Quantitative NMR Spectroscopy

1. Introduction

These notes summarise procedures for the acquisition and processing of quantitative ^{1}H, ^{19}F, ^{31}P, and ^{13}C NMR data. It is important to note that quantitative NMR (now referred to commonly as qNMR) is not simply a matter of collecting a 1D spectrum and comparing the integrals. A number of parameters must be optimised in order to obtain accurate and precise quantitative results.

There are essentially two types of quantitative NMR:

1. Relative concentration determination
   For relative concentration determination, you are comparing integrals of interest with one another. This will allow you to measure accurate ratios of the different species, if not the actual concentrations themselves.

2. Absolute concentration determination
   For absolute concentration determination, you are comparing the integrals of interest to a known concentration standard, and deriving from these absolute values of concentration, yield and purity. The concentration standard will be referred to as the *calibration compound* or *calibrant*, to distinguish it from a *reference compound*, which may be employed for chemical shift referencing.

2. Relative concentration determination

Relative concentration determination is the most common type of quantitative NMR experiment in organic chemistry, and the easiest to set up. This method will give you the ratios of compounds in a mixture. Typical applications include purity evaluation, and isomer ratio determination.

When preparing your sample, ensure that your compound is soluble in the solvent selected, the concentration is high enough to give good S/N, and that the sample volume is appropriate for NMR. No calibrant is required.

3. Absolute concentration determination

For absolute concentration determination, in addition to the points above concerning sample preparation, your sample should contain a known amount of a calibration compound. All compounds should be weighed as accurately as possible. The calibrant does not need to be structurally related to the analyte of interest, but it does need to contain the nucleus of interest and to have resonances that do not overlap with those of the analyte. It is also beneficial if the calibrant produces relatively simple NMR spectra, with only singlet resonances. Additional requirements for calibration compounds are that they should be chemically inert, highly soluble in the solvent used, have low volatility, and not have excessively long T_1 relaxation times. It is also beneficial if the

calibrant exists in pure form and is easily weighable. Several common ^{1}H quantitative NMR calibration compounds have been proposed, and some of the more popular examples are found in Table 1; further examples can be found at http://www.sigmaaldrich.com/analytical-chromatography/analytical-standards/application-area-technique/organiccrm.html.

Please speak to a member of the NMR staff if you require help in selecting an appropriate concentration calibration compound.

If there is no suitable choice of calibrant available, or if sample preparation is an issue, it may be possible to use an external calibrant. For this, consult a member of the NMR staff.

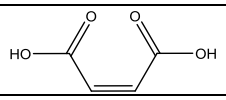
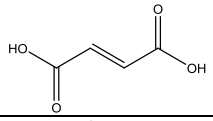
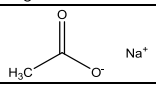
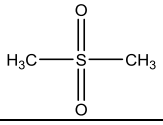
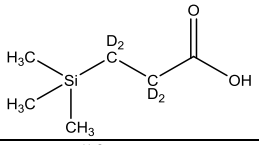
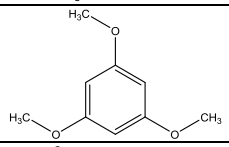
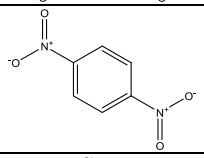
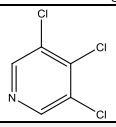
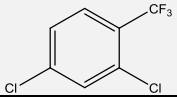
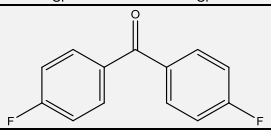
				Solvent solubility			
Calibration Standard	Structure	Molecular weight (g/mol)	δ (ppm)	D_2O	DMSO-d_6	CD_3OD	$CDCl_3$
Maleic acid		116.07	6.0-6.3	✓	✓	✓	✗
Fumaric acid		116.07	6.6-6.8	✓	✓	✓	✗
Sodium acetate		82.03	1.6-1.9	✓	✓	✓	✗
Dimethyl sulfone		94.13	3.0	✓	✓	✓	✓
TSP-d_4		150.29	0.0	✓	✓	✓	✗
1,3,5-trimethoxybenzene		168.19	6.1 3.7-3.8	✗	✓	✓	✓
1,4-dinitrobenzene		168.11	8.3-8.5	✗	✓	✓	✓
3,4,5-trichloropyridine		182.43	8.5-8.8	✗	✓	✓	✓
2,4-Dichlorobenzotrifluoride		215.00	-62 (19F)	✗	✓	✓	✓
4,4'-Difluorobenzophenone		218.2	-106 (19F)	✗	✓	✓	✓

Table 1: Properties of selected ^{1}H and ^{19}F calibration standards. Chemical shifts provide a guide but are solvent dependent within these ranges.

4. Signals to be considered for quantification

When analysing a spectrum with multiple peaks, the signal used for quantification should be unambiguously assigned. In addition, the signal should be as simple as possible, i.e. a singlet is superior to a multiplet, and have as little overlap as possible with other peaks. Exchangeable protons, such as amides or hydroxyls, should not be used for quantification due to their broadness and susceptibility to sample conditions. If signal overlap is an issue, it may be possible to address this by changing the solvent or pH, or through the use of auxiliary shift reagents.

5. Acquisition

5.1 Parameter sets

Specific parameter sets are available on the spectrometers for quantitative experiments. Please speak to the NMR staff if you require other nuclei to be defined.

The ^{1}H and ^{19}F experiments do not use ^{13}C decoupling by default. This means that when integrating your peaks (see below) one needs to be consistent in either including or excluding the one-bond satellites for every peak considered. The optional use of ^{13}C decoupling removes the satellite peaks and prevents their overlap with other peaks which may interfere with accurate quantitation; speak with the NMR staff if you think this is necessary- in most cases it is not required. The ^{13}C and ^{31}P experiments must use inverse-gated decoupling, thereby preventing signal enhancement through NOEs.

5.2 Shimming

After inserting the sample in the magnet, wait at least five minutes for the sample temperature to equilibrate. Tune and shim your sample then run an initial 1D spectrum. Accurate shimming is essential for quantitative NMR, so if the spectrum shows evidence of poor shimming (i.e. poor resolution, broad or unsymmetrical peaks), then re-do the shimming. It is advisable to turn spinning off to prevent artefacts known as “spinning sidebands”. If poor shimming is still observed after multiple attempts, remove your sample from the magnet and check that the sample volume is acceptable, the sample is properly mixed and that the tube is clean.

5.3 Relaxation delay

It is crucial to ensure that all signals have relaxed fully between pulses. Use a relaxation delay (“d1”) of at least five times the T1 of the slowest relaxing signal of interest in the spectrum. A major problem here is that the relaxation times of the nuclei in the sample are often not known, although they can be quickly estimated with an inversion recovery experiment. T1 values for ^{1}H nuclei in medium-sized molecules typically range from 0.5 to 4 seconds, while those for ^{13}C can range from 0.1 to tens of seconds for quaternary carbons. For very long ^{13}C relaxation times, it may be necessary to add a relaxation agent.

As an example, for a quantitative proton experiment, if the longest ^{1}H T_1 in the sample is 4 seconds, the recycle time should be set to at least 20s. In practice, however, the recycle time is the sum of the relaxation time and the acquisition time. So if the acquisition time is 5s, the relaxation delay can be set to 15 s, giving 20s in total.

If the relaxation times are not known, the following d1 values are suggested:

^{1}H: 30s
^{19}F: 30s
^{31}P: 30s
^{13}C: 60s

5.4 Spectral window

There should be sufficient "empty" space on either side of the spectrum so as to ensure that signals of interest are not affected by attenuation due to receiver filters. A large spectral width also helps with baseline correction. As a rule of thumb, the spectral width should be set so that the ends of the spectrum extend by approximately 10% "empty" space on each side.

5.5 Digital resolution

For accurate integration, each peak should be described by a minimum of four data points covering the linewidth, and preferably many more. The digital resolution (the spacing between data points) is defined by the spectral width ("SW") divided by half the number of acquired points ("TD"), which also equates to the inverse of the acquisition time (1/AQ). For a line-width of 1 Hz, which is typical for small to medium-sized molecules, the minimum digital resolution should therefore be 0.2 Hz, corresponding to an acquisition time of 5s.

The digital resolution in your spectrum can be determined using the command "fidres". Having previously defined an appropriate spectral width, the digital resolution should then be set to at least sw / (td/2) Hz using the parameter "td".

5.6 Signal to noise

Good signal to noise is essential for accurate integration. For integration errors < 1%, a S/N of at least 250:1 is required. The number of scans should therefore be set so as to ensure a good S/N. This will depend on the concentration of your sample. In order to calculate the signal to noise ratio in your spectrum, use the "sino" command in topspin.

5.7 Excitation bandwidth

The uniform excitation of resonances is also an essential requirement for qNMR. For ^{1}H spectroscopy this is readily achieved using standard parameters, but for nuclei displaying greater chemical shift dispersion some caution is required. This is a particular problem for ^{19}F

NMR due to its high frequency and larger chemical shift range, but can also be problematic for ^{13}C NMR. In such cases, it is advisable to compare resonances that have similar chemical shifts (and to choose calibrants that will enable this) and to place the centre of the spectrum (defining the transmitter excitation frequency "O1P") at the midpoint of the peaks to be compared. Alternative sequences employing excitation with frequency-swept adiabatic pulses may provide better results; speak to the NMR staff for assistance.

6. Processing

6.1 Zero filling

Ensure that you use zero filling, as this will result in increased integral precision. In order to use zero filling it is necessary to set the size of the real spectrum ("SI") to be a multiple of the number of data points used ("TD"). Setting SI = TD is the minimum amount of zero filling, and is usually sufficient.

6.2 Line broadening

It is advisable to use line broadening, particularly if the spectrum has low S/N. Normally an exponential weighting function is used. This is controlled by the parameter "LB", which by default is set to 0.3 Hz for ^{1}H and ^{19}F, and 1 Hz for ^{13}C.

Increasing the line broadening will increase the S/N, but decrease the resolution. Conversely, decreasing the line broadening will increase the resolution at the expense of the S/N. Values between 0.2 to 1 Hz are typical for ^{1}H and ^{19}F spectra. Higher values are used for ^{13}C spectra, usually in the range 1 – 5 Hz. Therefore, if increased resolution or S/N is needed, it may be possible to achieve this by changing the line broadening.

6.3 Accurate phasing

The spectra are phased automatically (eg. The Bruker "apk" command). However, the phasing should be checked to make sure that it is optimal (see figure 1). If not, the phasing should be altered manually.

6.4 Baseline correction

A flat baseline is important for accurate integration. The baseline is corrected automatically once the spectrum is completed (the Bruker "abs n" command). However, the baseline should be checked manually, and if necessary corrected (see figure 1).

6.5 Define appropriate integration regions.

In order to cover 99 % of the peak area, the integration region should cover at least 20 times the line width in each direction (figure 2). The integral limits can be decreased if signal overlap is an issue, however it is important to use the **same** integral limits for all peaks. For example, if a peak integral is measured to +/- 50 Hz on each side, all other peaks must also

be measured to +/- 50 Hz. In addition, if ^{13}C satellites or spinning sidebands are included in the integral region for one peak, they should be included for all other peaks measured. If signal overlap is too severe to allow appropriate integration regions, peak deconvolution can be used (for example in mestreNova).

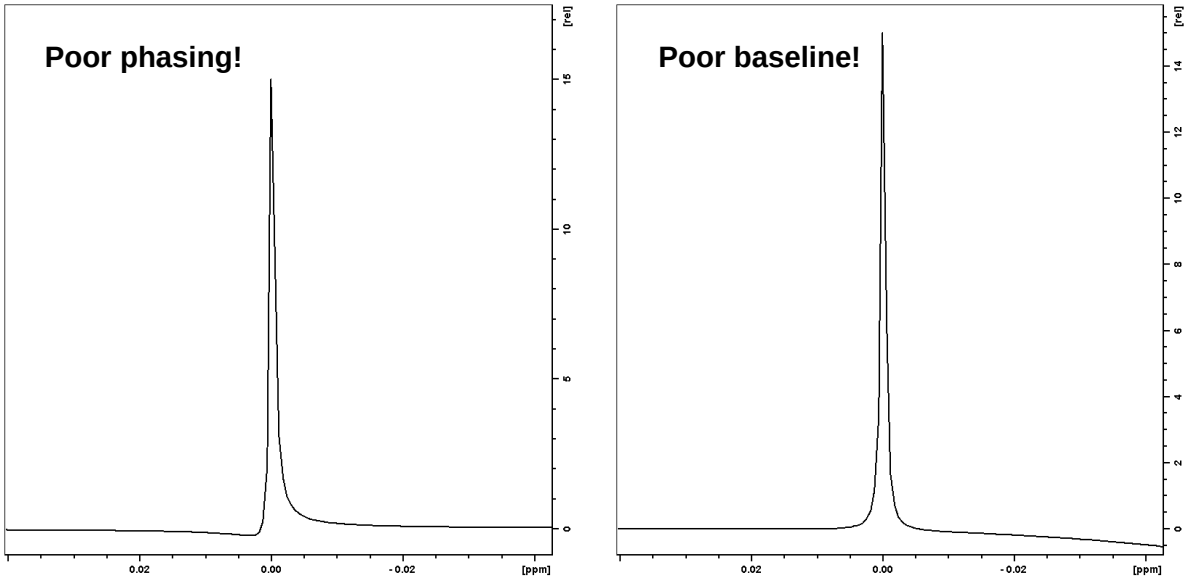


Figure 1: Examples of poor phasing and poor baseline.

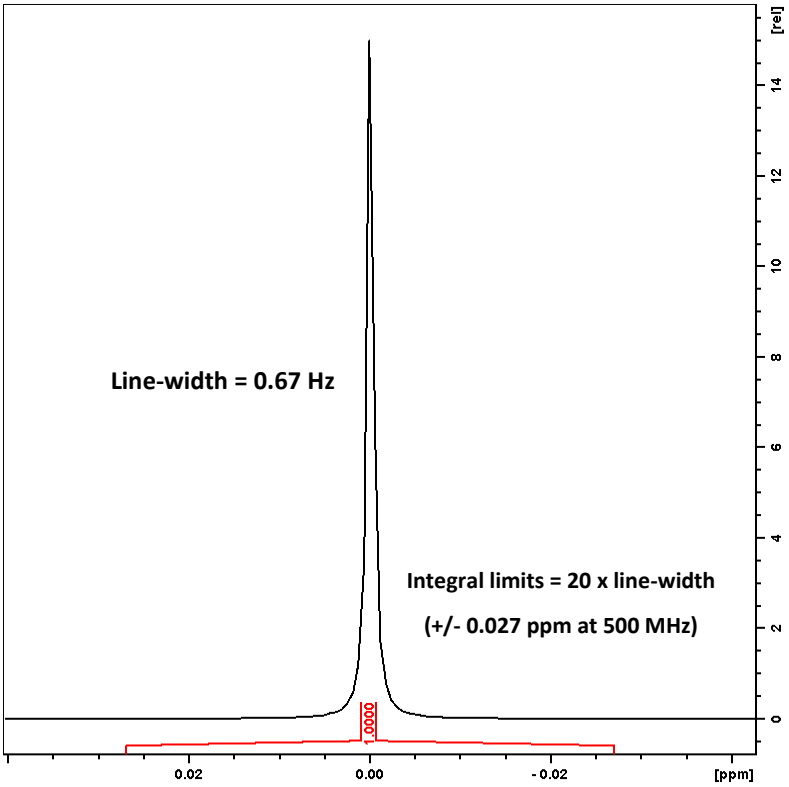


Figure 2: Peak integration- the integrated region should include at least +/- 20x peak half-height linewidth (Hz).

7. Analysis

7.1 Relative concentration determination

Essentially you need to integrate the signals of interest, normalise for the number of protons, and perform a simple ratio analysis. The molar ratio Mx/My between two compounds x and y can be determined using the formula:

$$\frac{Mx}{My} = \frac{Ix}{Iy} \cdot \frac{Ny}{Nx}$$

where I is the integral, and N is the number of nuclei giving rise to the signal.

7.2 Absolute concentration determination

This analysis can yield the concentration of the analyte or can directly provide a measure of its purity.

7.2.1 *Molar concentration*

The signal(s) corresponding to the calibration compounds must be integrated and normalised according to the number of protons giving rise to the signal. The integrals of the compound(s) of interest must then be compared with those of the calibration compound. The concentration of a compound x in the presence of a calibrant can be calculated using the following formula:

$$C_{\mathrm{x}} = \frac{I_{\mathrm{x}}}{I_{\mathrm{cal}}} \times \frac{N_{\mathrm{cal}}}{N_{\mathrm{x}}} \times C_{\mathrm{cal}}$$

where I, N, and C are the integral area, number of nuclei, and concentration of the compound of interest (*x*) and the calibrant (*cal*), respectively. This assumes that the purity of the calibration compound is 100%. If the purity of the calibrant is not known, it may be useful to do a purity analysis first.

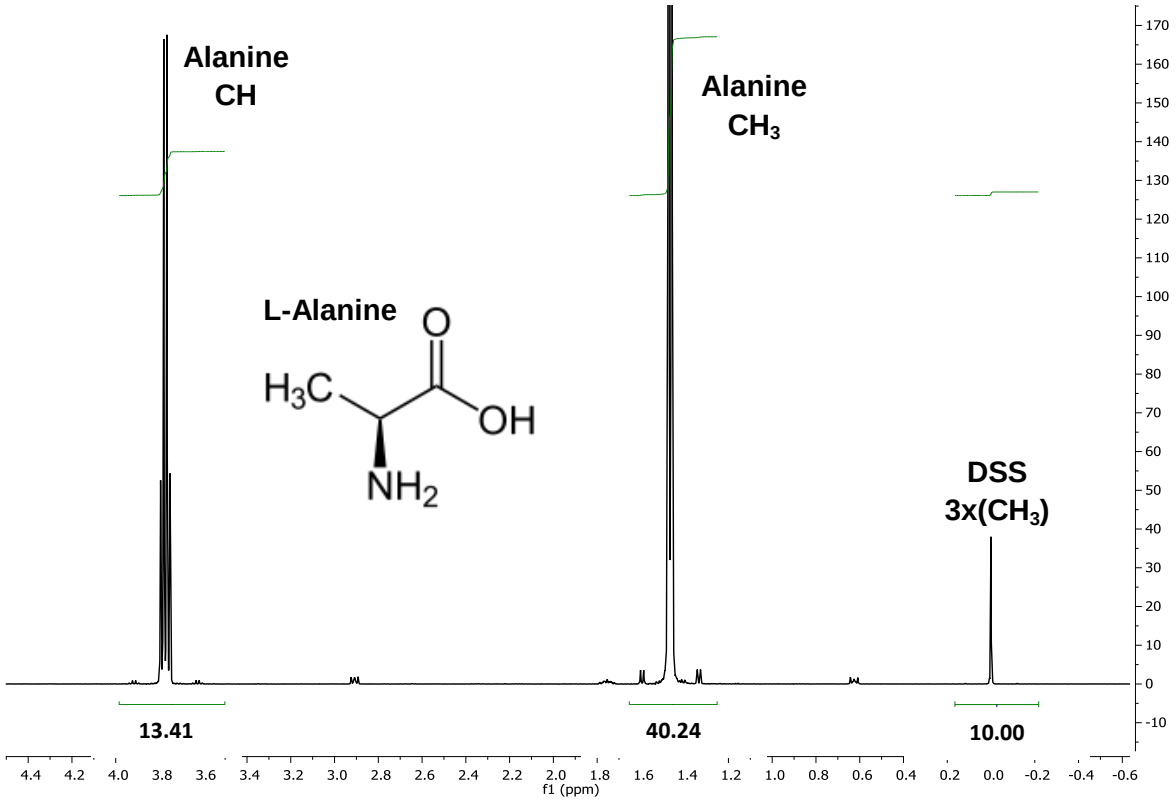
7.2.2 *Purity / yield determination*

The purity of a compound x, as a percentage of its nominal weight, can be determined using the following formula:

$$Px = \frac{Ix}{Ical} \times \frac{Ncal}{Nx} \times \frac{Mx}{Mcal} \times \frac{Wcal}{Wx} \times Pcal$$

where, I, N, M, W and P are the integrated area, number of nuclei, molecular mass, gravimetric weight and purity of the compound of interest (*x*) and the calibration compound (*cal*), respectively. Because this is a weight-based % purity, it allows you to evaluate the purity even if other components in the sample are invisible by NMR.

As an example, figure 3 illustrates a purity determination of a commercial sample of the amino acid alanine. A nominal mass of 1.78 mg alanine was tested, using 1.2 mg DSS (purity = 98%) as an internal calibration standard.



Compounds

Alanine: M_x = 89.09 g/mol, W = 3.56 mg. Nominal mass = 1.78 mg.
DSS: M_{cal} = 196.34 g/mol, W_{cal} = 0.64 mg, P_{cal} = 98%

Integrals

Alanine: I_x = 53.65 (Sum of C-H and CH3 proton signals), N_x = 4
DSS: I_{cal} = 10, N_{cal} = 9

Purity Evaluation

P_x = 96.5%

Figure 3: Example of purity evaluation using quantitative ^{1}H NMR.