1D Quantitative Experiments and Key Areas to Consider During Analysis

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Background

- Quantitative NMR (qNMR) is a vital tool in the pharmaceutical industry as it can be used to determine the purity of all stages of the process from raw materials to the final drug product.
  - It can also be used for in process checks (IPCs), reaction completion, residual solvents and molar concentration, ensuring efficacy and patient safety at all stages of the drug development lifecycle.
  - The main nuclei used in Almac Sciences for quantitative applications are ^{1}H, ^{13}C, ^{19}F and ^{31}P.

Relative *vs* Absolute Quantification

ABSOLUTE	RELATIVE
Calculates the absolute concentration, yield or purity of a compound	Analyses the ratio of components of the same spectrum
Uses a standard of known concentration	No external standard required
Result is dependent on weight and requires accurate weighing	Not weight dependent
PROS: Gives a true concentration and considers the entire sample composition	CONS: Only provides ratios and may not take the entire composition of the compound into consideration
CONS: Requires accurate weighing, careful standard prep and standard selection	PROS: No weighing or addition of standard necessary so it's easier to set up and a faster analysis process

Purity/ Yield Determination *via* absolute quantification:

$$\text{Result (\%w/w)} = \frac{Int_{sample} \times n_{std} \times Wt_{std} \times MW_{sample}}{Int_{std} \times n_{sample} \times Wt_{sample} \times MW_{std}} \times Purity_{std}$$

Std = standard, Int = integrated area, n = number of nuclei, Wt = mass of compound, MW = molecular weight

30° *vs* 90° Pulse

During analysis, the Radiofrequency (RF) pulse tips the net magnetisation vector away from the z-axis into the transverse plane (Figure 1):

- 90° Pulse: Rotates magnetisation fully into the xy-plane → maximum signal.
- 30° Pulse: Rotates magnetisation partially → smaller signal but faster recovery.

There are Pros and Cons of each type of pulse. With 30° best suited for maximizing throughput, and 90° best suited for maximizing signal to noise.

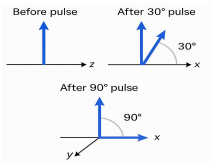

Figure 1: Diagram depicting 30° Pulse vs 90° Pulse

T_1 Analysis

Determines the amount of time taken for the net magnetisation to return to its equilibrium state after being disturbed by an RF pulse.

- 90º Pulse: Recovery to equilibrium is maximal and gives the maximum recovery of signal.
- 30º Pulse: Recovery is smaller and faster because the system starts closer to equilibrium.
- Can be determined via:
  - Peak intensity: The height of the peak is measured.
  - Peak area: The integrated area under the peak is measured; preferred for quantitative analysis.
- Depending on degree of quantification required, and whether a 30º or 90º pulse has been applied, a relaxation delay (D1) will be incorporated into the pulse sequence.
  - This D1 will be the T_1 value multiplied by a factor of ≥3.

Decoupling

In ^{1}H NMR, protons often couple with neighbouring nuclei (e.g. ^{13}C), resulting in ^{13}C satellite signals due to $^{1}J_{CH}$ coupling to the 1% naturally abundant ^{13}C nucleus.

Decoupling simplifies spectra by removing these satellites, resulting in less complex spectra for easier analysis, with better signal to noise.

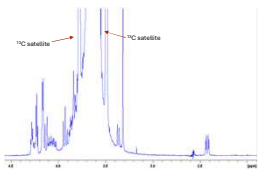

Figure 2: Partial ^{1}H spectrum without ^{13}C decoupling (zg30)

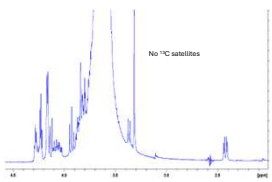

Figure 3: Partial ^{1}H spectrum with ^{13}C decoupling (zgig30)

Key Parameters for Setting Up Quantitative Experiments

Signal to Noise Ratio (S/N)

- An S/N >150:1 allows peak integrations with standard deviation of <1%, which is required for accurate quantification

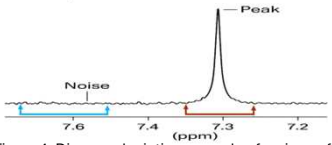

Figure 4: Diagram depicting example of regions of signal and noise in a spectrum

Acquisition Time (AQ)

- Length of time FID is acquired for
  - Longer AQ gives higher resolution and improves S/N

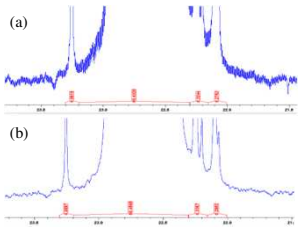

Figure 6: Spectra with (a) short AQ and (b) longer AQ

First Transmitter Frequency (O1P) & Spectral Width (SW)

- O1P is the midpoint of the observed spectrum
  - Important parameter to consider for nuclei with large chemical shift ranges (e.g. ^{19}F or ^{31}P)
- SW determines the bandwidth observed during the experiment
  - Ensure all peaks of interest are included in spectrum by setting SW wide enough

Figure 8: Correlation between O1P and SW

Number of Scans (ns)

- S/N increases with $\sqrt{ns}$

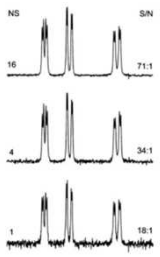

Figure 5: relationship between ns and S/N

Dummy Scans (ds)

- Ensures spin system is at a steady state before data collection

Relaxation Delay (d1)

- Delay between end of acquisition and next Rf pulse to ensure net magnetisation has returned to equilibrium
  - Set to at least 3x longest T_1 of the system

Receiver Gain (RG)

- Determines the amplification applied to FID signal
  - Correct RG setting ensures signal is strong enough for good S/N without distortion

Second Transmitter Frequency (o2p)

- This is the centre of the frequency range for the broadband decoupling.

Size of FID (TD)

- The FID is a time-domain signal that decays towards zero intensity at an exponential rate
  - The size of the FID directly affects the resolution of the NMR spectrum, with longer decay times resulting in narrower lines and higher resolution

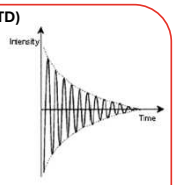

Figure 7: Example of FID

Size of Spectrum (SI)

- Determines the number of data points in the processed spectrum after Fourier Transform.
  - For quantitative analysis SI must be set to set least 128K, and the peaks of interest must have ≥10 points above the half height of the peak for accurate quantification.

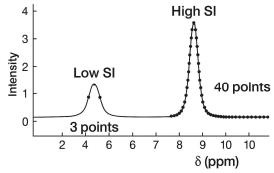

Figure 9: Diagram displaying effect of SI

Trouble Shooting

ISSUE	POTENTIAL CAUSE	POTENTIAL SOLUTION
FID truncation	There isn't enough data points collected during acquisition	Increase AQ or decrease SW
T_1 too long	All the components together in the matrix may affect T_1	Change solvent or standard
Replicate Agreement not within acceptable range	Sample may be inhomogeneous	Grinding may help
Assay value increasing with time (e.g. during 24-hour stability analysis)	Some standards such as 1,3,5-Trimethoxybenzene are susceptible to acid and perform a deuterium swap under these conditions	Change the standard
Aliasing	This happens when frequencies beyond half the sampling rate appear as false peaks within the observed range	Increase SW