



DRUGS WORKING GROUP

## GUIDELINE FOR qNMR ANALYSIS

DOKUMENT TYPE:	REF CODE:	ISSUE NO:	ISSUE DATE:
GUIDELINE	DWG-NMR-001	001	06.11.2019

**Author:** Torsten Schoenberger  
KT43 - Central Analytics II, Bundeskriminalamt, Germany

### Acknowledgment:

The author would like to thank the following persons for their very important contribution to this guideline. They have significantly improved the quality of the document through excellent technical suggestions and linguistic corrections:

- Mike Bernstein, Mestrelab, UK
- Constance Braouet, Fresenius University, Germany
- Ron Crouch, JEOL, USA
- Venita Decker, Bruker Biospin, Germany
- Takako Suematsu, JEOL RESONANCE Inc, Japan
- Claude Guillou, European Commission, Joint Research Centre, Italy
- Helge Klare, BWZ Cologne, Germany
- José G. Napolitano, AbbVie, USA
- Bernie O'Hare, GSK, USA
- Johann Panzer, Hessisches Landeskriminalamt, Germany
- Joseph G. Ray, University of Illinois at Chicago, USA
- Jenny Rosengren-Holmberg, National Forensic Centre, Sweden
- Dan Sørensen, Eurofins Alphora, Canada

## Table of Content

1	Aim.....	3
2	Scope.....	3
3	Definitions and Terms .....	3
4	 SCSSRS – The six commandments of qNMR.....	4
4.1	Selectivity .....	4
4.2	Chemical Inertness .....	5
4.3	Solubility.....	6
4.4	Stability .....	6
4.5	Relaxation .....	6
4.6	Sufficient resolution.....	6
5	General Requirements .....	7
5.1	Sample Preparation.....	7
5.1.1	Calibrants.....	7
5.1.2	Reference Material .....	8
5.1.3	Weighing .....	8
5.1.4	Number of Samples .....	9
5.1.5	Typical Weighing Amount.....	9
5.1.6	Insoluble Particles .....	9
5.1.7	Ready to use solutions with calibrants.....	9
5.2	Data Acquisition .....	10
5.2.1	Pulse angle .....	10
5.2.2	Acquisition time .....	10
5.2.3	Repetition Time.....	11
5.2.4	Time Domain.....	12
5.2.5	Transmitter Offset .....	12
5.3	Data Processing.....	13
5.3.1	Spectrum Size.....	13
5.3.2	Apodization .....	14
5.3.3	Phase- and Baseline-Correction.....	14
5.3.4	Partial Baseline Correction (only relevant in some cases) .....	15
5.4	Evaluation .....	17
5.4.1	Integration .....	17
5.4.2	Alternative: Line Fitting.....	19
6	Calculation .....	20
7	Quality Assurance .....	20
7.1	Validation .....	20
7.2	Critical Quality Attributes and Analytical Control Strategy.....	21
8	References.....	23
9	Annex.....	24
10	Amendments Against Previous Version.....	24

APPROVED BY THE ENFSI BOARD ON 02.01.2020

## 1 Aim

The aim of this practical guideline is to support the forensic community with special knowledge on quantitative Nuclear Magnetic Resonance (qNMR) analysis. qNMR has special requirements in comparison to chromatographic methods. The method is very universal and can be used in a variety of forensic applications. The number of applications in forensic laboratories is increasing rapidly. This guideline is intended to be a practical aid for users, enabling them to consider the special needs of the method from the outset.

## 2 Scope

The qNMR Guideline is especially intended for the use of qNMR spectroscopy of solvated analytes in forensic and customs laboratories. Since qNMR is a universal technique by nature, this guideline can be applied to other working fields as well. The focus is on straightforward analysis of the <sup>1</sup>H spectra. However, concrete applications will be described in additional “Application Notes” as annexes to this guideline.

The guideline refers to the analysis of drug chemistry exhibits which can be almost pure substances or mixtures of multiple compounds.

Any special sample preparation (e.g. extraction) or sampling strategies for inhomogeneous materials are not covered by this general guideline and must be considered separately.

Recommendations given here should be considered as “fit for purpose”. The general outline is designed for an overall method’s uncertainty level of approx. 1 % (coverage factor k=1 for 68 % confidence interval).

Typical parameters are given in grey boxes, such as the following:

Typical parameter [Bruker abbreviation]	x
---	---

When appropriate, the parameter abbreviation used by the Bruker acquisition software is given in square brackets. The corresponding abbreviations used by the JEOL software are listed in chapter “9 Annex”.

These typical parameters are intended to achieve accurate results, but they can also be modified to fit other purposes.

For increased certainty of the measurement, more effort and accuracy in the individual steps are generally required.

The qNMR guideline is also designed to help chemists to acquire knowledge and develop their skills in the application of qNMR with the aim of providing as much practical advice as possible in a concise manual.

## 3 Definitions and Terms

- **Acquisition Time** – Duration of the digitization of the FID.
- **Apodization** – Apodization refers to the mathematical processing technique by which the FID is multiplied pointwise by some appropriate function in order to improve the instrumental line shape.
- **Excitation** – Process causing the transition of the nuclear spin state from one level to another with higher energy.
- **Free induction decay (FID)** – The observable NMR signal.
- **Fourier Transformation** – A mathematical description from Fourier analysis of how continuous, aperiodic signals are decomposed into a continuous spectrum.

- **Imaginary Spectrum** – One of two equally sized blocks of frequency data produced by Fourier transformation of the time domain signal; usually the imaginary frequency data is not displayed and is used for phase correction of the real spectrum.
- **Internal Calibrant** – A substance added directly to the sample which is used for the calibration of analytes for quantitative analysis.
- **Pulse Angle** – Angle through which the magnetization vector is rotated.
- **Relaxation Delay** – The time allocated after each acquisition scan for the system to return to equilibrium.
- **Relaxation** – The process of getting back to the state of equilibrium after excitation.
- **Repetition Time** – Time from one pulse to the next.
- **Satellites** – Small peaks on both sides of and with the same distance to the main signal which are obtained by coupling with another adjacent NMR active nucleus.
- **Shift Reference Material** – A substance added directly to the sample which allows referencing of the spectrum's frequency axis (typically expressed in ppm).
- **Spectrum Size** – The size of the processed data after performing the Fourier Transformation.
- **Spin** – A quantum-mechanical property of a nucleus. In NMR, only nuclei with the spin  $I > 0$  are observable. Nuclei with a spin of  $\frac{1}{2}$  generate the simplest NMR spectra and are the most commonly studied.
- **Time Domain** – The number of raw data points to be acquired. A large value of the Time Domain enhances the spectrum resolution but also increases the acquisition time AQ. It is usually set to a power of 2, for example, 64k for a 1D spectrum.
- **Transmitter Offset** – Describes the middle of the excitation profile of a pulse.
- **Zero Filling** – Addition of zeros to the end of FIDs to adjust the size of the data, typically to a power of two and commonly used to improve the digital resolution of the transformed spectrum.

#### 4 SCSSRS<sup>1</sup> – The six commandments of qNMR

qNMR is generally accepted as a “relative primary” method. This means, that an SI unit (mol for the amount of substance) is directly measured with a very high metrological quality. It is “relative” because a calibrant (often referred to as “standard”) of known purity is needed to determine the absolute amount of the analyte.

The most important advantage of qNMR, which makes the method so universal, is that the signal intensity is directly proportional to the number of nuclei. This is valid as long as the “six commandments” (SCSSRS) are obeyed.

##### 4.1 Selectivity

With NMR, all NMR detectable components in a sample are normally covered in one spectrum. The signal associated with a certain atom must be discernible without the interference of other signals. Thus, selectivity is a critical quality attribute. This becomes easier to achieve with higher magnetic fields although selectivity can also be managed sometimes in other ways, such as using other solvents which can affect the signal position.

<sup>1</sup> SCSSRS = **S**electivity, **C**hemical Inertness, **S**olubility, **S**ufficient Resolution, **R**elaxation, **S**tability

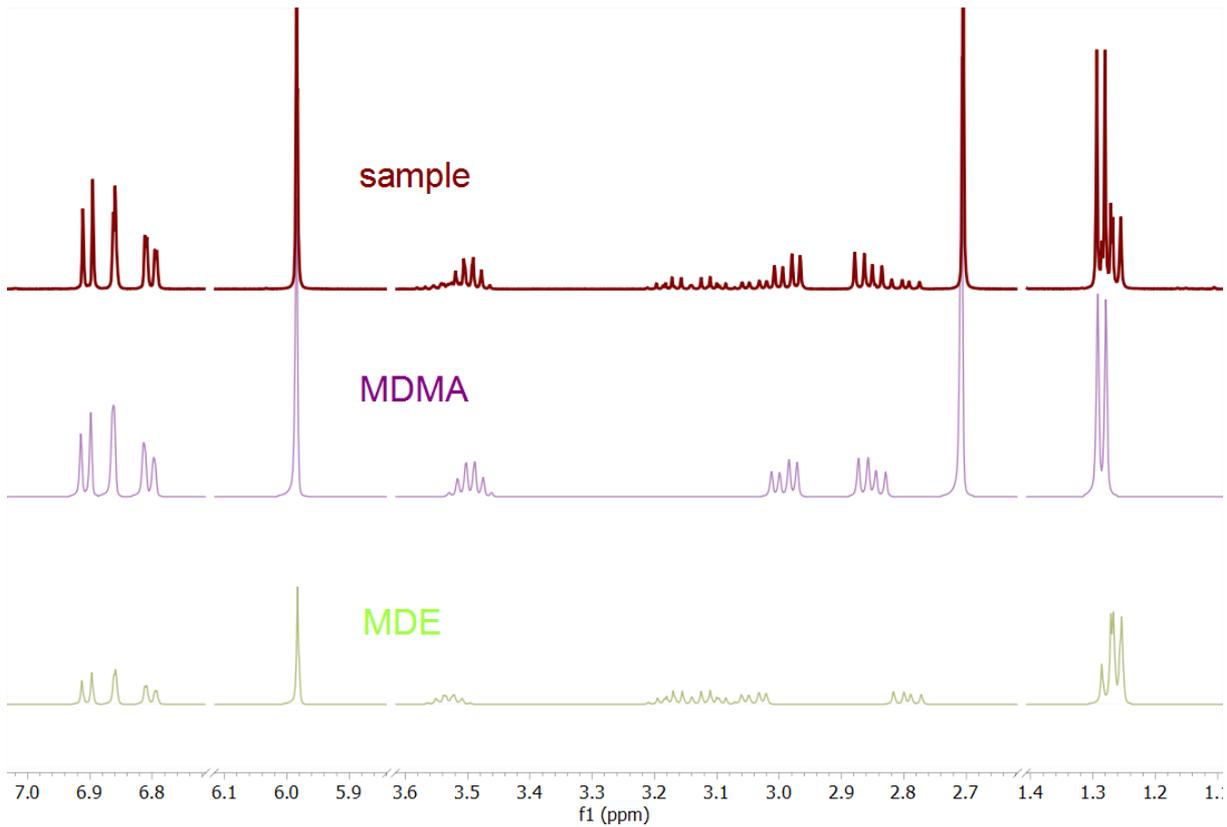


Figure 1: One critical example - A spectrum of a Mixture (top) with library spectra of components below - all regions are overlapped. (see also 3.5.1-3.5.2)

Selectivity also depends on the method of signal evaluation. While integration requires completely baseline separated signals, line fitting algorithms (Figure 2) can be used to evaluate overlapping signals.

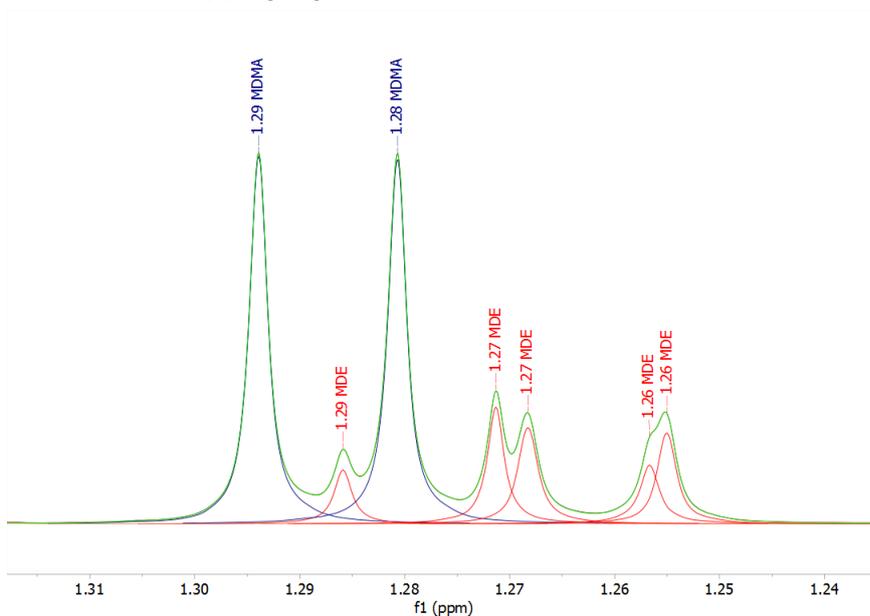


Figure 2: The same mixture spectrum as in Figure 1 - the overlapped methyl group's signals are individually evaluated by line shape analysis.

#### 4.2 Chemical Inertness

All components in the solvent (analyte, internal calibrant, shift reference material) must not react with each other.

### 4.3 Solubility

Since only soluble material is detectable, the material of interest must be completely dissolved in the solvent used.

### 4.4 Stability

The analyzed materials must be stable in the solution until the end of the measurement. This applies also to the individual atoms in the target molecule (e.g. whether labile protons are present).

### 4.5 Relaxation

Whenever spins of NMR-active nuclei are manipulated in the spectrometer by excitation pulses, they make a transition from ground to their excited state. As soon as the manipulation stops, the spins over time will return to their ground state due to relaxation. The strongest signal is achieved if all spins are in their ground state when being excited. Thus, if another excitation pulse is applied before relaxation is complete, the signal will be decreased compared to the first excitation. Therefore, it is essential to assure an (almost) complete relaxation for all analyzed spins in order to make signal intensities comparable.

### 4.6 Sufficient resolution

This refers actually to two criteria since the term itself is ambiguous:

- I. The signals should be as sharp as possible to allow a high signal to noise ratio and to avoid overlap with adjacent signals.
- II. The signal itself must be described by a sufficient number of data points (Figure 3) in order to enable accurate evaluation.

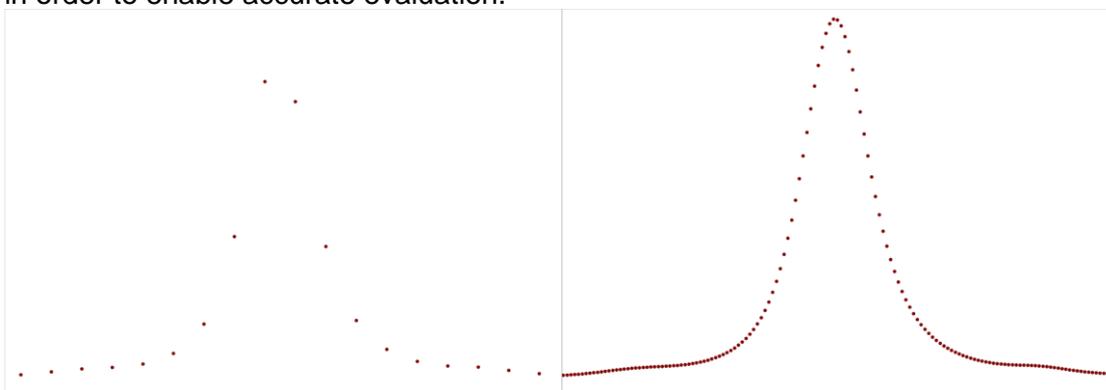


Figure 3: The left signal is described by too few data points which results in inaccurate integration. The right signal would result in more accurate integration.

## 5 General Requirements

### 5.1 Sample Preparation

#### 5.1.1 Calibrants

qNMR methods are often distinguished by the usage of internal calibrants (IC) or external calibrants (EC).

This guideline focuses on the use of IC where the calibrant is directly added to the solution of each sample. This is the most accurate method since signal intensities of analyte and calibrant can be directly compared in one spectrum, and only the weights of these materials in solution need to be known.

In contrast to this, qNMR can also be performed by using EC, which means that they are separated from each other in different solutions. An external calibration can be applied by using, for example, a coaxial inner tube [1] (Figure 5) or by using the PULCON<sup>2</sup> method [2,3] (Figure 4) where the signal intensity of the calibrant is detected in a separate sample and measurement. For the relatively modern PULCON method, very stable spectrometers are required which provide almost constant signal intensity over a long period of time. This is generally the case for modern spectrometers with superconducting magnets. Even by applying external calibrants uncertainty levels below 2 % (k=1) can be achieved. However, one must know both the weights and the volumes of each solution, which increases the overall error relative to the IC method.

APPROVED BY THE ENFSI BOARD ON 02.01.2020

<sup>2</sup> PULCON = "Pulse Length-based Concentration Determination"

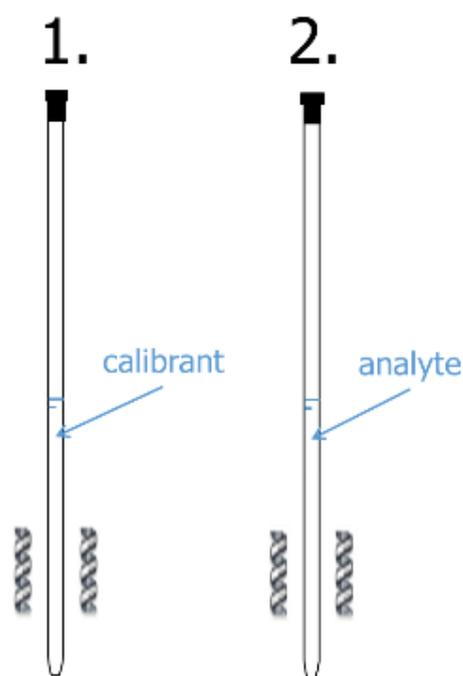


Figure 4: For external calibration, the calibrant and the analyte are measured in two different tubes as used for PULCON.

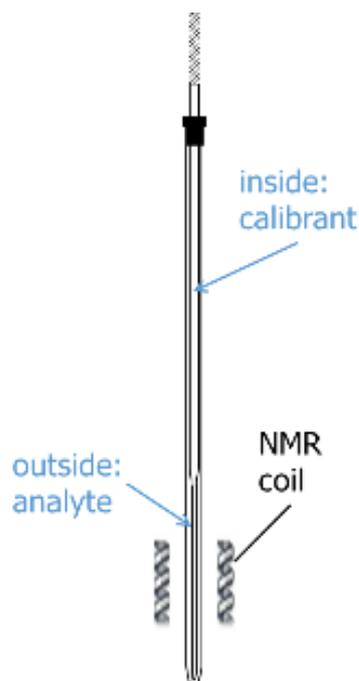


Figure 5: External calibration by coaxial insert. The inner tube separates the calibrant from the actual sample solution.

### 5.1.2 Reference Material

Reference materials used as calibrants should be selected according to the following criteria:

- Solubility
- Signal's position in the spectrum (no overlap with analyte components)
- Sufficient purity and uncertainty level for the purity value (provided on the certificate when certified reference material is used)
- No interaction with an analyte or other material (inertness)

The qNMR reference materials must not be confused with “chemical shift reference standards”, which are used only for referencing the spectrum scale (e.g. to ‘0 ppm’ for TMS).

A good and up to date overview, also with links to commercially available special qNMR certified reference materials, is given on the Wiki of the ValidNMR homepage [4]

### 5.1.3 Weighing

Of course, the weighing procedure for the analyte and the calibrant has a major impact on the method's uncertainty.

Valuable recommendations for accurate weighing are listed in weighing guidelines, such as the one given here in reference [5].

The balance, the minimum weight, and the whole weighing procedure must be chosen and adapted according to the method's uncertainty. The uncertainty contribution from the weighing process should be below 10 % of the combined uncertainty of the entire method.

The quickest way is to weigh the compounds directly into the NMR tube. Special spatulas are available which fit into a standard NMR tube with 5 mm diameter. Once the analyte and reference material have been weighed into the NMR tube the solvent can be added and the closed tube can be shaken or treated with ultra-sound in order to dissolve the compounds. This procedure is sufficient to reach the 1 % uncertainty level.

Weighing of the compounds in a suitable vessel (e.g. vial) is also possible. However, in this case, an additional step by using a pipette is needed to transfer the solution into the NMR tube. This should be the method of choice when an even lower uncertainty level is required.

#### 5.1.4 Number of Samples

As long as the sample is homogenous, only a duplicate analysis (two samples with one measurement for each sample) is necessary to exclude incidental sample preparation errors. The qNMR method itself is extremely robust. The uncertainty should be determined once during the validation and does not need to be redefined for each sample by using more than two analyses.

Number of samples	2
-------------------	---

#### 5.1.5 Typical Weighing Amount

The lowest weighing amount is limited by the minimum weight that assures the least uncertainty contribution of the weighing process. The upper limit is determined by the solubility of the analyte and reference material. Typical weighing amounts for the use of a 5 mm NMR tube with 0.5 mL solvent are roughly in the range of 5 to 15 mg.

Weighing amount	5 – 15 mg
-----------------	-----------

A detailed description of the requirements for the minimum sample amount with regard to the uncertainty contribution is given in the USP<sup>3</sup> chapter <1251> [6].

Current data sheets of balances refer to that Chapter and provide minimum “USP” sample amounts for different uncertainty levels.

#### 5.1.6 Insoluble Particles

For exhibits consisting of mixtures, it is quite normal that not all components are completely soluble in the NMR solvent. This is acceptable as long as the insoluble particles do not belong to the analytical target components of the measurement (e.g. diluents or adulterants) or the reference material. Insoluble particles cannot be detected in the applications described here anyway but they can affect the sample’s homogeneity which would cause shimming problems and eventually a bad line shape. The insoluble material can stay in the solution in the NMR tube as long as the actual NMR measuring area, which is defined by the coil position, is not affected. In most cases, this insoluble material precipitates at the bottom of the tube or agglomerates at the top surface of the liquid solvent.

The presence of insoluble material requires a careful assessment of whether the actual target components are completely dissolved or not. This can also be facilitated by applying a duplicate measurement with significantly differing weighing amounts (e.g. 6 and 10 mg) or with different solvents.

#### 5.1.7 Ready to use solutions with calibrants

The internal calibrants can also be dissolved directly in a larger volume of the solvent to create a stock solution. Then, the concentration of the IC needs to be determined accurately, e.g. by qNMR analysis or by weighing the IC and pipetting the solvent. By knowing the

<sup>3</sup> USP: The United States Pharmacopeial Convention

concentration, defined amounts of the IC can be easily added to the NMR tube by adding a well-defined amount of the solution. This can be done by using a calibrated and very accurate pipette or dispenser, or by weighing the solution.

This method is mainly limited to solutions in deuterated water (D<sub>2</sub>O) or DMSO. Other normal NMR solvents are too volatile to keep the IC's concentration constant.

DMSO is a quite hygroscopic solvent. Thus, DMSO solutions should only be used for a limited time period (a few days).

The D<sub>2</sub>O can also evaporate to a certain extent which results in a higher concentration of the IC. The solvent's loss should be checked even in capped vessels if the solution will be in use for a longer period of time (months), e.g. by checking the weight of the complete vessel.

## 5.2 Data Acquisition

### 5.2.1 Pulse angle

For NMR used for the 1D-<sup>1</sup>H experiment for non-quantitative identification purposes, pulse duration corresponding to a 30° flip angle of the magnetization is often recommended<sup>4</sup> because data are acquired without considering the complete relaxation of the spins. However, complete relaxation is essential for qNMR (one of the six SCSSRS commandments).

The use of a pulse duration corresponding to a 90° flip angle delivers double intensity in comparison to the 30° angle (which means one-fourth of the number of scans is needed to get to the same signal intensity). To allow full relaxation, one must wait 7 times the longest T<sub>1</sub> of the signals of interest before triggering again a pulse for recording of the next scan. When using the 30° instead of the 90° pulse, this waiting time can only be shortened to ~ 6 times T<sub>1</sub>. Therefore, the 90° pulse angle is the best choice to get the most signal intensity per time period.

*[The selection of the 90° pulse is a "must" for applying external referencing methods since other pulses would increase the measurement uncertainty.]*

Pulse angle [P1]	90°
------------------	-----

### 5.2.2 Acquisition time

The acquisition time [AQ] must be long enough to cover the fully decayed signal (FID) but should not be much longer in order to avoid just collecting noise when no signal is present. The acquisition time can be shortened somewhat if the FID is to be multiplied by an exponential decay (the so-called line-broadening).

The acquisition time is effected by the selection of the time domain and the spectral width (see also 5.2.4).

<sup>4</sup> The 30° pulse is a usual simplified assumption. The actual "best" solution is defined by the "Ernst angle" [7] which needs to be adopted for the individual sample. However, the Ernst angle do not apply for the special qNMR needs (full relaxation with 7xT<sub>1</sub>)

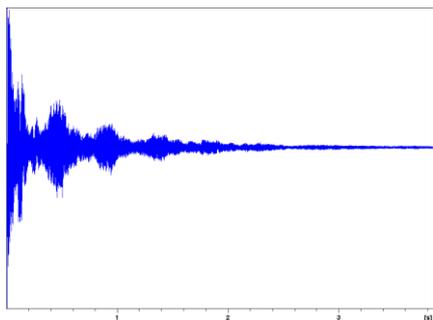


Figure 6: Fully decayed NMR signal (FID).

Typical acquisition time is something like 7 seconds (5 – 8 seconds).

Acquisition time [AQ]	7 s
-----------------------	-----

### 5.2.3 Repetition Time

The repetition time is the time from the beginning of one scan to the beginning of the next one. In the case of qNMR, where only one pulse per scan is being applied, the time can also be described as the one between two pulses.

In the qNMR pulse sequence, the repetition time is mainly defined by the acquisition time and a delay [D1] during which the excited nuclei from the previous scan relax back to thermal equilibrium.

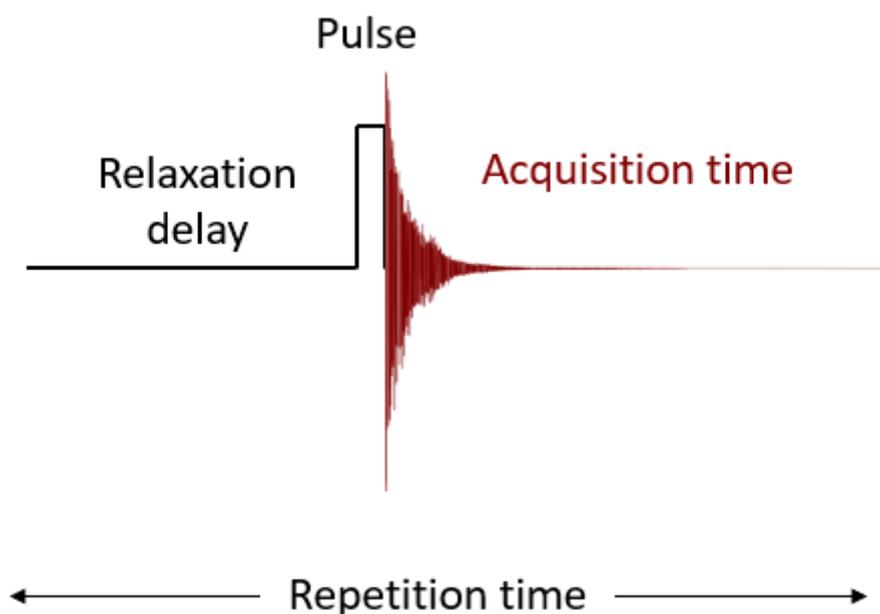


Figure 7: Description of timings for each pulse element in an NMR experiment.

The repetition time must be long enough to assure (almost) full relaxation (one of the commandments to obey). This is defined by the spin-lattice relaxation time,  $T_1$ . After waiting for 7 times  $T_1$ , approx. 99.9 % of the spin magnetization is relaxed (in comparison: 5 times  $T_1 \rightarrow 99.3$  %). This means that the error caused by incomplete relaxation is reduced from 0.7 to

0.1 %, a critical consideration when attempting to obtain data with an overall accuracy of 1.0 %.

Thus, for qNMR experiments, the repetition time should be at least 7 times as high as the slowest  $T_1$  value of all spins evaluated.

The  $T_1$  can be determined by using the “inversion recovery experiment”, which is quite time consuming regarding measurement and evaluation. Please note, that a longer repetition time is possible, but prolongs the overall experiment time. Thus, it should be kept as long as needed, but as short as possible.

Much faster, fully automated tools are in development (November 2019), e.g. by Bruker, and should be available soon.

As a rule of thumb,  $T_1$  values for  $^1\text{H}$  spins in organic molecules are typically below 10 s. Exceptions only exist for very small molecules, such as Ca-formate (used as certified reference material for qNMR!) and some rare special cases of symmetrical molecules. Symmetric molecules give simple spectra, and are, therefore, useful reference materials, but can add to the length of the experiment.

Thus, a repetition time of 70 s ( $[D1] = 63$  s, with  $[AQ]=7$ s) is generally sufficient.

Delay [D1]	63 s { 7 times $T_1$ – [AQ]}
------------	------------------------------

#### 5.2.4 Time Domain

The time domain [TD] describes the observed NMR signal over time which is encoded in the number of data points provided for the measured signal [TD]. Note that actually the time - domain FID is composed of both a real and an imaginary part. After Fourier Transformation, the frequency domain consists of two separate parts: a real spectrum and an imaginary spectrum. The NMR user only deals with the real spectrum after transformation, but it is essential to realize that a FID composes of TD data points results in two spectra, each composed of TD/2 data points. The time domain must be big enough to provide sufficient signal resolution (see also 3.4.1 Spectrum Size).

The time domain is directly related to the spectrum width [SW] and the acquisition time [AQ]:

$$AQ = \frac{TD}{2 \cdot SW} \quad \text{Equation 1}$$

A good combination of these parameters must be found to fulfill all requirements.

For a  $^1\text{H}$  spectrometer frequency of 500 MHz and a spectrum width of 15 ppm (= 7,500 Hz, as 1 ppm @ 500 MHz corresponds to 500 Hz), a typical value for TD is 128k, which will provide a real frequency domain spectrum of 64k data points.

Time Domain [TD]	128k
------------------	------

#### 5.2.5 Transmitter Offset

The transmitter offset describes the frequency at which the pulse is applied, and this will occur at the center of the final spectrum. However, the excitation profile of the pulse is not really rectangular. It decays slightly and symmetrically on both sides of the center and is mainly affected by the length of the excitation pulse – being smaller for shorter pulses. This effect can lead to a relative deviation in the expected signal intensity in the range of a few ‰. It can be neglected as long as the method’s uncertainty is in the percent range. However, the transmitter offset should be set approx. to the middle of the evaluated signals.

Note, that the effect of the excitation profile can be more impactful for the detection of other nuclei, such as  $^{19}\text{F}$ , because their chemical shift range is much larger.

Transmitter Offset [O1P]	4.5 ppm
--------------------------	---------

### 5.3 Data Processing

#### 5.3.1 Spectrum Size

The spectrum size [SI] is defined as the number of data points in the real spectrum after Fourier Transformation. It must be high enough to provide sufficient resolution of the narrowest signal. As a rule of thumb, more than 5 points should describe the signal above the half-height (Figure 8).

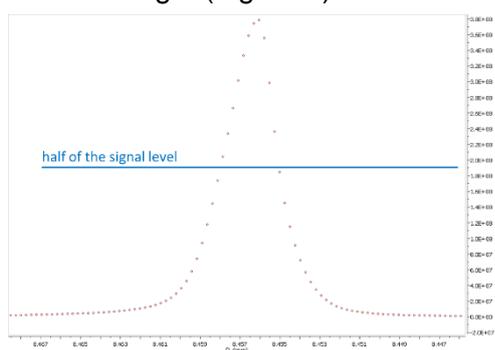


Figure 8: More than 5 points describe the signal above half height.

This is usually achieved by applying a zero filling (that is increasing SI) to the FID which results in adding additional, interpolated points to the signal.

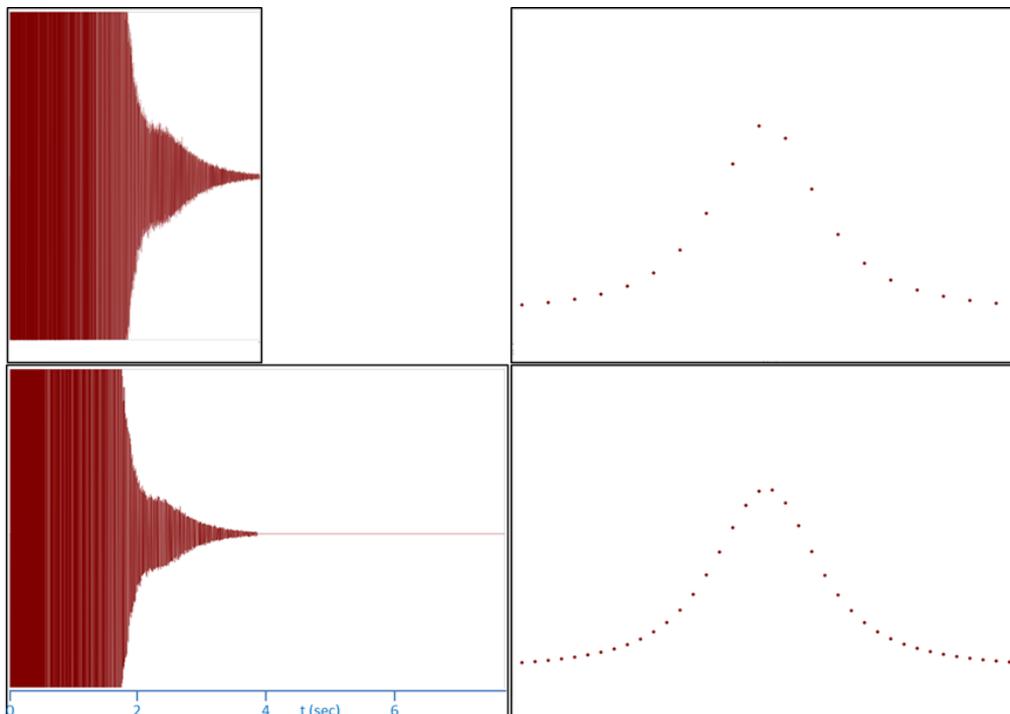


Figure 9: Effect of zero-filling, top: no zero-filling, bottom: zero-filling with a factor of two.

As mentioned above, TD counts the imaginary and real parts of the spectrum as individual data points. In contrast, SI is working in complex points, combining one imaginary and one real data point. Therefore, if SI is set to the same value as TD, a zero-filling by a factor of two is automatically applied.

APPROVED BY THE ENESI BOARD ON 02.01.2020

### 5.3.2 Apodization

Before applying the Fourier transform, the FID is usually manipulated by an apodization function (Figure 10, top) to improve the signal to noise level at the expense of signal resolution (Figure 10, bottom). The exponential multiplication is normally used, where the strength is controlled by the parameter “line broadening” factor [LB], [width].

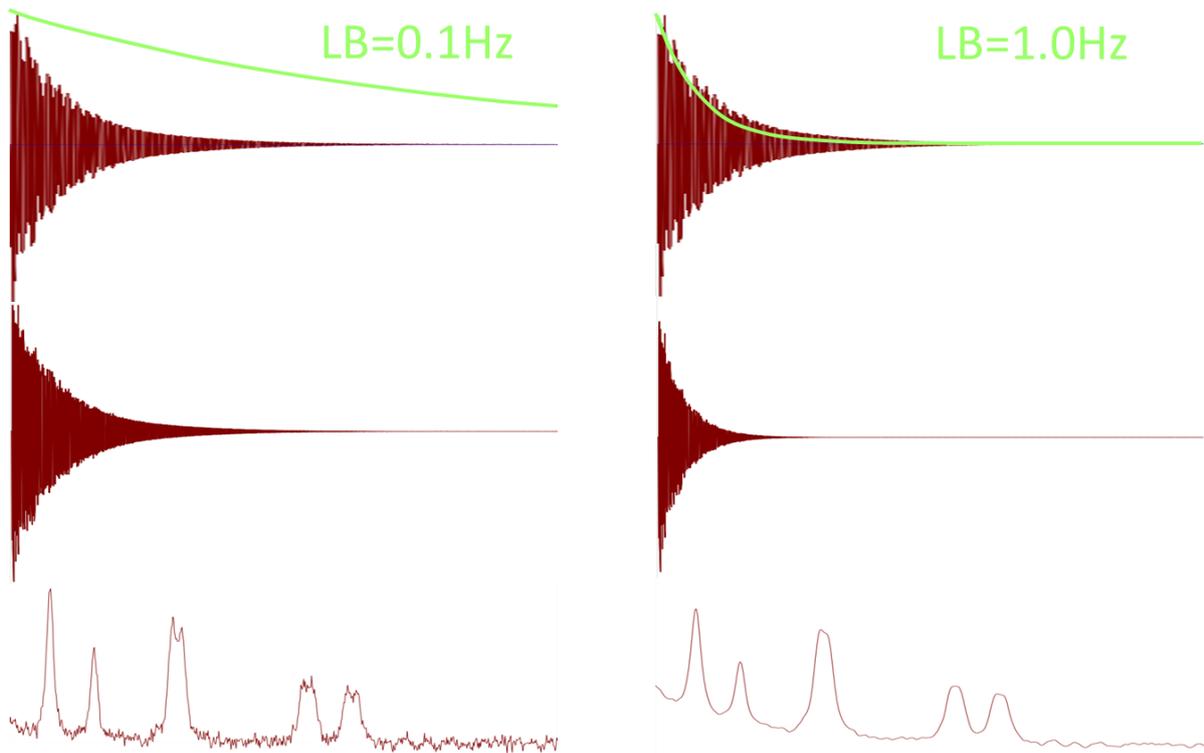


Figure 10: Two different line broadening factors (left column: 0.1 Hz, right column: 1.0Hz) are applied for exponential multiplication on a FID:

Top: Original FIDs with indicated exponential functions.

Middle: FIDs after exponential multiplication.

Bottom: The resulting spectral regions.

A [LB] value of 0.2 Hz is very common for high-field NMR. This leads to a signal to noise gain by factor two without substantially decreasing the signal’s resolution.

[For low-field NMR, even higher LB values can be applied, since the signal’s line width is much broader.]

Apodization Function	[EM]
Line Broadening Factor [LB]	0.2 Hz

### 5.3.3 Phase- and Baseline-Correction

Correct phase- and baseline corrections are essential for qNMR. The current algorithms are usually able to calculate the phase correction properly in automation mode. If incorrect phasing is observed, it needs to be corrected manually. Polynomial fit algorithms are commonly used to correct baselines. However, there is no guarantee that one specific

algorithm will work in all cases. Applying digital filters such as [BASEOPT] can simplify the baseline correction during the data acquisition.

Baseline correction algorithms, which also compensate parts of the signal, must not be used (e.g. the “Whittaker Smoother” algorithm in MNova (Figure 11)).

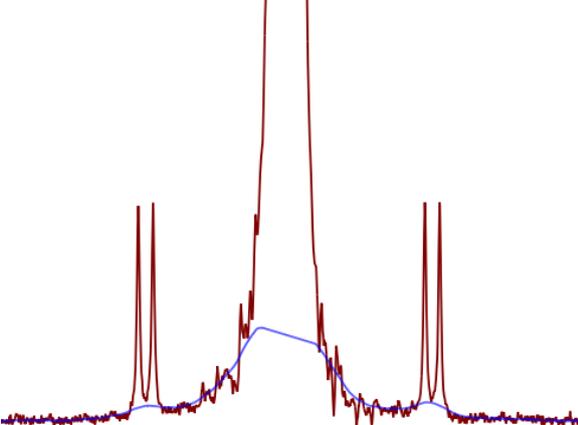


Figure 11: Example of an inadequate baseline correction which also compensates part of the signal.

5.3.4 Partial Baseline Correction (only relevant in some cases)

It is sometimes necessary to apply an additional partial baseline correction (e.g. by applying [absf] in Bruker software), for example, if signals to be evaluated are overlaid by the foot of very broad neighbor signals (Figure 12).

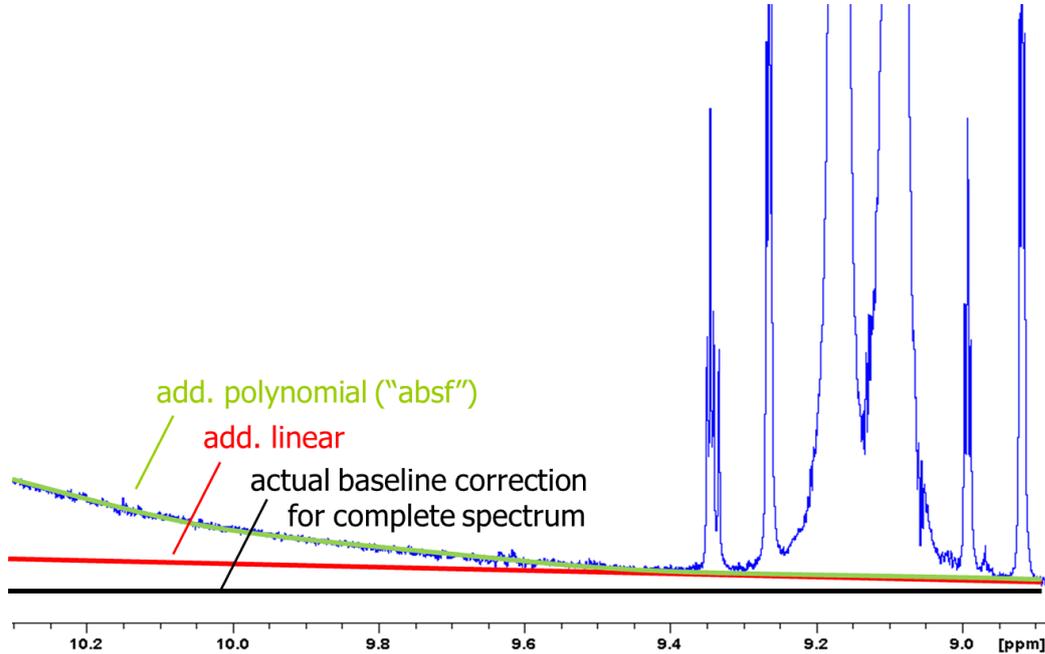


Figure 12: The effect of using different partial baseline correction.

Alternatively, “Multipoint Baseline Correction” in MNova can also be used (Figure 13).

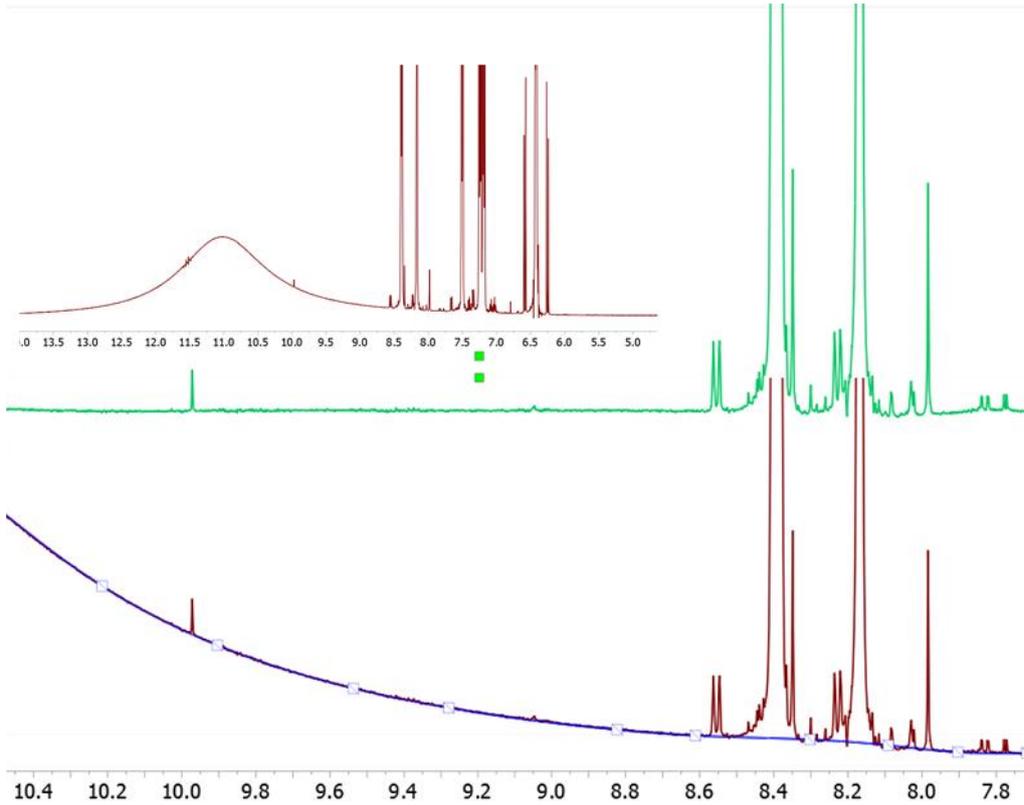


Figure 13: Application of Multipoint Baseline Correction for partial spectral regions.  
 Top: Typical situation where additional partial correction is required: very broad signal affects actual signals for integration.  
 Bottom: Actual spectrum region with placed points for the partial correction.  
 Middle (green): Spectrum after application of the "Multipoint Baseline Correction".

Correction of the integral's bias and slope must **never** be applied at any means since this cannot deliver objective numerical values.

## 5.4 Evaluation

### 5.4.1 Integration

The shape of the NMR signal is Lorentzian. To capture 99 % or 99.9 % of the total intensity of a signal with an integral, the integration limits have to be set to 64 or 636 times the half-width of the signal, respectively [8] (Figure 14).

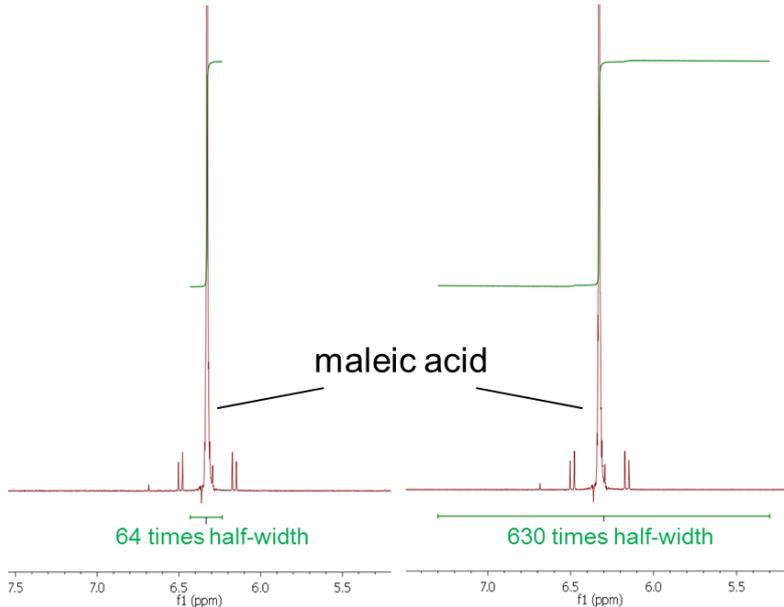


Figure 14: Illustration of the integration region related to the half-width of the maleic acid signal (at 500 MHz). Set to 64 times the half-width (left) covers 99 % of the signal intensity, respectively set to 636 times (right) covers 99.9 %.

$^{13}\text{C}$ -satellites appear for each signal of a proton attached to a carbon. According to the natural abundance of the  $^{13}\text{C}$  isotope both satellites together provide ~1.1 % of a signal's total area (Figure 15).

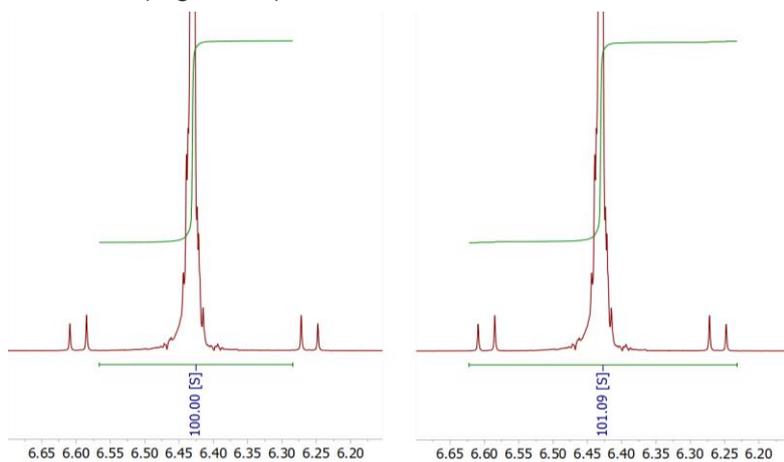


Figure 15: Integral region without (left) and with (right) covering the  $^{13}\text{C}$  satellites with a difference of 1.1 % for the integral value.

As a result, the integration regions must be set in a consistent way for all integrals (calibrants and analytes).

For the 1 % uncertainty level, it is acceptable to set the limits in-between the satellites in the region with a low slope (Figure 16). This provides some options to exclude impurity signals in the integral regions.

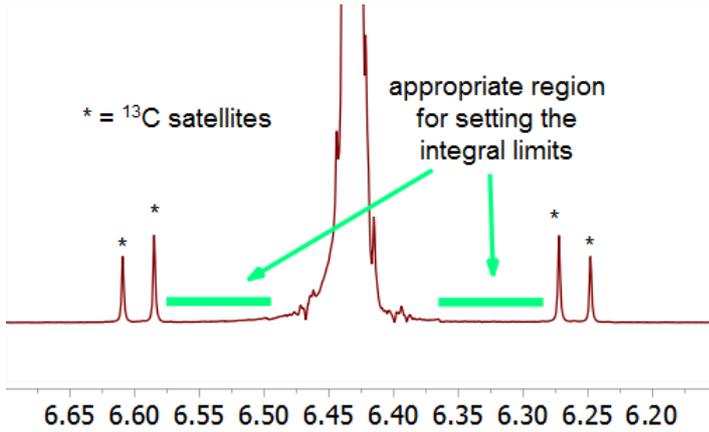


Figure 16: Appropriate regions for setting the integral limits.

[For achieving lower uncertainty levels, the aspects mentioned above must be considered even more carefully.]

Another option to avoid the problem is to broadband decouple the <sup>13</sup>C spectrum during the acquisition of the <sup>1</sup>H spectrum. Pulse sequences such as Bruker's zgig make doing this routine.

### The impact of the signal to noise ratio (SN)

The signal to noise ratio has a direct impact on the integral's precision. The relative standard deviation (RSD) can be assessed by using equation 2, which is based on the evaluation of experimental data [9]:

$$RSD = \frac{86}{SN} \quad \text{Equation 2}$$

RSD = relative standard deviation  
 SN = signal to noise ratio

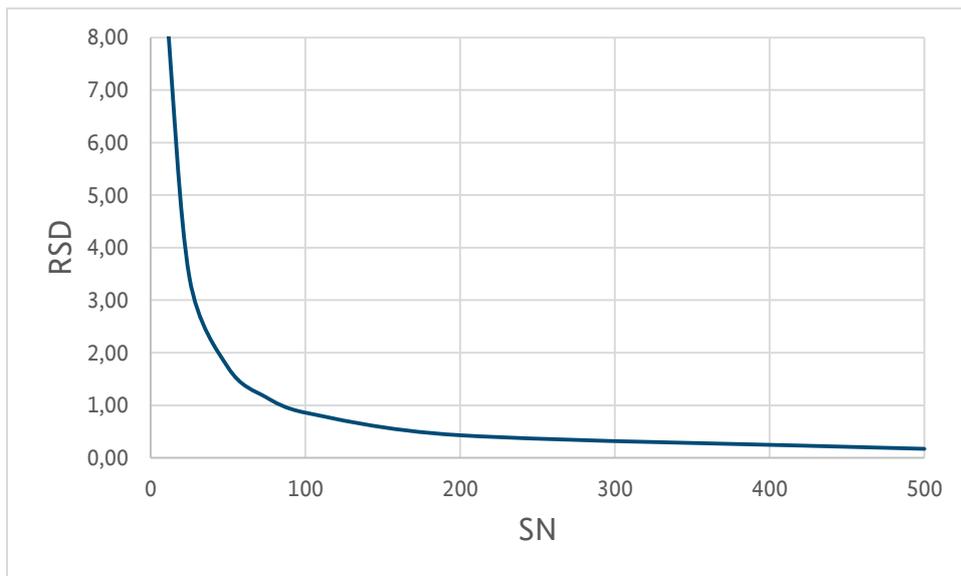


Figure 17: Relative standard deviation of integral values as a function of the signal to noise value.

This means that a SN of 86 would deliver an uncertainty contribution to the integration of already 1 %.

As a rule of thumb for the 1 % method's uncertainty level, the SN should be above 150 since there are also other error sources that contribute to the combined uncertainty.

SN	> 150
----	-------

#### 5.4.2 Alternative: Line Fitting

There are several line fitting tools available for the NMR spectrum evaluation. Very accurate results (< 0.5% uncertainty) can be achieved for example with the qGSD<sup>5</sup> tool of the Mestrelab software MNova.

However, the use of these algorithms requires more experience and control in order to assess the uncertainty contribution since they can only be applied on a case by case basis.

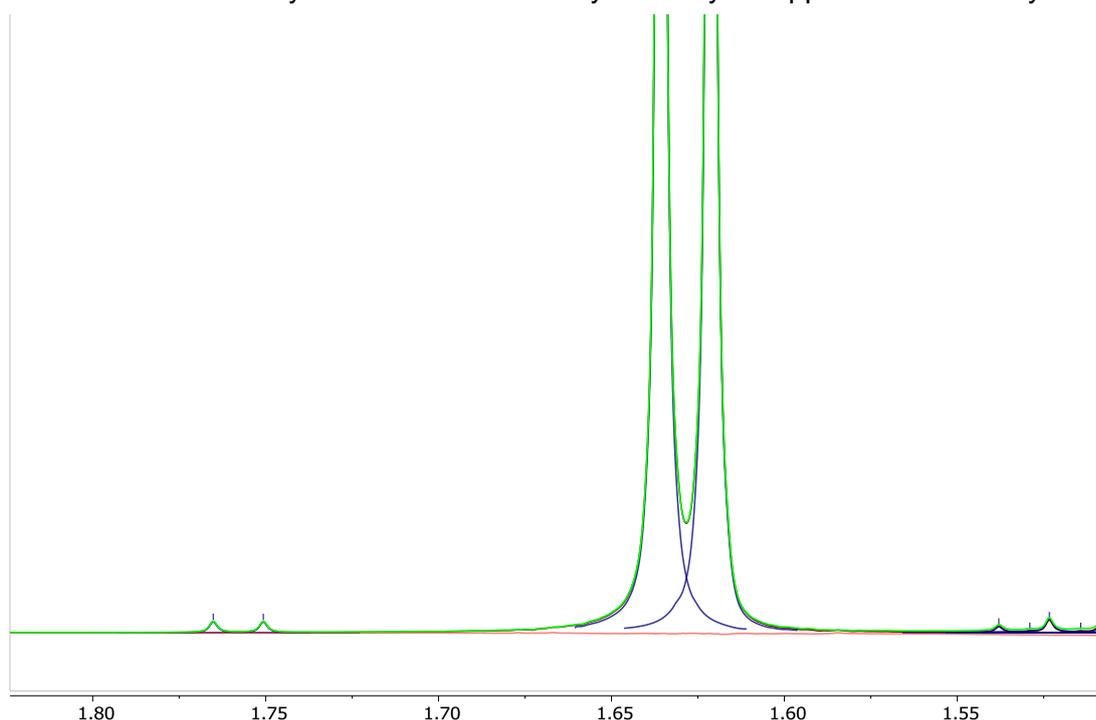


Figure 18: Signal group well fitted by qGSD in MNova. The actual signal (maroon) can no longer be recognized since it is covered by the sum of the fits (green). The single fits are in blue. The residual line (between actual signal and fit) is in red and it is in the same range as the baseline.

For example, the qGSD performance can be controlled by checking the residual line visually. In a good fit, the residual line should be almost equivalent to the baseline.

Alternatively, numerical values are given, e.g. “qGSD residuals (%)” which describe the overall difference between the intensity of the fitted signals and the actually detected intensity for a whole multiplet.

These residuals should be distinctively below 1.0 % for a good fit.

<sup>5</sup> qGSD = quantitative Global Spectrum Deconvolution

## 6 Calculation

The purity of the main component  $P_X$  (expressed in % g/g) can be calculated from the NMR intensity of peaks due to that component and the intensity of a calibrant peak from a reference material with known purity  $P_{Cal}$  according to equation 3:

$$P_X = \frac{I_X}{I_{Cal}} \cdot \frac{N_{Cal}}{N_X} \cdot \frac{M_X}{M_{Cal}} \cdot \frac{m_{Cal}}{m_{sample}} \cdot P_{Cal} \quad \text{Equation 3}$$

$I$  = integral  
 $N$  = number of spins belonging to the respective molecular unit  
 $M$  = molar mass  
 $m$  = mass  
 $P_{Std}$  = purity of the standard in % g/g

## 7 Quality Assurance

### 7.1 Validation

A detailed guideline for NMR validation is given by the EUROLAB “Guide to NMR Method Development and Validation – Part I: Identification and Quantification” [10]. This guide details how each validation parameter can be determined.

It is important to note that the NMR validation can be applied universally, as long as it can be assured that the six commandments for qNMR (chapter 2) are obeyed. One must keep in mind that, qNMR is a relative primary method!

Validation parameters, such as **limits of detection (LOD) and quantification (LOQ)**, just need to be determined for one substance exemplarily, since the NMR sensitivity is substance independent.

For transferring LOD/LOQ values from one substance to another substance, two things must be considered:

- 1) The molar masses of both substances
- 2) The multiplet pattern:  
The split of signals to multiplets leads to a reduction of the signal height and the signal to noise ratio, which is the relevant factor for the LOD/LOQ definition.

Table 1: Relative heights for different multiplets

number of lines	intensity distribution	relative multiplet height (RMH) of the largest multiplet peak
1 (singlet)	1	100 %
2 (doublet)	1:1	50 %
3 (triplet)	1:2:1	50 %
4 (quartet)	1:3:3:1	37.5 %

Example:

The validation was performed for the methyl group (doublet) of amphetamine ( $M=135.21$  g/mol).

The LOD/LOQ-values can be transferred to the methyl group (triplet) of methamphetamine ( $M=149.23$  g/mol) by using equation 4:

$$LOD_{new} = \frac{M_{new}}{M_{example}} \cdot \frac{RMH_{example}}{RMH_{new}} \cdot LOD_{example} \quad \text{Equation 4}$$

*LOD* = limit of detection  
*M* = molar mass  
*RMH* = relative multiplet height

Naturally, the multiplet's height depends also on additional factors, such as smaller long-range couplings and line-broadening due to different reasons. However, this has just a minor effect and can vary from sample to sample.

To be on the safe side, it is possible to multiply the LOD/LOQ values with a safety factor of "2" which should cover these (sometimes not assessable) influences.

The **linearity** is actually always a given for modern spectrometers. It should be proven just once for a hardware combination (spectrometer-magnet-probe) and this hardware test is typically conducted before the NMR console leaves the production factory. This test can be easily reproduced with one's exact probe and magnet combination in the laboratory. The linearity study only needs to be done using one compound.

The **working** range is covered by the LOQ and the solubility limit. The NMR measurement has no impact on this parameter.

**Trueness:** There is no systematic error for quantitative NMR spectroscopic analysis as long as the recommendations of this guideline are followed. This can be demonstrated once during the method validation by quantifying one substance with exactly defined purity.

The **precision** can also be determined completely substance independent.

The **selectivity** can be proven for some typical mixtures found in a standard forensic laboratory. Normally, a signal overlay can be recognized visually on the 1D-<sup>1</sup>H spectrum. The selectivity is not critical for a high-field spectrometer (500 MHz or more) and becomes more critical with lower frequency. In cases of doubt, additional NMR experiments (e.g. 2D-CH-correlation or COSY) can be performed to add another dimension to facilitate the recognition of impurity signals.

**The uncertainty** is the crucial validation parameter, as its specification always completes the measurement result. The uncertainty considers the trueness and precision. Since the trueness is usually given (no systematic error), the uncertainty is only defined by (and equals to) the precision.

## 7.2 Critical Quality Attributes and Analytical Control Strategy

The critical quality attributes for the whole analytical process need to be considered for planning the analytical control strategy.

Since NMR spectroscopy is generally a very robust technique, the amount of necessary controls is usually moderate.

The type of controls and the frequency of their performance must be adapted according to the laboratory's specific situation, for example:

- Sample preparation tools (balances, pipettes, etc.)
- Experience of staff
- Type of spectrometer (field strength, age, etc.)
- Evaluation software
- Calculation tools

Some tools already come with automatic control. This can be, for example, a self-calibrating balance or evaluation software that checks for the signal's quality on the actual analysis spectrum. These tools can reduce the amount of manual controls even more.

Some attributes, such as phase and baseline correction, may be checked by visual inspection of the spectrum.

For the control of the spectrometer performance, two **system suitability tests** are usually considered essential:

- 1) HUMP test: checks for correct shimming parameters
- 2) <sup>1</sup>H Sensitivity Test: checks the spectrometer's hardware performance

Analyzing a **control sample** which goes through the entire analytical process (with sample preparation) can cover a lot of quality attributes at once, including the weighing procedure. A control sample can be any substance or mixture with well-defined purity which can be quantified with the standard method.

The period of analyzing a control sample can be adapted according to the situation in the laboratory. For example, the period should be shorter if many different people are running the analysis. In general, the period can be expanded to some days or a few weeks.

As for all analytical procedures, the validity of the qNMR analysis should be proven by proficiency tests. There are just a few specific qNMR tests which are conducted irregularly. Information on new tests is posted on the webpage [ValidNMR.com](http://ValidNMR.com).

It can be recommended to participate in quantitative proficiency tests that are not initially designed for qNMR analysis, such as the tests organized by the ENFSI Drug Working Group. These tests are not designed for a specific technique. It is not necessary to participate in proficiency tests for every single analyte as long as the same validated method is used for the analysis of various substances.

APPROVED BY THE ENFSI BOARD ON 02.01.2020

## 8 References

- [1] Henderson, T. J.: "Quantitative NMR Spectroscopy Using Coaxial Inserts Containing a Reference Standard: Purity Determinations for Military Nerve Agents", *Analytical Chemistry*, 2002, 1, 191-8.
- [2] Wider, G.; Dreier, L.: "Measuring Protein Concentrations by NMR Spectroscopy", *Journal of the American Chemical Society*, 2006, 8, 2571-6.
- [3] Dreier, L.; Wider, G.: "Concentration measurements by PULCON using X-filtered or 2D NMR spectra", *Magnetic Resonance in Chemistry*, 2006, 44, S206-12.
- [4] ValidNMR : "Main Page - Welcome to the validNMR Wiki!", [http://www.validnmr.com/w/index.php?title=Main\\_Page](http://www.validnmr.com/w/index.php?title=Main_Page), 27. March 2019.
- [5] Mettler Toledo: "Weighing the Right Way", Greifensee, <http://lab.mt.com/gwp/waegefibel/waegefibel-e-720906.pdf>, 2008.
- [6] The United States Pharmacopeial Convention: General Chapter "Weighing on an Analytical Balance", 2018.
- [7] Wikipedia : "Ernst angle", [https://en.wikipedia.org/wiki/Ernst\\_angle](https://en.wikipedia.org/wiki/Ernst_angle), 02. April 2019.
- [8] Malz, F.: "Quantitative NMR-Spektroskopie als Referenzverfahren in der analytischen Chemie", Dissertation, Berlin, Humboldt-Universität, Mathematisch-Naturwissenschaftlichen Fakultät I, 2003.
- [9] Hays, A.P.; Schoenberger, T.: "Uncertainty measurement for automated macro program-processed quantitative proton NMR spectra", *Analytical and Bioanalytical Chemistry*, 2018, 28, 7397-400.
- [10] EUROLAB Technical Report 1/2014: „Guide to NMR Method Development and Validation – Part 1: Identification and Quantification”, [http://www.eurolab.org/documents/EUROLAB%20Technical%20Report%20NMR%20Method%20Development%20and%20Validation%20May%202014\\_final.pdf](http://www.eurolab.org/documents/EUROLAB%20Technical%20Report%20NMR%20Method%20Development%20and%20Validation%20May%202014_final.pdf), 27. March 2019

## 9 Annex

Comparison of abbreviations for parameters used in Bruker (TopSpin) and JEOL (Delta) software:

Parameter	Bruker	JEOL (Delta)
pulse angle	P1	x_pulse
acquisition time	AQ	x_acq_time
relaxation delay	D1	relaxation_delay
time domain	TD	x_points <sup>6</sup>
spectral width	SW	x_sweep
transmitter offset	O1P	x_offset
spectrum size	SI	FN
apodization function	EM	sexp
line broadening factor	LB	width

## 10 Amendments Against Previous Version

Not used

<sup>6</sup> „x\_points“ corresponds to the number of data points just for the real part, whereas “TD” corresponds to the real and imaginary parts of the same size, so:  $x\_points = TD / 2$