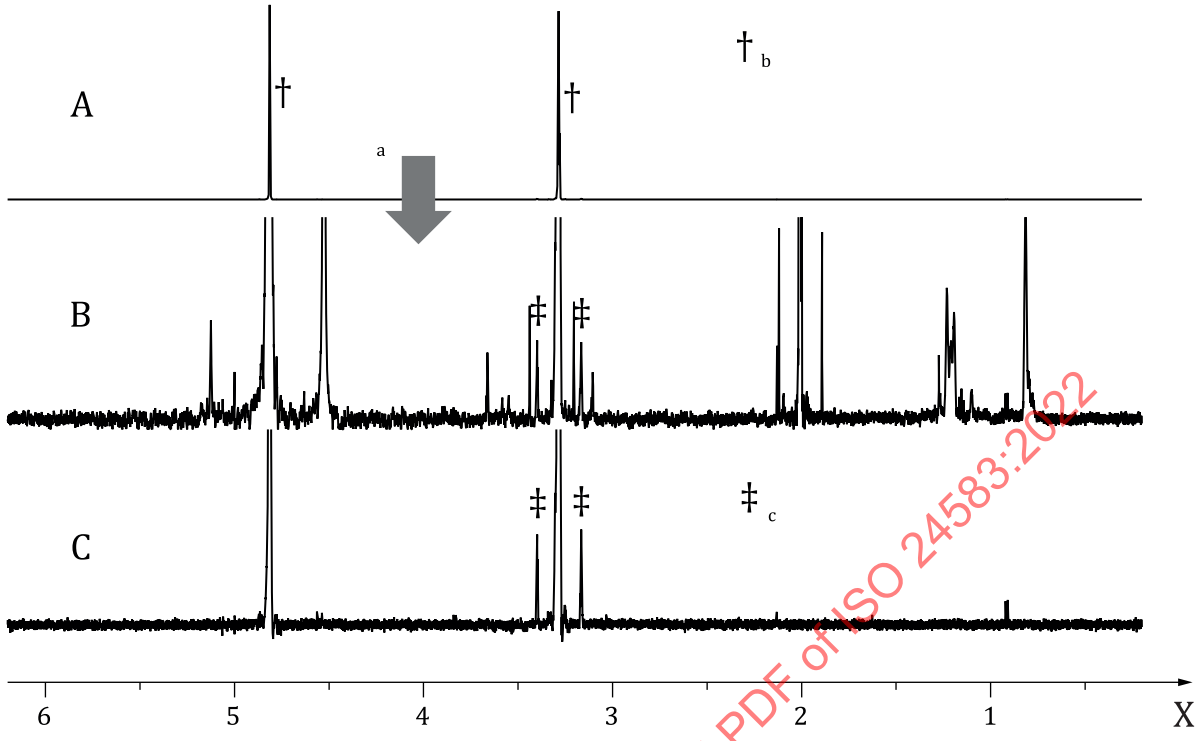
B.4.3 Consideration of stability

Time-dependent changes of the analyte or the internal standard, or both, including decomposition and isomerization, can influence the accuracy of the analytical results. If there are changes in spectral patterns or the analytical results for the sample solution over time, it is necessary to consider using a different solvent, a different internal standard, and/or acquiring qNMR data soon after sample preparation.

B.4.4 Consideration of impurities

To check for impurities in the solvent (as shown in Figure B.2), a blank solvent qNMR spectrum should be acquired and evaluated. When impurity signals can overlap with signals from the sample or the internal standard, those signals should not be used for the purity estimation. It can be avoided by using other solvents.

Impurity signals not originated from the solvent should also be considered. One example is a broad water signal from air moisture that can be removed by using a membrane filter or similar material to dry compressed air used for the NMR instrument.



Key

X chemical shift (ppm)

A full-scale ^{1}H NMR spectrum of conventional (D_4)methanol whose quality was ensured by deuteration ratio

B expanded view of A

C expanded view of ^{1}H NMR spectrum of high purity grade (D_4)methanol whose quality was ensured by not only the deuteration ratio, but also chemical purity with GC-FID and limit test for impurities with ^{1}H NMR

a Vertical enlargement.

b Resonance signals of protonated methyl group of (D_4)methanol.

c ^{13}C satellite signals of protonated methyl group of (D_4)methanol.

NOTE 1 The full-intensities of B and C were magnified by 500 times to illustrate clearly the comparison of the ^{13}C satellite signals and the impurity signals included in the conventional (D_4)methanol.

NOTE 2 See References [23], [24] and [25] for further information.

Figure B.2 — Comparative ^{1}H NMR spectra of conventional and high purity grade deuterated solvents

B.4.5 Consideration of pH and chemical shift

The pH value of the prepared sample influences the chemical shift of (some) signals, thus it needs to be controlled in protic/"deuteric" solutions.

By precise (and reproducible) adjustment of the solution's pH with an (inorganic) buffer, signals intended for quantitation can be shifted to a preferable region, e.g. to avoid signal overlap.

Impurities and moisture in the solvent can cause overlapping of peaks which can be an additional source of bias.