Guide to NMR Method Development and Validation – Part I: Identification and Quantification

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Foreword

Nuclear Magnetic Resonance (NMR) spectroscopy is one of the most versatile methods of analysis. Till the early 1970s, NMR spectroscopy was exclusively used for structure elucidation and purity testing of synthesized compounds. Nowadays, however, the range of successful applications covers identification and structure elucidation of organic and biochemical molecules, precise quantitative determination of individual analytes and multicomponent analysis as well as so-called "non-targeted screening" in combination with different chemometric techniques [1, 2]. Since NMR is a very selective analytical tool (each molecule has its own spectral fingerprint), not only quantification of ingredients is possible, but also comparison, discrimination or classification of foods, beverages and other consumer products can be achieved (e.g., authenticity evaluation, determination of origin and botanical variety of certain products) [1]. "Non-targeted" NMR analysis allows a fast and highly selective sample screening, from which much more information can be obtained than by using any other analysis technique that was previously used for this purpose [1].

NMR techniques have been an integral part of scientific research for a long time and are increasingly used in the monitoring of foods, beverages, cosmetics and pharmaceuticals. The special criteria proposed in this report should facilitate development of any practical application of a NMR method in these areas of applications. Furthermore, our suggestions are in accordance with quality management (QM) system required for governmental food control [3-5]. It should be mentioned, however, that the conditions listed in this report must always be adapted for the specific analytical problem, the most recent QM requirement and the matrix under investigation.

1. General conditions

For NMR analysis, the operating manuals of the respective equipment manufacturer have to be followed and the operation must be performed by qualified personnel.

1.1 Acquisition parameters

1.1.1 Homogeneous magnetic field

To obtain meaningful NMR spectra, a homogeneous magnetic field must be achieved with suitable shimming routines.

1.1.2 Temperature stability

A constant temperature with a maximum drift of 0.1 K has to be maintained during the acquisition of NMR spectra.

1.1.3 Data acquisition time

The data acquisition time must be chosen so that truncation of the Free Induction Decay (FID) is avoided.

1.1.4 Pulse angle

In NMR spectroscopy, pulse angles up to 90 degrees can be chosen for acquisition. The employment of 90 degree pulse in quantitative NMR leads to the maximum signal intensity with respect to the measurement time spent. In quantitative NMR using external calibration, the 90 degree pulse angle is preferably used.

The determination of the pulse angle must be uniform and can be done either by using an iterative, visual method or by automated methods [6].

1.1.5 Number of data points

In quantitative NMR (qNMR), the number of acquired data points must be large enough to describe the shape of a signal accurately. Ideally, more than 10 data points should be above the half of the peak height [7, 8].

1.1.6 Relaxation time

In qNMR, the longitudinal (spin-lattice) relaxation time T_1 has to be considered. T_1 is different for each signal of every compound and can also vary depending on the matrix (pH, salt content, etc.). To be on the safe side, the sum of the relaxation delay and the acquisition time should exceed five times the highest T_1 value of all evaluated signals. Theoretically, the factor 5 will lead up to 99.0 % of the maximum signal intensity, the factor 7 will lead up to 99.9 % [9].

1.1.7 Electronic amplification

The receiver gain (RG) must be set for each sample to be analyzed, so that no truncation of the FID occurs. This is particularly important for quantitative analysis. For chemometric methods and quantification by external calibration, it must be ensured that always the same signal amplification is used.

1.2 Processing

1.2.1 Smoothing

The exponential multiplication is widely used as apodization function for 1D NMR spectra. In the quantitative ^{1}H NMR, low smoothing factors (lb, line broadening) of 0.1-0.3 Hz are suitable. They result in an increase of the signal-to-noise (S/N) ratio without distortion of signal intensities ratio [9].

1.2.2 Phase correction

Phase correction can be performed automatically or manually. Automatic correction may not be sufficient for qNMR in some cases. Exact phase correction has to be ensured for accurate quantification.

1.2.3 Baseline correction

For qNMR, baseline correction is fundamental. A number of mathematical functions are available for this purpose. It is necessary to pay attention that only the baseline itself, but not the actual signal is corrected. In addition, partial baseline correction may be useful to correctly evaluate signals situated near very broad signals.

1.3 Quantitative analysis

1.3.1 Signal integration

To achieve accurate integration of signals, no interference of the analyte of interest with other signals must occur. The integral width should be chosen so that the same parts of the analyte and reference signals are used for integration (especially with/without ^{13}C satellites in ^{1}H NMR).

A manual correction of the integral parameter, known as “slope” and “bias”, should be avoided. However, this is typically not necessary if the baseline has been accurately corrected.

1.3.2 Alternative techniques

There are other alternative methods for signal evaluation. These include, for example, the evaluation of the signal heights [10] or signal deconvolution [11]. The necessary parameters of these methods as well as their limitations and uncertainties are not described in this report. However, to use these alternative methods one has to be aware of their application range and limitations.

2. Verification and identification

2.1 Single analytes

If only NMR spectroscopy is used for structure verification (e.g. comparing of sample composition with the list of ingredients), it is usually sufficient that all the characteristic signals are detected and assigned. The measurement of a single pulse 1D spectrum (^{1}H or ^{13}C -NMR) is sufficient in most cases. For identification and verification in complex cases the application of 2D correlation spectra (e.g. J-Resolved (J-Res), H,H-COSY, HSQC, HMBC) might become necessary. Depending on the analytical problem it could be also helpful to observe different nuclei (e.g. ^{15}N, ^{19}F, ^{31}P).

Ideally, a spectrum of a reference material should always be recorded under the same conditions as the analyte. In cases of spectral overlap between analyte and reference compound, the identification can be carried out optically by stacking both spectra. Reference spectra from databases and scientific publications can also be used for identification. Theoretical calculations of spectra using tables or prediction programs can be used as an auxiliary tool, but typically is not sufficient for unambiguous identification. In cases when no reference data are available, a complementary technique should be used. Mass spectrometry is especially suitable for this purpose [12]. The application of the complementary technique might be unnecessary if useful a priori information about the sample is available (synthesis control).

2.2 In mixtures

In mixtures or in natural matrices some characteristic signals of an analyte may be overlapped in the spectrum. Ideally, all signals of an analyte should be identified and assigned, for example, by comparison with reference spectra.

Depending on the matrix under investigation, it may be sufficient if only single signals of a particular compound can be clearly assigned. This is especially the case if extensive information about matrix ingredients is available (e.g. in the case of fruit juices). Identification of substances in mixtures can be facilitated by the application of 2D spectroscopy (especially HSQC and J-Res) or Diffusion Ordered Spectroscopy (DOSY) experiments.

Regarding the number of required identification points no general rule exists. For example, for sugars in fruit products one signal of an anomeric proton may be sufficient for identification. However, for the differentiation of synthetic drugs with similar structures almost all signals may be needed.

3. Quantitative NMR (qNMR)

Since NMR technique is a quantitative technique per se, it can be also applied for quantitative analysis considering the requirements of DIN/ISO 17025 [5]. Many common methods in analytical chemistry such as external quantification, internal quantification or standard addition are also applicable for NMR [13]. A great advantage of the NMR technique is the possibility of quantifying an analyte without reference substance by applying another reference standard.

3.1 Internal calibration

For internal calibration a defined amount of a reference material is added to the sample solution. The quantification is performed by comparing the integrals (or an equivalent response) of analyte (X) and reference (Std). The analyte concentration is calculated according to the following equation:

$$C_X = \frac{I_X}{I_{Std}} \cdot \frac{N_{Std}}{N_X} \cdot \frac{M_X}{M_{Std}} \cdot \frac{m_{Std}}{m_{sample}} \cdot P_{Std}$$

C Concentration
I Integral
N The numbers of protons generating the selected signals for integration
M Molecular weight
m Weight
P Purity

The utilized reference materials must meet the following minimum requirements [9]:

• known purity
• no overlapping signals in a given matrix
• precisely weighable (non-hygroscopic / non-volatile)
• chemically inert with respect to the solvents used and the matrix analyzed

The traceability of a certified reference material has to be ensured.

3.2 External calibration

Quantification can be also carried out by external calibration. A number of different methods are available for this purpose (e.g. coaxial tubes [14], ERETIC [15, 16], PULCON [17, 18], Quantas [19]). In the course of method validation all features and characteristics of the method used must be evaluated and analyzed. External calibration by a calibration curve with different analyte concentrations can be also used [20].

Increasingly PULCON is used for quantification in chemical and biological applications [17]. This principle method can also be used for quantification of analytes in food like fruit juice, wine or dietary supplements.

3.3 High-precision determination of analytical standards purity

The high-precision analysis (uncertainty of measurement < 0.3 %) of pure substances demands further requirements. All parameters must be optimized in terms of precision. As the precision is highly dependent on S/N, an extremely high S/N of more than 10,000:1 is required for such quantification [8].

4. Parameters for validation

Parameters that have to be validated for the specific method are summarized in the following table:

	Qualitative analysis	qNMR	determination of analytical standards purity (assay)
4.1 Trueness		X	X
4.2 Precision		X	X
4.3 Selectivity (Specificity)	X	X	X
4.4 Limit of detection	X	X	
4.5 Limit of quantification		X	
4.6 Linear range		X	X
4.7 Working range		X	X
4.8 Robustness	X	X	X
4.9 Measurement uncertainty		X	X

4.1 Trueness

If all important parameters are taken into account, no systematic errors should be found. For qNMR, the proof of trueness could be performed by comparing the purities of several certified reference materials or by the comparison with an independent method of known trueness.

4.2 Precision

Precision of the NMR method is determined by multiple measurements within a short time of several re-weighted samples of one matrix (including sample preparation).

For the determination of laboratory precision one sample should be analyzed several times in one laboratory by systematically changing parameters, such as operator, device or time.
If inter laboratory precision (reproducibility) is required, it can be determined by comparative measurement of a sample in different laboratories.

4.3 Selectivity

NMR spectroscopy provides very detailed structural information and, therefore, it possesses in principle, high specificity. However, specificity may not be achieved, if, for example, necessary signals for identification cannot be assigned due to signal overlap.
qNMR is considered to be selective when it can be ensured that the analyzed signal does not overlap with other components. It is sufficient to prove the specificity of the method for some most critical examples.
If alternative methods are used for the quantitative evaluation (1.3.2), the selectivity requirements can be met despite the signal overlap. This must be proved in each particular case.

4.4 Limit of detection (LOD)

Limit of detection (LOD) is an important validation parameter relevant for the identification of an analyte. At the concentration of LOD positions and shapes of all relevant analytes' signals in the spectrum can be visually recognized [21]. The detection limit can be determined mathematically or visually from the spectrum. Mathematically LOD can be determined for the relevant signal as intensity for which S/N equals 3. The visual way for LOD determination is shown on Fig.1 on the ephedrine-HCl as an example:

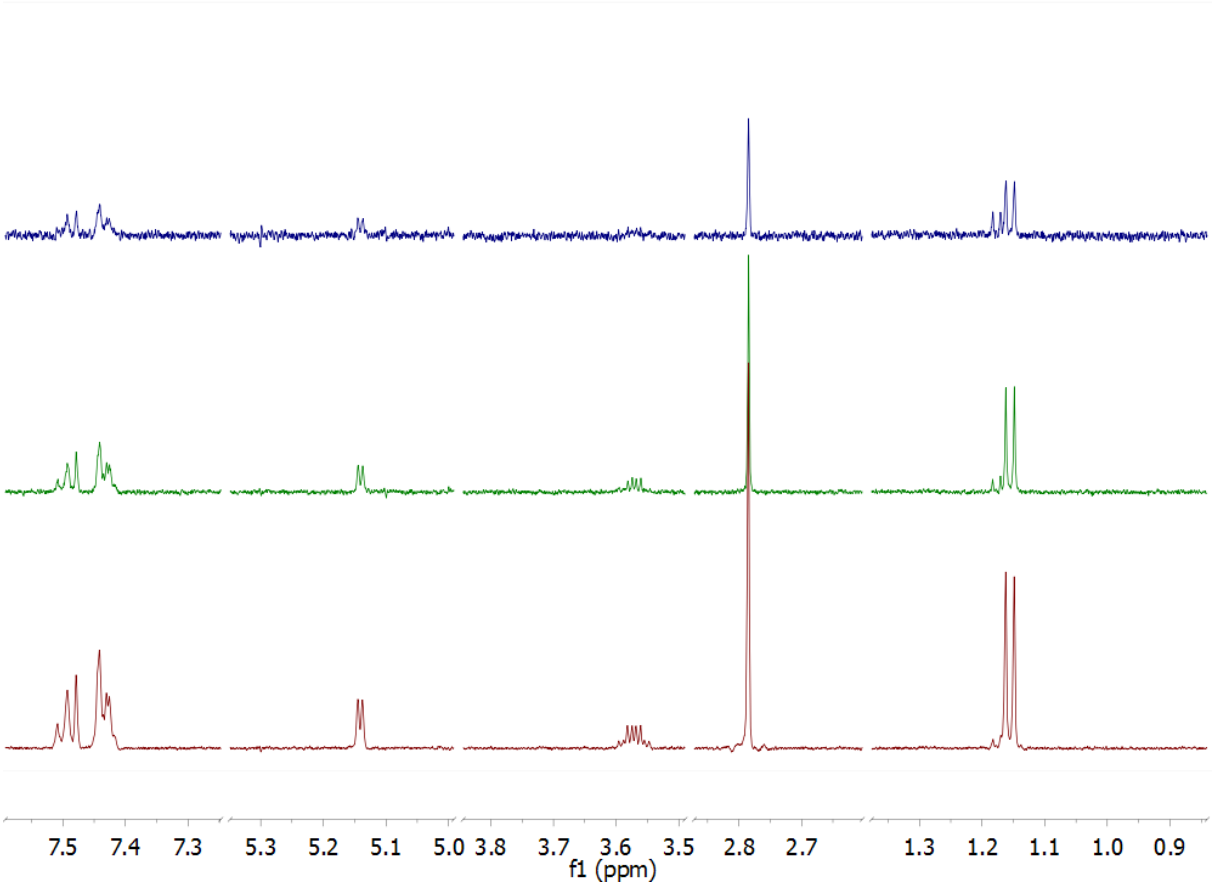

Fig.1: Signals of ephedrine-HCL of 0.084 mg (bottom), 0.025 mg (middle) und 0.007 mg (top).

It can be seen that ephedrine signals can be clearly visually identified at 0.025 mg (Fig.1). Therefore, this amount can be regarded as the detection limit for this compound.

It should be noted that the signal intensity is dependent on the signal's shape (line width, coupling pattern) and on the number of corresponding nuclei. Apart from that, signal intensity is proportional to the concentration of substance (if all requirements for qNMR are fulfilled).

If common quantification routines are used (valid for any analyte in a given matrix), it is sufficient to determine the LOD (and also limit of quantification, LOQ) for a single analyte in a matrix. The detection limit for another analyte can be assessed from this value by considering different signal shapes.

The limits of detection (LOD) and the limits of quantification (LOQ) can be also calculated based on the standard deviation of the response and the slope of the calibration curve. This procedure is also approved by the German Institute for Standardization and is regarded as a method, from which reliable and practically meaningful values can be obtained [22]. These calibration curves can be constructed based on the integration of specific resonances for each

compound and known analyte concentration (if matrix effect should be taken into account, spiked solutions can be used).

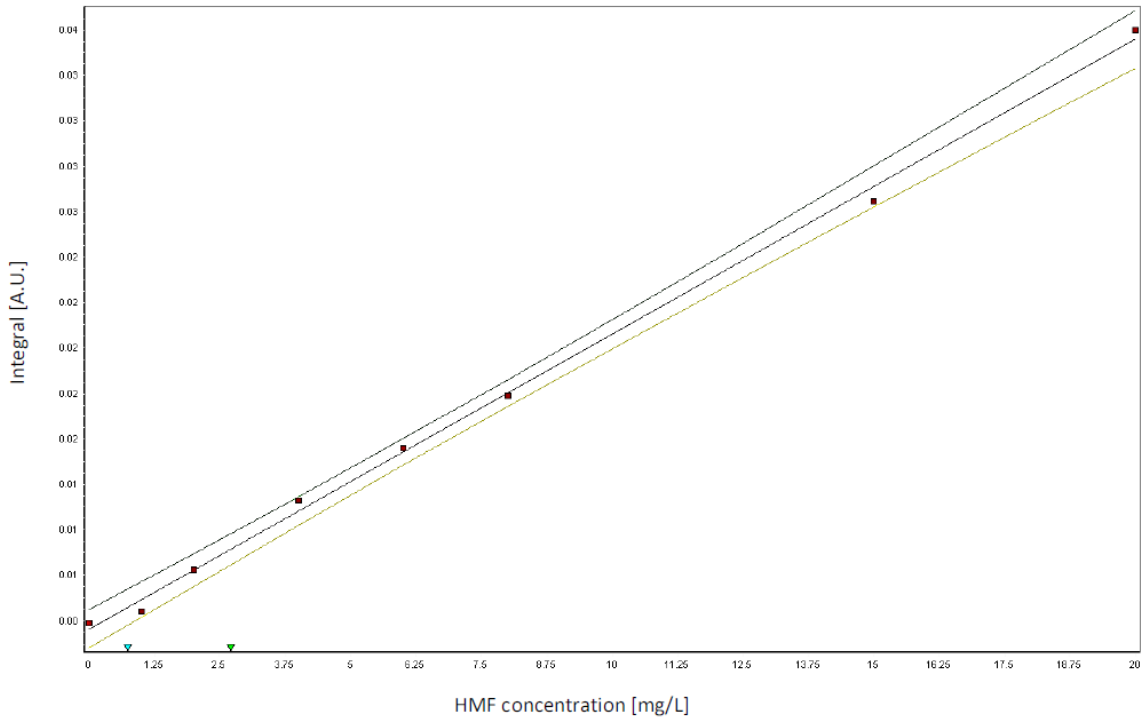

Fig.2: Calibration curve for HMF in apple juice matrix near LOD/LOQ values with 95% prediction limits.

4.5 Limit of quantification (LOQ)

Limit of quantification (LOQ) is the concentration of analyte, for which the selected signal exhibits the minimal required S/N for the given method precision [21]. The minimum required S/N can be determined experimentally by means of multiple measurements at different dilutions. The relative standard deviations are determined in each case. The minimum S/N is then equal to the concentration, for which acceptable standard deviation has been obtained. This standard deviation should be oriented on the standard measurement uncertainty of the method (Table 1).

See also 4.4 for calibration curve method [22].

Table 1 Values of measurement uncertainty as the function of S/N as an example [23].

S/N	Relative measurement uncertainty in %
30	10
60	3
150	1
400	0.5
1,200	0.1
10,000	0.07
20,000	0.05

4.6 Linear range

Due to the high dynamic range of modern NMR spectrometers the linearity is ordinarily an uncritical parameter. The signal intensity is proportional to the number of nuclei.
The linearity of the NMR spectrometer used must be validated only once by using at least 5 different concentrations of any readily soluble substance. The chosen concentrations should cover the working range of the method. The linearity should be evaluated by appropriate statistical methods, such as calculation of a regression line by the method of least squares [21]

4.7 Working range

The working range of an NMR method usually starts from the LOQ (lower limit) to the analyte solubility (upper limit) for an analyte in a given matrix. This standard procedure is suitable for each analyte in a given matrix. For another matrix, it must be ensured that the analyte has been completely dissolved and that no complex formation / aggregation / degradation takes place. This can be done by visual inspection of the solution or, if insoluble residues of other components remain in solution, through multiple measurements at different concentrations and by comparison of actual and reference line widths of the signals of interest. For critical analytes or in complex matrices the linearity of the working range should be additionally checked.

4.8 Robustness

For the validation of the robustness, possible influences of different sources must be considered and consequently examined (e.g. pH values, high salt concentrations of the sample matrix, reactive chemicals). The robustness checks for such possible impacts on the final result.

4.9 Measurement uncertainty

The measurement uncertainty results from the previously mentioned performance characteristics such as trueness and precision [24,25]. The uncertainty of measurement predicted by the uncertainty budget must correspond to the uncertainty of multiple determinations of concentration of analyte in sample. The method is said to be in statistical control when this condition is accomplished

When no systematic error is detected, the measurement uncertainty is equal to the precision. In other cases, the calculated analyte concentration should be adjusted by estimated recovery values. For example, degradation up to 50% of spiked concentration for some substances (acetaldehyde, acetoin, lactic acid and pyruvic acid) in apple juice matrix was observed, leading to unacceptable recovery values. The calculated concentrations were corrected using results from the degradation studies in water [26].

Alternatively, the measurement uncertainty can be calculated from the combination of specific individual factors (e.g., weighing, preparation, measurement, calculations, etc.) [24,25].

The uncertainty of reference material´s purity must be also taken into account when it is of the same order of magnitude as the overall method´s uncertainty.

The confidence interval has to be reported together with the uncertainty value.

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