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Purity by absolute qNMR[1,2]

The following experimental conditions provide *general guidelines for quantitative 1D ^{1}H NMR (qHNMR) experiments*, assuming a compound with ~500 amu, with application to absolute value quantification procedures. After sample preparation (A), the quantification methodology is performed on the appropriate sampling methodology (B-E), and documented (F). Sample size guidelines for typical spectrometers are shown below (A). The quantity of sample depends on the field strength and the nature of the probe (see B).

A. Sample Preparation

Samples are weighed (0.01 mg accuracy) into 5 mm or 3 mm standard NMR tubes. A predetermined volume of solvent is added to achieve a constant solvent height for all measurements, matched to or centered on the probe coil, to facilitate shimming. For oily samples, external dissolution is recommended. To minimize evaporation and prevent moisture pickup, the tubes may be either sealed with a torch or capped and wrapped with PTFE tape and subsequently with paraffin tape.

	5 mm tubes	*3 mm tubes*
Solvent Volume	600 μL	170 μL
Weight of Sample (~500 amu)	4 – 12 mg	2 – 6 mg

B. NMR Instrument/Software Controlled Parameters

Pulse Program: Single pulse, without carbon decoupling ('s2pul' [Agilent/Varian]; 'zg' with 90^o pulse [Bruker]; "single pulse" [Jeol])

Sample Temperature: 25 °C (298 K, regulated ± 0.1 K)

Data Points (acquired): 64 K

Zero-Filling (SI or FN): to 256 K

Dummy Scans: 4

Scans (NS or NT): The number of scans (transients) to be used depends on: (i) the sample size and molecular weight (see A); (ii) the type of probe [direct or indirect ^{1}H detection]; room temperature [RT] or cryogenic probe [CP]); (iii) the field strength, and (iv) the pulse width. The table summarizes recommended general conditions.

Pulse Width (P1 or PW)	**90°**		**10°**	
	RT	**CP**	**RT**	**CP**
Relaxation delay (D1)	60 s	60 s	0	0
Acquisition time (AQ or AT)	4 s	4 s	4 s	4 s
Spectral Window (SW)	30 ppm	30 ppm	30 ppm	30 ppm
Transmitter Offset	7.5 ppm	7.5 ppm	7.5 ppm	7.5 ppm
Number of Scans for 300–600 MHz	64	16	512	64
for 700 MHz and above	32	8	256	32

The number of scans can be appropriately adjusted (up or down) depending on factors (i)-(iii). For mass limited samples and molecules with different molecular weights (significantly less than 300 or greater than 700 amu), the sensitivity of the measurement should be adjusted based on the molarity ratio, considering that the sensitivity is proportional to the square root of the relative number of scans (NS; transients).

C. Hardware dependent parameters

Preacquisition Delay: will vary with instrument and probe (alpha [Agilent/Varian]; DE [Bruker]; delay [Jeol]); document the probe including the model and the preacquisition delay used.

90° Pulse Width (P1 [Bruker]; PW(90) [Agilent/Varian]; pulse [Jeol]): The values depend on the instrument, NMR solvent, and probe, and need to be calibrated and documented.

Tuning and 90° Pulse Determination: Prior to determining the 90° pulse, the probe's frequency tune and impedance match must be optimized. The 90° pulse should be calibrated prior to the quantification experiment by determination of the 360° pulse on the sample. Document that tuning and matching were performed as well as the value of the 90° pulse.

Temperature Regulation: The probe temperature should be regulated (fluctuation <0.1°C) and documented.

D. Post-Acquisition Processing and Measurement of Integrals

The processing of 1D NMR data routinely uses some line broadening (LB) as an apodization (weighing) function, together with zero-filling (256 K). This can be used for qHNMR quantification as well. Application of Lorentzian-Gaussian (LB + GB) apodization together with zero-filling (256 K) may also be applied. Recommended values for these two processing conditions in qHNMR are as follows:

Processing Using Line Broadening:	LB = 0.1 Hz
Processing Using Lorentzian-Gaussian:	LB = -0.3 Hz, GB = 0.05
Zero Filling:	to 256K real data
Phasing:	manual phasing
Baseline Correction:	5th order polynomial

The signals of interest to be used for the quantification are selected, integrated (quantitative measure), and both values (integral value and range [ppm/ppb]) documented for all the signals used for quantification.

E. Calculation

An adequately high S/N (see B for generally suitable acquisition parameters) is necessary to properly account for minor constituents (showing signals with S/N>~10).

*Absolute Quantification: T*his involves the use of an added internal calibrant (IC), and the calculations constitute an assay of purity of the major compound of interest (or of any identified impurity). The sample preparation should conform to the description given above, except that a ***second*** substance (the IC) is also weighed into the NMR tube; its weight should be documented. Alternatively to weighing the IC directly into the NMR tube, stock solutions of the IC and/or the analyte can be used to prepare near equimolar mixtures of the analyte and the IC. The calculations are as follows:

$$P\ [\%] = \frac{n_{IC} \cdot Int_t \cdot MW_t \cdot m_{IC}}{n_t \cdot Int_{IC} \cdot MW_{IC} \cdot m_s} \cdot P_{IC}$$

Where: m_{IC} = weight (mass) of the internal calibrant (IC)
m_S = weight mass) of the sample
Int_{IC} = area (integral) of the IC resonance signal being used for quantification
Int_t = area (integral) of the target analyte (t) resonance signal being used for quantification
n_{IC} = number of protons that give rise to Int_{IC}
n_t = number of protons of the target analyte that give rise to Int_t
MW_{IC} = molecular weight of the internal calibrant
MW_t = molecular weight of the target analyte
P_{IC} = purity of the internal calibrant, as percent value

Other components within the sample being analyzed can be quantified in exactly the same manner provided: (i) the MW is known; (ii) the integrated signals correlate to a known equivalent of protons; (iii) the signals are isolated and their integrals free of overlap. The results of all calculations should be documented. Advantages of absolute quantification are that the purity of the major component can be evaluated, regardless of whether other components are observable or "NMR silent," and that the result is a weight based % purity.

Caveats of the Absolute Quantification Method: The absolute method cannot be applied to mixtures unless at least one specific proton signal can be assigned to each of the components within the mixture, and the number of protons for the signals it represents is known. If the MW of the minor components is unknown, only mole % but not weight % content can be determined. Note that the internal calibrant (IC) should be distinguished from the internal standard (IS) used for NMR chemical shift referencing (typically TMS or DSS).

F. Documentation

In general, all parameters that contribute to the quantification conditions of the qHNMR experiment should be documented, with detail addressing sample preparation (A), acquisition (B and C), post acquisition processing and quantitative measurement of integrals (D), and the calculation (E).

Step-by-Step Workflow for the Absolute qHNMR Method with Internal Calibration (IC)

Step 1 Using quantitative acquisition and processing parameters, acquire and prepare the baseline corrected, well-phased and properly referenced qHNMR spectrum of the sample. Document the exact weights of the sample (m_s) and the internal calibrant (m_{IC}). Determine the purity of the internal calibrant (P_{IC}) by one of the following methods, in the order of priority listed below:

(a) In the case of a traceable reference material (e.g., NIST), use the documented purity.
(b) Determination by gravimetry or other primary analytical method.
(c) Determination by absolute qHNMR; note that this approach eventually requires the use of methods (a) or (b) to yield accurate results.
(d) Determination by the qHNMR normalization (100%) method.
(e) If items a-d are unfeasible, set $\mathbf{P_{IC}}$ to 100.0%.

Comment: The accuracy and precision of the determination of P_{IC} impacts the result of absolute qHNMR analysis directly. At the very minimum, the value of P_{IC} used for the calculation should be documented for reproducibility.

Step 2 Identify the purest signal of the **target analyte**, assign its integral as the integral of the target analyte (Int_t), and determine the number of protons that give rise to this signal (n_t).

Alternatively, for multiple signals: Identify the purest signals of the target analyte. Calculate the normalized integrals values per proton equivalent by dividing each integral by the corresponding number of protons. Calculate the integral of the analyte (Int_t) as the average of all normalized integrals. Set the total number of protons (n_t) to one.

Step 3 Identify the purest signal of the **internal calibrant**, and assign its integral as the integral of the internal calibrant (Int_{IC}), and determine the number of protons that give rise to this signal (n_{IC}).

Alternatively, for multiple signals: Identify the purest signals of the internal calibrant. Calculate the normalized integrals values per proton equivalent by dividing each integral by the corresponding number of protons. Calculate the integral of the analyte (Int_{IC}) as the average of all normalized integrals. Set the total number of protons (n_{IC}) to one.

Step 4 Determine the molecular weights of the target analyte (MW_t) and the internal calibrant (MW_{IC}).

Step 5 Determine the purity (***P***) of the target analyte using the following equation:

$$P\ [\%] = \frac{n_{IC} \cdot Int_t \cdot MW_t \cdot m_{IC}}{n_t \cdot Int_{IC} \cdot MW_{IC} \cdot m_s} \cdot P_{IC}$$

DERIVATION OF THE EQUATION

Relationship of mole ratio to mass ratio	$\frac{mol_{IC} \cdot MW_{IC}}{mol_t \cdot MW_t} = \frac{[m_{IC} \cdot P_{IC}]}{m_t}$
Molar ratio by integrals	$\frac{mol_{IC}}{mol_t} = \frac{\frac{Int_{IC}}{n_{IC}}}{\frac{Int_t}{n_t}} = \frac{Int_{IC} \cdot n_t}{n_{IC} \cdot Int_t}$
...merge...	$\frac{Int_{IC} \cdot n_t}{n_{IC} \cdot Int_t} \frac{MW_{IC}}{MW_t} = \frac{[m_{IC} \cdot P_{IC}]}{m_t}$
...solve for m_t...	$m_t = \frac{n_{IC} \cdot Int_t \cdot MW_t \cdot [m_{IC} \cdot P_{IC}]}{Int_{IC} \cdot n_t \cdot MW_{IC}}$
Purity is % of target analyte in sample	$P = \frac{m_t}{m_s} \cdot 100$

Variables & Values
Int = integral
mol = moles
MW = molecular weight
P = purity (as percent value)
m = mass
n = number of protons giving rise to a given NMR signal

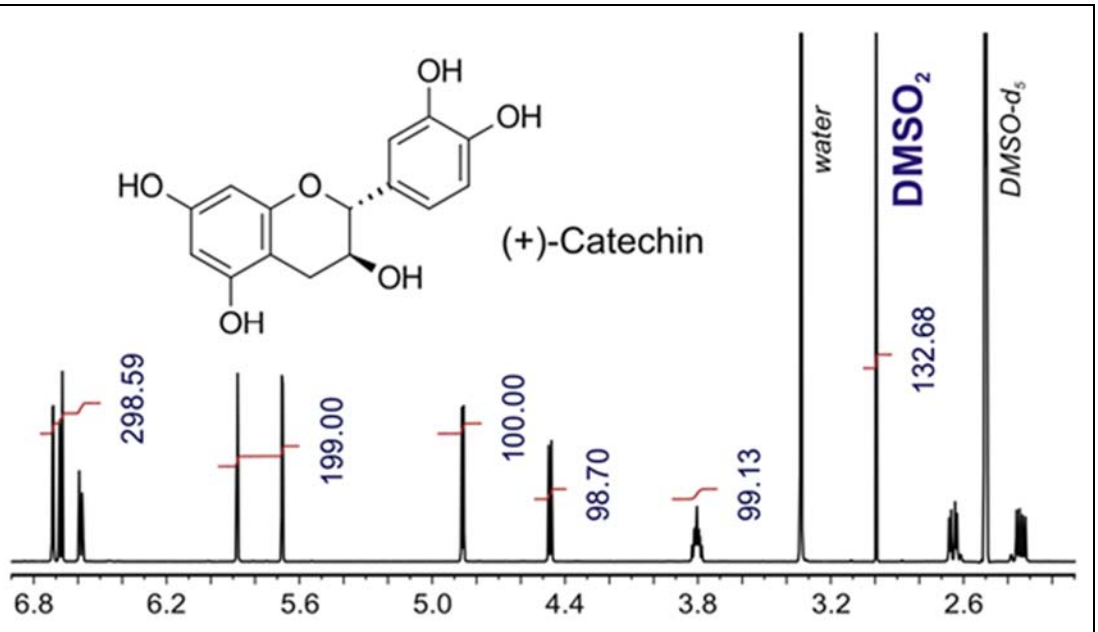
Indices
t: target analyte/molecule
IC: internal calibrant
s: sample

1. These guidelines were developed by Guido F. Pauli, Shao-Nong Chen, David C. Lankin, Tanja Gödecke, Birgit U. Jaki, J. Brent Friesen, James B. McAlpine, and José G. Napolitano. The guidelines were tested by Erick Carlson, Subhashree Francis, Daniel Beck, Huaping Mo, Johannes Wiest, Mark Cushman, Ulrike Holzgrabe and Gunda I. Georg.

2. See also: Pauli, G. F.; Chen, S.-N.; Simmler, C.; Lankin, D. C.; Gödecke, T.; Jaki, B. U.; J. Friesen, B.; McAlpine, J. B.; Napolitano, J. G.. 57Importance of Purity Evaluation and the Potential of Quantitative ^{1}H NMR as a Purity Assay. *J. Med. Chem.* **2014**, *57*, 9220-9231. *See Correction* DOI: 10.1021/acs.jmedchem.5b01667.

Example 1 Absolute IC Method

Commercial Sample of (+)-Catechin (5.33 mg/mL) in DMSO-$d_6$with the Addition of Dimethylsulfone ($DMSO_2$, 99.4% pure) as Internal Calibrant

Step 1	
Step 1	m_s = 3.20 mg, m_{IC} = 0.228 mg [a], P_{IC} = 99.4%
Step 2	Int_t = 99.37 [b,c], n_t = 1
Step 3	Int_{IC} = 132.68, n_{IC} = 6
Step 4	MW_t = 290.27 g/mol, MW_{IC} = 94.13 g/mol
Step 5	$P\,[\%] = \frac{n_{IC} \cdot Int_t \cdot MW_t \cdot [m_{IC} \cdot P_{IC}]}{n_t \cdot Int_{IC} \cdot MW_{IC} \cdot m_s} \cdot 100 = 98.2\%$

Notes

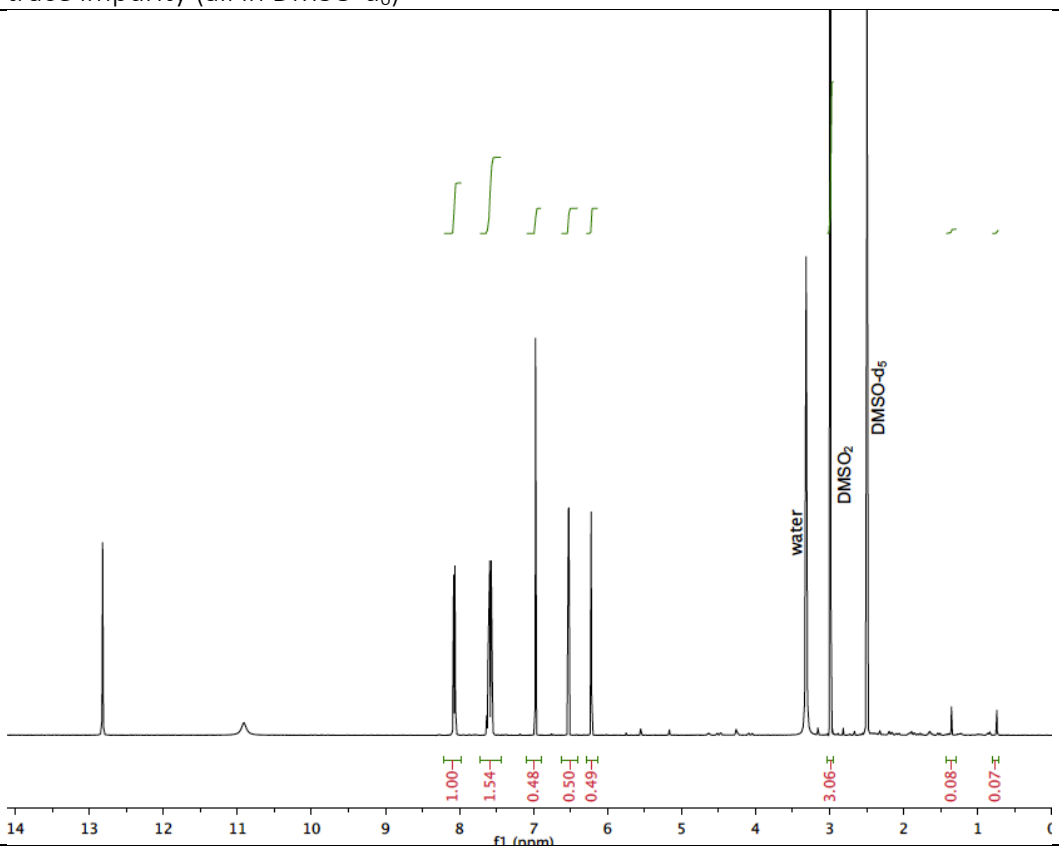
[a] The amount of calibrant was calculated taking into account the exact mass of $DMSO_2$ in a 2.28 mg/mL stock solution and the dilution factor during sample preparation (×1/10).

[b] The integral of the target analyte was calculated as the average of signals at 3.80, 4.47, 4.86, 5.60 – 5.98, and 6.50–6.77 ppm.

[c] The signals of the methylene protons were not included/integrated due to overlap with the ^{13}C satellites of the residual solvent signal (DMSO-d_5).

Example 2 Absolute IC Method

Chrysin Sample (10.08 mg/mL) with addition of 0.0956 mL of a solution of Dimethylsulfone ($DMSO_2$, 10.01 mg/mL) as Internal Calibrant, and 0.035 mL of a Solution of Hydrocortisone (3.74 mg/mL) as trace impurity (all in DMSO-d_6)

Step 1	
Step 1	$\boldsymbol{m_s}$ = 2.52 mg, $\boldsymbol{m_{IC}}$ = 0.956 mg, $\boldsymbol{P_{IC}}$ = 100.0%
Step 2	$\boldsymbol{Int_t}$ = 0.5 [a], $\boldsymbol{n_t}$ = 1
Step 3	$\boldsymbol{Int_{IC}}$ = 3.06, $\boldsymbol{n_{IC}}$ = 6
Step 4	$\boldsymbol{MW_t}$ = 254.24 g/mol, $\boldsymbol{MW_{IC}}$ = 94.13 g/mol
Step 5	$P\ [\%] = \frac{n_{IC} \cdot Int_t \cdot MW_t \cdot m_{IC}}{n_t \cdot Int_{IC} \cdot MW_{IC} \cdot m_s} \cdot P_{IC} = 100.5\%$ chrysin[b] $P\ [\%] = \frac{n_{IC} \cdot Int_t \cdot MW_t \cdot m_{IC}}{n_t \cdot Int_{IC} \cdot MW_{IC} \cdot m_s} \cdot P_{IC} = 1.1\%$ hydrocortisone

Notes

[a] The average one proton integral was determined from the qHNMR spectrum and used for calculation of percentage purity.

[b] The +0.5% difference between the determined qHNMR purity (100.5%) and the theoretical maximum of 100% is well within the accuracy of typical laboratory settings (NMR and balance validation). Other potential reasons for >100% purity values are differences in residual water content between the internal calibrant and the analyte, or the use of an internal calibrant that is significantly less pure than the target analyte.