# The Importance of Purity Evaluation and the Potential of Quantitative $^1$H NMR as a Purity Assay

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## ■ SUPPORTING INFORMATION

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## S1: Terminology in qHNMR

### PREFACE

There are several numerical components in NMR and qHNMR which required some form of "standardization" to certain values. Examples for these components are: the x-axis ($\delta$ scale) of the spectra; the intensity of the signals and their integrals; the arbitrary values of integrals. Several words describing this "standardization" process (e.g., referencing, calibrating, normalizing) are commonly and often interchangeably used, standardization of the terminology helps avoiding confusion.

### TERMINOLOGY USE IN THIS WORK

The present work uses the following terminology:

| | |
|---|---|
| **reference, referencing** | Setting of the chemical shift ($\delta$) scale of the NMR spectrum; performed with TMS or similar additives |
| **calibration, calibrant calibrating** | Process of standardizing the quantitative measure in qHNMR; performed by the use of internal calibrants (IC), external calibrants (EC), or combinations of both (ECIC) |
| **normalization, normalized** | Setting of integral values (or other quantitative measures) to a certain reference value that is characteristic of the analyte and ideally related to values in SI dimensions (e.g., molecular weight, mass).<br>**Example:** normalization of an integral to the number of protons giving rise to it, to yield a "normalized 1-proton integral" (***nInt***). |
| **adjustment adjusting** | Process of correcting for a known control value or measure that has not yet been included in the purity calculation.<br>**Example:** adjustment of purity values by multiplication with a correction factor that reflects the purity of the internal or external reference standard |

## S2: The qHNMR Normalization (100%) Method

### ■ STEP-BY-STEP WORKFLOW 100% METHOD

**Step 1** Acquire and prepare the baseline corrected, well-phased and properly referenced qHNMR spectrum using quantitative acquisition and processing parameters.

**Step 2** Define all integrals in the qHNMR spectrum and establish a set of **distinct integrals** of signals ($\boldsymbol{Int_1 ... Int_i}$, where $\boldsymbol{i}$ is the total number of integrals. Each distinct integral (*syn.* integration range) can include individual signals or groups of overlapped signals. Omit the signals corresponding to the NMR solvent (residual solvent signal) and water (if any).

**Step 3** Determine the number of protons ($\boldsymbol{n_t}$) in the **target analyte** that give rise to each of the integrated signals of the target analyte.

**Step 4** Assign each of the integrals ($\boldsymbol{Int_1 ... Int_i}$) to all known chemical species in the sample, including the target analyte and all of the impurities detected in the sample ($\boldsymbol{Imp_1 ... Imp_u}$, where $\boldsymbol{u}$ is the total number of impurities.

**Step 5** Determine the **normalized integral of the target analyte** ($\boldsymbol{nInt_t}$) as follows:

(a) Identify the purest signal of the target analyte.
(b) Determine how many protons give rise to this signal.
(c) Set its integral value ($\mathbf{Int_t}$) to an arbitrary number ($\boldsymbol{X}$), such that it reflects an integral value per one proton (1H) equivalent; this determines the normalized [1H] integral of the target analyte ($\boldsymbol{nInt_t}$).

> **Recommendations**: (I) An arbitrary $\boldsymbol{X}$ value of 100 (unity in %) can be used routinely for the integral of signals corresponding to one proton. Similarly, if the integral of the purest signal represents two protons of the target analyte, $\boldsymbol{X}$ would be set to 200 arbitrary units. (II) In order to avoid rounding errors, **Int** values with four significant figures should be used for the calculation of three significant figures for the expression of the purity values.

**Step 6** For each impurity ($\boldsymbol{Imp_1 ... Imp_u}$), do the following:

(a) Determine their molecular weights ($\boldsymbol{MW_1 ... MW_u}$).

> **Recommendation**: Use the exact masses to account for differences between molecules of varying MW ranges and elemental composition (different isotope patterns).
>
> **Comment**: For the determination of the molecular weights of the impurities, consider other analytical methods: particularly useful are MS and hyphenated MS data (e.g., LC-MS). Additional 1D and 2D NMR data, knowledge about potential synthetic side products, other spectroscopic information, and general knowledge about (residual) solvents can be valuable to establish the identity of impurities. If unknown impurities are present, a reasonable purity estimate is achievable by proposing probable molecular weights based on similarities to known compounds. In these cases, it is essential to document the proposed molecular weight values including the rationales behind them. One generally accepted approach (which is applied widely in pharmacopoeias and chromatography-based impurity evaluation) is to assume that the target analyte and the unknown impurities have the same molecular weight. This is equivalent to setting the relative response factors in to unity in chromatography based impurity evaluation. In this case, the result (% purity) will represent the impurities in the form "impurities calculated as isomers of the target component".

(b) Determine the number of protons that give rise to each of the integrals corresponding to impurities ($\boldsymbol{n_1 ... n_u}$).

**Step 7** Break down each integral value ($\boldsymbol{Int_1 ... Int_i}$) into as many subintegral values ($\boldsymbol{sInt_1 ... sInt_j}$) as needed such that each subintegral value ($\boldsymbol{sInt_1 ... sInt_j}$) is assigned to one or a minimum number of component(s) that give(s) rise to it (target analyte and/or impurities).

**Step 8** Assign to each subintegral value (***sInt*₁…*sInt*$_j$**) the combination of the number of protons (***n*₁…*n*$_k$**) and the component(s) that give(s) rise to them (target analyte and/or impurities).

**Step 9** Determine the normalized, one proton (1H) integral values of all impurities (***nInt*₁…<u>nInt$_u$</u>**). For this purpose, perform the following for each impurity (***Imp*₁…*Imp*$_u$**):

(a) Count the number of its subintegral values (***sInt*₁…*sInt*$_k$**) to yield the value (***k***).
(b) Calculate the **normalized subintegral value** (***nsInt*$_u$**) of each impurity (***Imp*₁…*Imp*$_u$**) by dividing all its subintegral values (***sInt*₁…*sInt*$_k$**) with the number of protons (***n*₁…*n*$_k$**) that correspond to each subintegral.
(c) Compute the sum of all **normalized subintegral values** (***nsInt*$_u$**) for each impurity (***Imp*₁…*Imp*$_u$**).
(d) Divide this sum by the number of the impurity's subintegral values (***k***).

> **Comment on steps 6-9**: The use of only the purest signals is recommended. However, in most cases it is necessary to deal with overlapped signals. If signals of multiple known impurities are included in the same integration area, break down the integral value by subtracting the individual contributions of each of the impurities. An estimate of these contributions can be commonly obtained by analyzing other integration areas. Once the contributions of each impurity to the integral value have been established, calculate the individual contribution values per proton equivalent by dividing each contribution by the corresponding number of protons. This finally enables calculation of the normalized integrals of each of the impurities (***nInt*₁…*nInt*$_u$**) as the average of all of the available contributions per proton equivalent.

**Step 10** Determine the purity of the target analyte (***P***) from the normalized integrals and molecular weights of the target analyte (***nInt*$_t$**, ***MW*$_t$**) and all defined impurities (***nInt*₁** *to* ***nInt*$_u$**; ***MW*₁** *to* ***MW*$_u$**) using the following equation:

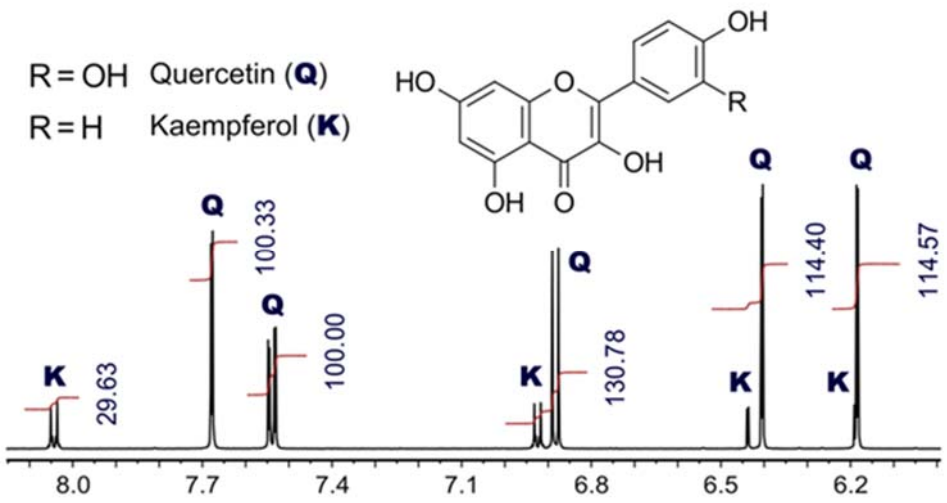
$$P\ [\%] = \frac{nInt_t \cdot MW_t}{nInt_t \cdot MW_t + \sum_1^u (nInt_u \cdot MW_u)} \cdot 100$$

**Variables & Values**
Int = integral
nInt = normalized integral (integral value normalized to 1H)
sInt = subintegral
nsInt = normalized subintegral (subintegral value normalized to 1H)
MW = molecular weight
P = purity
i: number of distinct integrals
j: number of subintegrals per integral
k: number of subintegrals per component
n = number of protons
u = number of impurities

**Indices**
t: target analyte/molecule
Imp: impurity

## ■ EXAMPLE 100% METHOD

**Commercial Sample of Quercetin (Q, 24.67 mg/mL)[a] in DMSO-$d_6$ Containing Kaempferol (K) as Impurity (600 MHz)**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Step 1** |  | | | | | | t = Q<br>Imp1 = K<br>**Comment** |
| **Step 2** | 29.63 | 100.33 | 100.00 | 130.78 | 114.40 | 114.57 | $Int_{o...i}$ |
| **Step 3** | 0 | 1 | 1 | 1 | 1 | 1 | $n_t$=5 |
| **Step 4** | **K** | **Q** | **Q** | **Q,K** | **Q,K** | **Q,K** | assign |
| **Step 5** | | | $nInt_t$ | | | | $nInt_t$=100 |
| **Step 6a** | $MW_t$ = 302.24, $MW_{Imp1}$ = 286.23 | | | | | | $MW$ |
| **Step 6b (K)** | 2 | 0 | 0 | 2 | 1 | 1 | $n_1$=6 |
| **Step 7** | 0 (Q)<br>29.63 (K) | 100.33 (Q)<br>0 (K) | 100.00 (Q)<br>0 (K) | 100.17 (Q)<br>30.61 (K) | 100.17 (Q)<br>14.23 (K) | 100.17 (Q)<br>14.40 (K) | B |
| **Step 8 (Q)** | 0 | 100.33 | 100.00 | 100.17 | 100.17 | 100.17 | $sInt_t$ |
| **Step 8 (K)** | 29.63 | 0 | 0 | 30.61 | 14.23 | 14.40 | $sInt_{Imp1}$ |
| **Step 9** | $n_{sInt1}$=[(29.63/2)+(30.61/2)+(14.23/1)+(14.40/1)]/4 = 14.69 | | | | | | $k$=4 |
| **Step 10** | $$P[\%] = \frac{nInt_t \cdot MW_t}{nInt_t \cdot MW_t + \sum_1^u (nInt_u \cdot MW_u)} \cdot 100 = 87.8\%$$<br>Impurity 1 (**K**) = 12.20% | | | | | | |

[A] Note that for the 100% Method, the weight of the sample does not enter into the calculation.
[B] The normalized integral of the target analyte (**Q**) was calculated as the average of the signals at 7.54 and 7.68 ppm

**Remarks**

1. If the qHNMR spectrum is acquired without $^{13}C$ decoupling, it is typically best to exclude the $^{13}C$ satellites from the integrals. In the case of unavoidable overlap, there are two potential approaches:
    a. Include the satellites in all integrals.
    b. Subtract 0.55% of the integral value of the main $^{1}H$ resonance integrals from the integral that includes them; in this case, make sure the satellites' integral values add properly to the theoretical 1.10%.

2. When acquiring qHNMR spectra in DMSO-$d_6$ or other non-protic solvents, keep in mind that hydroxyl and other exchangeable protons may (over time) result in less than unity proportional integrals - due to exchange. Accordingly, as a general rule, hydroxyl and amine protons are not suitable for quantitation.
3. Water signals depend not only on the sample but also on sample preparation including the NMR tube, quality of the NMR solvent used, storage conditions, atmospheric humidity, and other factors. Thus, in general 100% qHNMR applications, the water signal is an unreliable measure and should not be taken into consideration.
   However, the absolute qHNMR method is suitable for the indirect determination of the water content of a sample.

## S3: Absolute qHNMR with Internal Calibration (IC)

### ■ STEP-BY-STEP WORKFLOW ABSOLUTE IC METHOD

**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 (a)-(d) are unfeasible, set $P_{IC}$ 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 $\boldsymbol{m_t}$... $$m_t = \frac{n_{IC} \cdot Int_t \cdot MW_t \cdot [m_{IC} \cdot P_{IC}]}{Int_{IC} \cdot n_t \cdot MW_{IC}}$$

Purity is % of target analyte in sample $$P = \frac{m_t}{m_s} \cdot 100$$

**Variables & Values**

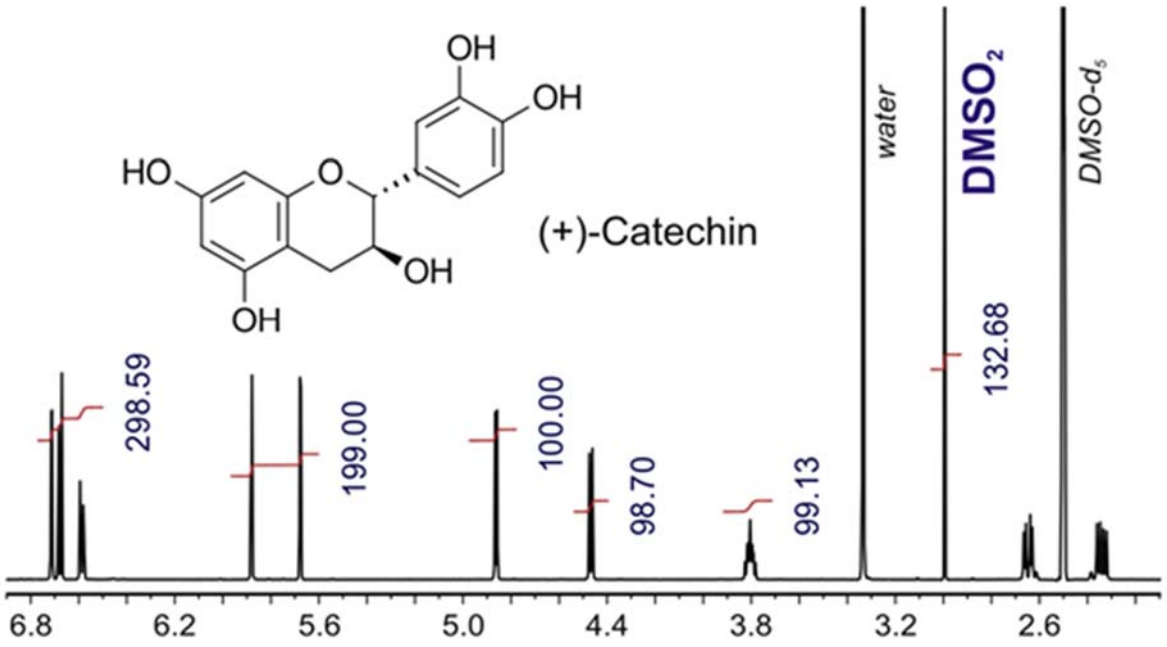
*Int* = integral
*mol* = moles
*MW* = molecular weight
*P* = purity (as percent value)
*m* = mass
*n* = number of protons giving rise to a given NMR signal

**Indices**

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

## ■ EXAMPLE 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 (600 MHz)**

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

**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-$d_5$).

## S4: Absolute qHNMR with External Calibration (EC)

### ■ STEP-BY-STEP WORKFLOW ABSOLUTE EC METHOD

**Step 1** Using quantitative acquisition and processing parameters, acquire and prepare the baseline corrected, well-phased and properly referenced qHNMR spectra of the analyte sample and the calibration sample. Document the exact weights of the sample ($\boldsymbol{m_s}$) and the external calibrant ($\boldsymbol{m_{EC}}$).

**Step 2** **In the qHNMR spectrum of the analyte sample:** Identify the purest signal of the **target analyte**, assign its integral as the integral of the analyte ($\boldsymbol{Int_t}$), and determine the number of protons that give rise to the signal ($\boldsymbol{n_t}$).

*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 ($\boldsymbol{Int_t}$) as the average of all normalized integrals. Set the total number of protons ($\boldsymbol{n_t}$) to one.

**Step 3** **In the qHNMR spectrum of the calibration sample:** Identify the purest signal of the **calibrant**, assign its integral as the integral of the external calibrant ($\boldsymbol{Int_{EC}}$), and determine the number of protons that give rise to the signal ($\boldsymbol{n_{EC}}$).

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

**Step 4** Determine the molecular weights of the target analyte ($\boldsymbol{MW_t}$) and the external calibrant ($\boldsymbol{MW_{IC}}$).

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

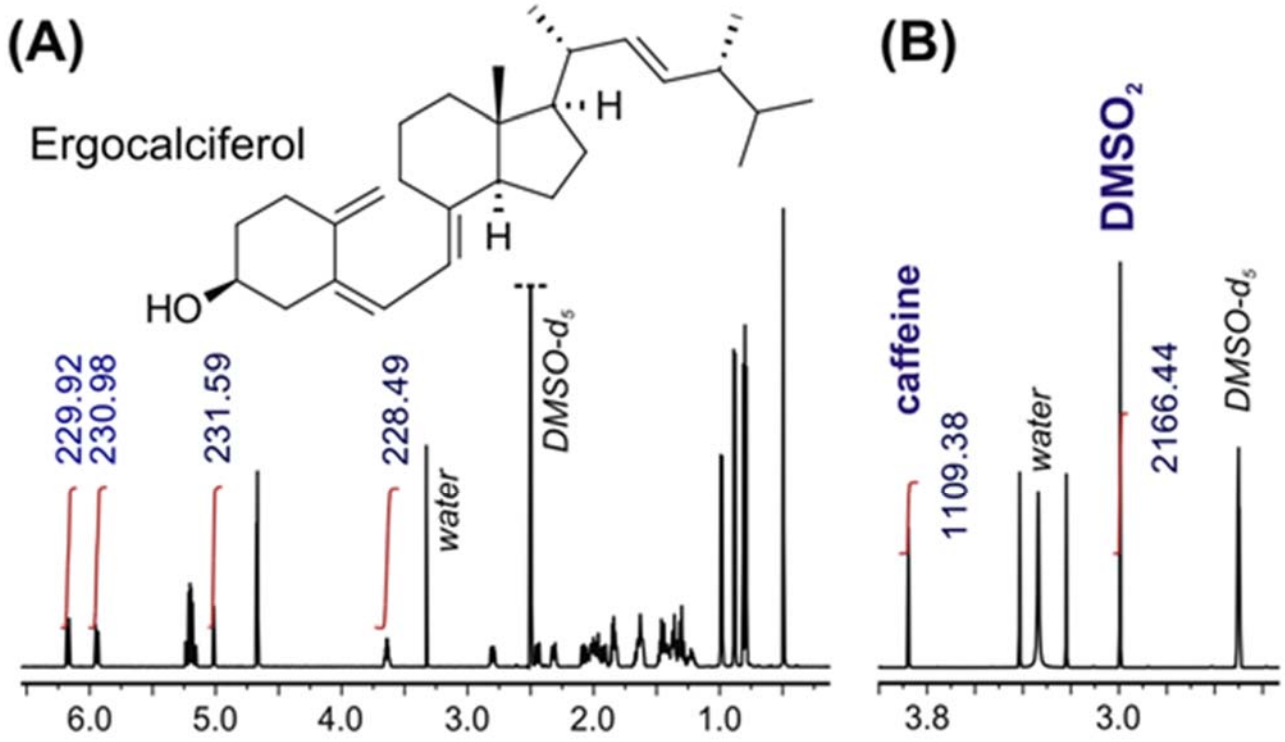
$$\boldsymbol{P}\ [\%] = \frac{n_{EC} \cdot Int_t \cdot MW_t \cdot m_{EC}}{n_t \cdot Int_{EC} \cdot MW_{EC} \cdot m_s} \cdot P_{EC}$$

**Variables & Values**
*Int* = integral
*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
EC: external calibrant
s: sample

## ■ EXAMPLE ABSOLUTE EC METHOD

**Commercial Sample of Ergocalciferol (11.11 mg/mL) Compared to an Equimolar Mixture of Two Calibrants, caffeine (98.7% pure) and Dimethylsulfone ($DMSO_2$, 99.4% pure, Recorded in DMSO-$d_6$ under Identical Experimental Conditions (600 MHz)**




### Values

$MW_t$ = 396.65 mg/mol $MW_{Caffeine}$ = 194.19 mg/mol $MW_{DMSO_2}$ = 94.13 mg/mol

*Calibration Spectrum*

| | | | |
|---|---|---|---|
| $m_{Caffeine}$ = 5.23 mg | $Int_{Caffeine}$ = 1109.38 | $n_{Caffeine}$ = 3 | $P_{Caffeine}$ = 98.7% |
| $m_{DMSO_2}$ = 2.47 mg | $Int_{DMSO_2}$ = 2166.44 | $n_{DMSO_2}$ = 6 | $P_{DMSO_2}$ = 99.4% |

*Analyte Spectrum*

| | | |
|---|---|---|
| $m_s$ = 6.66 mg | $Int_t$ = 230.25 [a] | $n_t$ = 1 |

### Calculation

$$P\ [\%] = \frac{n_{Caffeine} \cdot Int_t \cdot MW_t \cdot m_{Caffeine}}{n_t \cdot Int_{Caffeine} \cdot MW_{Caffeine} \cdot m_s} \cdot P_{Caffeine} = 98.6\%$$

$$P\ [\%] = \frac{n_{DMSO2} \cdot Int_t \cdot MW_t \cdot m_{DMSO2}}{n_t \cdot Int_{DMSO2} \cdot MW_{DMSO2} \cdot m_s} \cdot P_{DMSO2} = 99.1\%$$

Average purity based on both calibrations: $P$ = 98.8%

**Notes** [a] The integral of the target analyte was calculated as the average of signals at 3.64, 5.02, 5.94, and 6.17 ppm.

## S5: Absolute qHNMR with Combined External and Internal Calibration (ECIC)

### ■ STEP-BY-STEP WORKFLOW ABSOLUTE ECIC METHOD

**Step 1** Using quantitative acquisition and processing parameters, acquire and prepare the baseline corrected, well-phased and properly referenced qHNMR spectra of the analyte sample and the calibration sample. Document the exact weights of the sample ($\boldsymbol{m_s}$) and internal calibrant ($\boldsymbol{m_{IC}}$).

**Step 2** **In the qHNMR spectrum of the calibration sample:** identify the purest signal of the **calibrant**, assign its integral as the integral of the calibrant ($\boldsymbol{Int_C}$), and determine the number of protons that give rise to the signal ($\boldsymbol{n_C}$).

*Alternatively, for multiple signals:* Identify the purest signals of the calibrant, calculate the normalized integrals values per proton equivalent by dividing each integral by the corresponding number of protons. Calculate the integral of the calibrant ($\boldsymbol{Int_C}$) as the average of all normalized integrals. Set the total number of protons ($\boldsymbol{n_C}$) to one.

**Step 3** Identify the signal of the **residual protonated solvent**, assign its integral as the integral of the residual solvent ($\boldsymbol{Int_R}$), and determine the number of protons that give rise to the signal ($\boldsymbol{n_R}$).

**Step 4** Determine the molecular weights of the calibrant ($\boldsymbol{MW_C}$) and the residual solvent ($\boldsymbol{MW_R}$).

**Step 5** Assuming the purity of the residual solvent ($\boldsymbol{P_R}$) as 100%, determine the amount of residual protonated solvent in the calibration sample ($\boldsymbol{m_R}$) using the following equation:

$$m_R = \frac{n_C \cdot Int_R \cdot MW_R \cdot P_C}{n_R \cdot Int_C \cdot MW_C \cdot P_R} \cdot m_C$$

**Step 6** Taking into account the volumes of solvent used to prepare the samples, determine the amount of residual protonated solvent in the analyte sample ($\boldsymbol{m_R}^*$). If both samples were prepared using the same volume of solvent, then $\boldsymbol{m_R}^* = \boldsymbol{m_R}$.

**Step 7** **In the qHNMR spectrum of the analyte sample:** Identify the purest signal of the target analyte, assign its integral as the integral of the analyte ($\boldsymbol{Int_t}$), and determine the number of protons that give rise to the signal ($\boldsymbol{n_t}$).

*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 ($\boldsymbol{Int_t}$) as the average of all normalized integrals. Set the total number of protons ($\boldsymbol{n_t}$) to one.

**Step 8** Identify the signal of the **residual protonated solvent** in the qHNMR spectrum of the analyte sample. Assign its integral as the integral of the residual solvent ($\boldsymbol{Int_R}$), and determine the number of protons that give rise to the signal ($\boldsymbol{n_R}$).

**Step 9** Determine the molecular weights of the target analyte ($MW_t$).

**Step 10** Determine the purity of the target analyte ($\boldsymbol{P}$) using the following equation:

$$P\ [\%] = \frac{n_R \cdot Int_t \cdot MW_t \cdot m_R^*}{n_t \cdot Int_R \cdot MW_R \cdot m_s} \cdot P_R$$

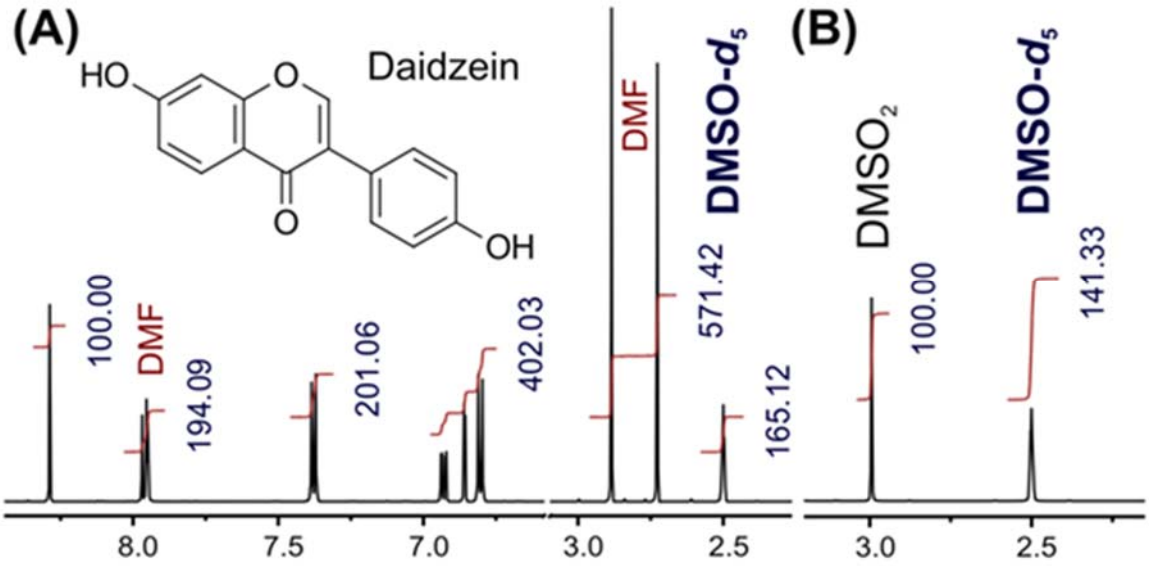
**Variables & Values**
*Int* = integral
*MW* = molecular weight

$P$ = purity (as percent value)
$m$ = mass
$n$ = number of protons giving rise to a given NMR signal

**Indices** t: target analyte/molecule
EC: external calibrant
s, *: sample

## ■ EXAMPLE ABSOLUTE ECIC METHOD

**Commercial Sample of Daidzein (17.78 mg/mL) in DMSO-$d_6$ was Analyzed by Establishing External Calibration with a Certified $DMSO_2$ Standard (99.4% Pure) and Using the Residual Protonated Solvent (DMSO-$d_5$) as Calibrant (600 MHz)**




## Values

| | | | |
|---|---|---|---|
| $m_S$ = 10.67 mg | $m_C$ = 0.605 mg [a] | $P_C$ = 99.4% | $P_R$ = 100% |
| $MW_t$ = 254.23 mg/mol | $MW_C$ = 94.13 mg/mol | $MW_R$ = 83.04 mg/mol | |
| **Calibration Spectra** | | | |
| $Int_C$ = 100.00 | $n_C$ = 6 | $Int_R$ = 141.33 | $n_R$ = 1 |
| **Analyte Spectra** | | | |
| $Int_t$ = 100.35 [b] | $n_t$ = 1 | $Int_R$ = 165.12 | $n_R$ = 1 |

## Calculation

$$m_R = \frac{n_C \cdot Int_R \cdot MW_R \cdot P_C}{n_R \cdot Int_C \cdot MW_C \cdot P_R} \cdot m_C = 4.50 \text{ mg}$$

The same volume of solvent was used to prepare both samples, therefore: $m_R^* = m_R$

$$P\,[\%] = \frac{n_R \cdot Int_t \cdot MW_t \cdot m_R^*}{n_t \cdot Int_R \cdot MW_R \cdot m_s} \cdot P_R = 78.4\%$$

**Notes** [a] The amount of calibrant was calculated taking into account the exact mass of $DMSO_2$ in a 6.05 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 6.76–6.98, 7.38, and 8.29 ppm.

## S6: The qHNMR Experiment

### ■ EXPERIMENTAL PARAMETERS & WORKFLOW

***A. Sample Preparation***

Samples are weighed (0.01 mg accuracy recommended) into 5-mm or 3-mm standard NMR tubes. If the nature of the sample precludes this approach, alternative methods of delivering the sample to the NMR tube (e.g., stock solutions) are suitable as long as the sample mass can be determined accurately. To facilitate shimming, a preset volume of solvent (see recommendations below) is added to achieve a constant solvent height, matched to or centered on the probe coil. 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 ( for an approximate MW of 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° pulse [Bruker]; "single pulse" [Jeol])

*Spinning status*: Non-spinning

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

Acquired *Data Points*: 64 K[a]

*Zero-Filling* (SI or FN): To 256 K data points

*Dummy scans*: 4

*Scans* (NS or NT): The number of scans (transients) to be used depends on: (i) the sample mass 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 | | 0 s | |
| *Acquisition time* (AQ or AT)[a] | 4 s | | 4 s | |
| *Spectral Window* (SW)[a,b] | ~30 ppm | | ~30 ppm | |
| *Transmitter Offset* | 7.5 ppm | | 7.5 ppm | |
| *Number of Scans/Transients*for 300–600 MHz | 64 | 16 | 512 | 64 |
| for 700 MHz and above | 32 | 8 | 256 | 32 |

[a]At any given magnetic field, only two of the three parameters (data points, acquisition time, and spectral window) are independent. Their combination should be chosen to match the listed values as follows: acquisition time to match most closely; spectral window to be >~25ppm, acquired data points to be adjusted accordingly.
[b]This recommendation facilitates the achievement of a flat baseline. Smaller spectral windows can be employed provided that related parameters are adjusted accordingly.

The number of scans can be appropriately adjusted depending on factors (i)-(iii). For mass limited samples and molecules with significantly different molecular weights (e.g., <300 or >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/transients.

***C. Hardware dependent parameters***

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

*90° pulse width:* The value (P1 [Bruker]; PW(90) [Agilent/Varian]; pulse [Jeol]) depends on the instrument, probe, and NMR solvent. It should be calibrated and documented. The 90° pulse width can be calibrated by determination of the 360° pulse on the sample.

*Tuning*: The probe's frequency tune and impedance match must be optimized. Document that tuning and matching were performed.

*Temperature:* The probe temperature should be regulated within <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 (to 256 K data points) 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 points |
| *Phasing:* | Manual phasing |
| *Baseline correction:* | 5$^{th}$ order polynomial with manual adjustment as needed |

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.

## S7: Digital Supporting Information and Data

### ■ RAW AND PROCESSED qHNMR DATA

The raw and processed NMR data for the examples in this study are made available in electronic form, using the following descriptive file names with release versions (at the writing of this manuscript, the release date was [20140511]).

| | Description | Files |
|---|---|---|
| 1 | Raw qHNMR data (FIDs) of the four examples (Figures 1 -4); format: Bruker; compressed ZIP file | `PurQnmr_RawData_[release date].zip` |
| 2 | Processed qHNMR data (spectra) of the four examples (figures 1 -4); formats: | |
| | PDF (with integrals), | `PurQnmr_Examples_[release date].pdf` |
| | MestReNova (with integrals) [*.MNOVA], as compressed ZIP file | `PurQnmr_Examples_[release date]_mnova.zip` |
| | Galactic [*.SPC] as compressed ZIP file | `PurQnmr_Examples_SPC_[release date].zip` |
| | JCAMP-DX [*.JDX] as compressed ZIP file | `PurQnmr_Examples_JDX_[release date].zip` |