Lifting the lid on qNMR: absolute quantitation of organic compounds

Quantitative NMR – principles and practice

In the analytical armoury of the scientist, few techniques are as flexible – or as powerful – as Nuclear Magnetic Resonance (NMR) spectroscopy.

First described in 1938, with the first recognisable instruments built in the mid-1940s, NMR spectroscopy was quickly established as one of the principal techniques used to obtain physical, chemical, electronic, and structural information about molecules. The technique exploits the principle of a chemical shift in resonance frequencies of the nuclear spins in the sample.

More than 75 years later, NMR spectroscopy routinely provides detailed information on the functional groups, topology, dynamics, and three-dimensional structure of molecules in solution and the solid state. It is used in many research and industrial fields, for example: pharmaceuticals, food, and agriculture.

From relatively early on (first papers surfaced around 30 years ago), NMR has been thought of as a quantitative technique too; since the area under an NMR peak is usually proportional to the number of spins involved, peak integrals can be used to determine composition quantitatively. Importantly, quantitative NMR (qNMR) does not require calibration to determine response factors – the ratio between the concentration of a compound being analysed and the response of the detector to that compound – as other analytical techniques do.

Recently, there has been an upsurge of interest in qNMR and a new generation of analysts and application scientists have become aware of the potential of the technique.

In this paper we look at why this is, giving particular emphasis to new advances in data processing that look set to widen the appeal of qNMR further.

Why qNMR?

qNMR can provide both relative quantitation – that is, the measurement of ratio of a target component contained within the sample being measured – and absolute quantitation – that is, the measurement of the actual amount of the target component contained within the sample being measured.

Absolute quantitation has some unique characteristics and advantages over other analytical methods. Chromatography detects the characteristics of the molecule itself, such as absorbance, refractive index, and fluorescence. This means that for quantitative analysis, chromatographers need a standard substance that is identical to the component that is being quantified in order to have a yardstick for making a measurement of the target molecule.

Furthermore, to ensure reproducibility in sample measurements, a method of eliminating variability in the response factor must be used. One of the easiest ways to do this is to use relative response factors and an internal standard to calibrate the instrument. In practice, a set of standards are measured, and a calibration curve is calculated against which samples are compared.

In contrast, qNMR detects the nuclei that form molecules. Therefore, if there is a proton in the molecule, we don't need a reference yardstick substance that is identical to the target component. It may be any suitable compound. In addition, calibration curves are not required.

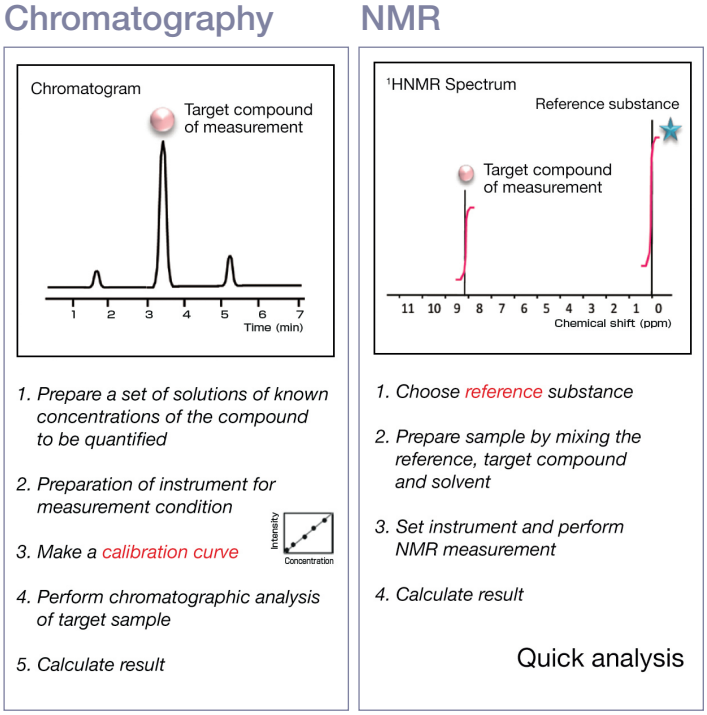


Figure 1: Comparison of chromatography and qNMR workflows

We can summarize the important features of qNMR with 4 key words:

Versatility – can be used for almost any organic compound that can be made into a solution.

Efficiency – don't need standard substance that is identical to the analysis target to perform quantification. Substances difficult to analyze quantitatively using chromatography as no standard substance is available, such as new compounds, can be quantitatively analyzed using qNMR. It's possible to use one reference substance for quantitative analysis of many measurement targets.

Speed – there is no need to create calibration curves for qNMR. No conditioning is required for performing a measurement either. For a low molecular weight compound, several milligrams are required to make a measurement, but each measurement can be completed in about 10-15 mins.

Reliability – if an appropriate protocol is followed, qNMR can be used to perform Si traceable purity assessments, therefore reliability of the results can be assured.

Optimized NMR measurement conditions

The measurement conditions employed for routine proton NMR are not ideal for quantitative analysis. You must use specific quantitative conditions when performing measurements for quantitative analysis.

Figure 2 shows an NMR spectrum of ethyl crotonate acquired with ordinary, routine conditions, meaning the default measurement conditions on JEOL NMR instruments. They allow us to collect proton spectra quickly as required at many NMR laboratories. Figure 3 was acquired using measurement conditions optimized for quantification.

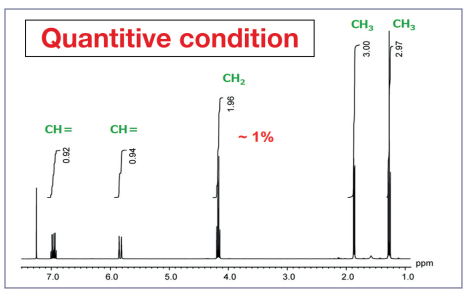


Figure 2: NMR spectrum of ethyl crotonate acquired with ordinary, routine conditions

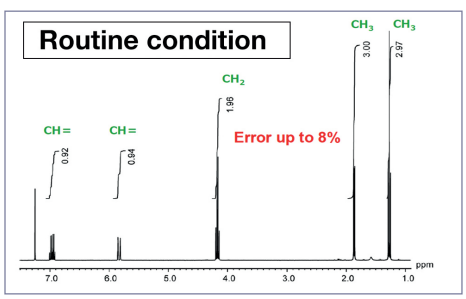


Figure 3: NMR spectrum of ethyl crotonate acquired using measurement conditions optimized for quantification

If we compare the peak areas, in Figure 2, from the left, the values are 0.92, 0.93, 1.96, 3.0 and 2.97. For a structural analysis, this can be interpreted as 1 to 1 to 2 to 3 to 3, but you can see that there is an error in the integral values of as much as 8%. This is unacceptable in high-precision qNMR analysis.

In comparison, in Figure 3, you can see that the area values and the proton counts match. The error is around 1%. This demonstrates the importance of using quantitative conditions when performing measurements for quantitative analysis.

But what are quantitative conditions?

Table 1 shows a comparison of the typical parameters for routine conditions and for quantitative conditions. The routine conditions are the default settings of the JEOL instrument for proton measurement. The quantitative conditions are based on the conditions specified in the Japanese Pharmacopoeia[1]. The parameter differences can be very broad, but there are six specific parameters that should be considered.

Typical parameters	Routine	Quantitative
Pulse repetition time	~7 sec	>T_1 x 7
Pulse flip angle	45°	90°
Scans	8	S/N>100
Digital resolution	0.5 Hz	<0.25 Hz
Sample spinning	On	Off
^{13}C decoupling	Off	On

Table 1: Typical parameters for routine and quantitative measurement conditions

Now let's look in more detail at the pulse repetition time and the number of scans which are two of the most important parameters.

Pulse repetition time

As shown in Figure 4, the pulse repetition time is the length of time from the irradiation of one pulse until the irradiation of the next pulse. For quantitative conditions, this should be at least seven times longer than T_1 (longitudinal relaxation time).

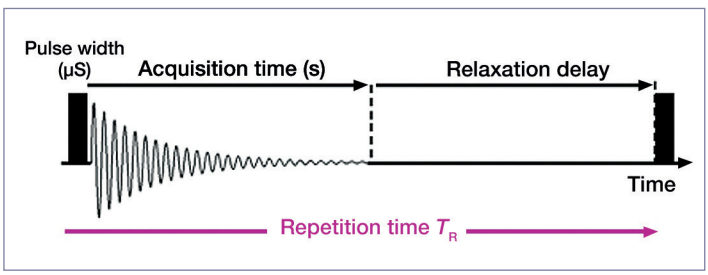
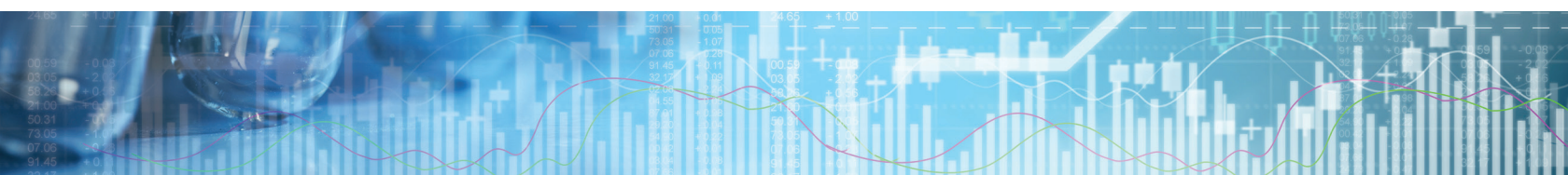


Figure 4: Pulse repetition time overview



The magnetization behavior on the sample side corresponds to the pulse sequence of the instrument when the magnetization is perturbed by the application of a pulse. If we wait long enough to allow the magnetization to recover completely before applying the next pulse, the quantitativeness of the signals can be ensured. Therefore, the parameter settings must ensure sufficient delay between pulses to ensure quantitativeness while making the measurements.

The index for setting this time is T_1 – time constant that is a characteristic of the signal. The relaxation time can be determined by making an inversion recovery measurement. In order to determine how much time is required for the pulse repetition time, Figure 5 shows the theoretical relationship between the signal strand and the ratio between the repetition time, and the relaxation time. The vertical axis indicates normalized signal strength, and the horizontal axis is the ratio of the repetition time to the longitudinal relaxation time.

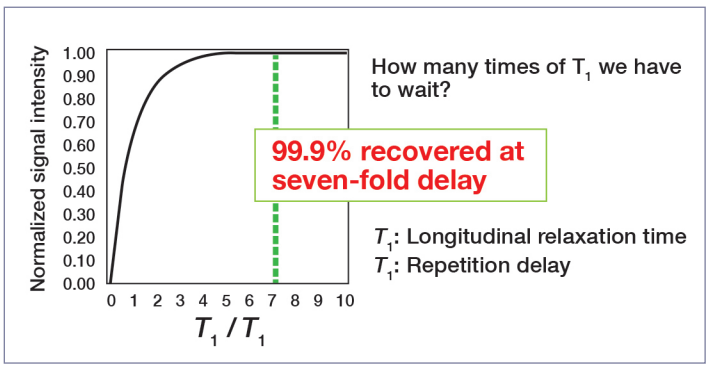


Figure 5: Pulse repetition time analysis

We can see the point at which the signal intensity turns to 100% expressed as a multiple of T_1. According to this, setting the pulse repetition time to 7 x T_1 or longer will mean the signal is nearly completely returned to the original state. This is why this is defined as the condition for quantification. In ordinary conditions, the main focus is on the signal to noise ratio in order to confirm the signal as fully as possible, meaning that the acquisition conditions emphasize the integration efficiency, so the goal is different.

The pulse repetition time is the most important parameter because it is the setting that theoretically improves quantitative performance. Other parameters are mainly for minimizing the integration error as we obtain the peak areas. There are no mandatory settings for the other parameters and there should be no problems if these are varied according to the situation.

Number of scans

For quantitative conditions, the signal to noise (S/N) ratio should be 100 or more. Figure 6 shows the theoretical relationship between S/N ratio and the accuracy. According to this, if the S/N is 100 or more, the integration error can be kept to an accuracy within 1%.

In other words, to obtain an integration with a slightly better accuracy, the S/N ratio must be higher. If this cannot be obtained because of the sample amount, it is important to understand that this will be a factor contributing to the quantification error. Thus, the setting for the number of scans is not a specific number. It is a setting to obtain a target accuracy for the available S/N ratio.

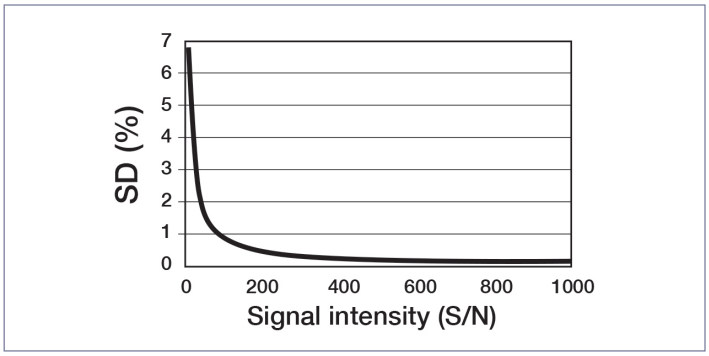


Figure 6: Influence of signal intensity (S/N) to repetitive accuracy (SD) of integration

The challenge of data analysis

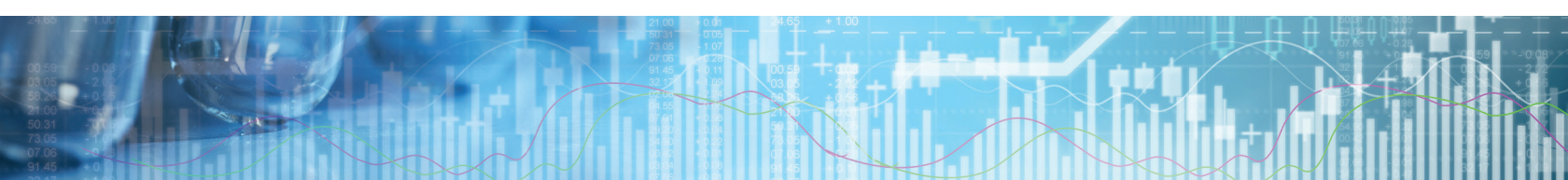
Conventional high-throughput, automated NMR analysis (be it for quantitative or structural/dynamic studies) has historically focused on increasing the throughput of the data collection and/or its reduction to tabular format. Bottlenecks can exist in the reduction of the spectral domain to the tabular domain, particularly for high-complexity systems, where critical, manual intervention, and guidance become necessary from the user.

Time-domain analysis (i.e. extraction of frequency, amplitude, linewidth and phase directly from the FID) has been proposed in the past for data reduction to a table. Now, the emerging CRAFT (Complete Reduction to Amplitude Frequency Table)[2] approach looks set to redefine the conventional steps in data processing.

It is important to note however, that the applicability of CRAFT for qNMR measurements is not about it being inherently better than existing, very well implemented approaches in NMR processing software such as JEOL's Delta NMR software and third-party offline NMR processing packages. For many analytical scenarios, this established methodology will remain in place. Rather, the power of the direct 'Spectrum to Spreadsheet' function in CRAFT is that it lends itself very well to easy, full automation for problems of real complexity.

CRAFT

First proposed as a robust and time-efficient Bayesian approach for quantitative mixture analysis in 2013, CRAFT uses a Bayesian statistical approach to convert NMR time-domain data directly to the tabular domain (Figure 7), an approach which is potentially a new paradigm for spectral analysis.



With CRAFT, the frequency domain data (the 'spectrum') is no more than a visualization tool for the tabular domain, in contrast to 'conventional processes' where the tabular domain is derived from the spectrum by an experienced spectroscopist.

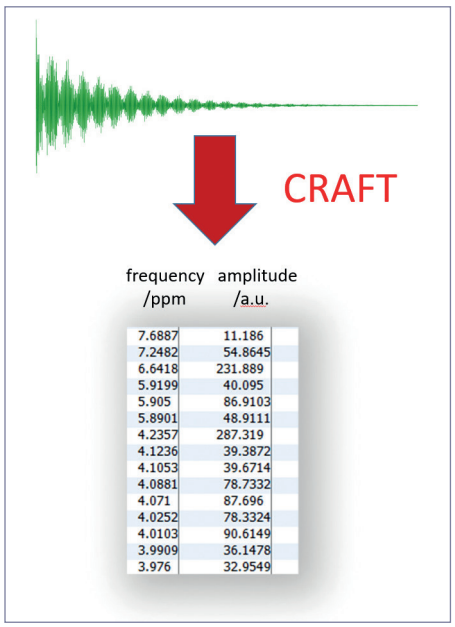


Figure 7: Output from CRAFT: data directly in tabular form

The reconstruction, after the fact, of model data taken from each component of the amplitude frequency table of an example ^{1}H-NMR measurement of brucine is shown in Figure 8. Note that the reconstructed trace (green) is almost identical to the original, measured, trace (blue) – the small differences are shown on the residual (brown) trace.

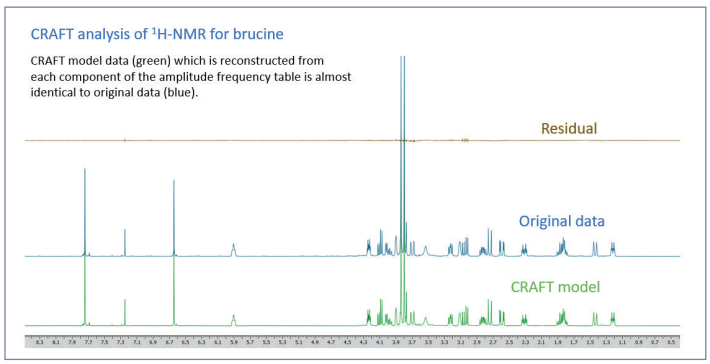


Figure 8: CRAFT analysis of ^{1}H-NMR for brucine

An example of a basic CRAFT result is shown in Figure 9. The saved CRAFT line list result can be viewed in a saved CRAFT result jdf file, or data may be visualized with the Delta data slate tools. It is important to understand that the "Amplitudes" reflect the total area of each reported frequency not peak height. By selecting a value in the line list table with a mouse click, its associated CRAFT model can be displayed.

A user can choose as many peaks as desired and normal shift-click, control-click options are respected. To remove the display of selected models control-click the selected lines in line list report to unselect.

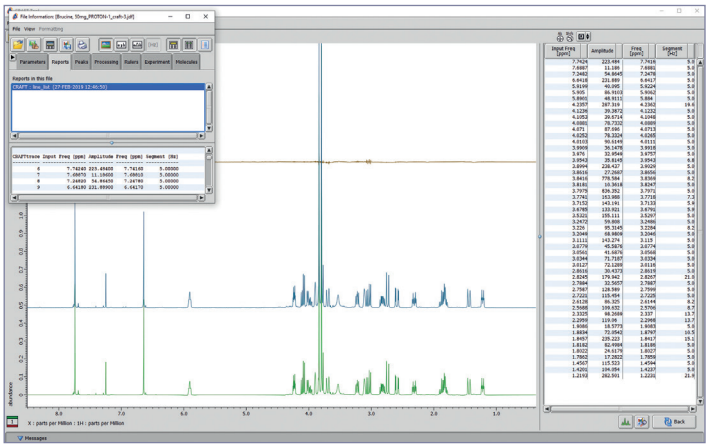


Figure 9: CRAFT analysis screen from JEOL CRAFT tool software shows line list table (right panel) reconstructed models (large panel), and the pop-up menu box to choose data export and display options

Summary:

qNMR can be relied on as a versatile, flexible, fast, and robust analytical tool. With conditions optimised for quantitative analysis, qNMR offers several advantages over alternative techniques. It can be used for almost any organic compound that can be made into a solution. Importantly, NMR does not require calibration to determine response factors, nor a standard substance that is identical to the analysis target to perform quantification.

In addition, there is no need to create calibration curves for qNMR. No sample conditioning is required for performing a measurement and, for low molecular weight compounds, analysis can be completed in around 10-15 mins. With an appropriate protocol, qNMR can be used to perform Si traceable purity assessments, and it is well suited to automation and high-throughput analysis.

With improved data processing options, using CRAFT for the analysis of complex mixtures, for example, qNMR looks set to find even wider application in the coming years.

Contact details

For more information about qNMR, CRAFT and the JEOL NMR solutions, please contact your local representatives, details here:
www.jeolusa.com

REFERENCE: 1. The Japanese Pharmacopoeia, Seventeenth Edition, P2519, 2016
2. Amplitude Frequency Table – robust and time-efficient Bayesian approach for quantitative mixture analysis by NMR, Krish Krishnamurthy, Magn. Reson. Chem, 51, pp 821-829 (2013).