# 12

# Quantification

## 12.1 Introduction

NMR offers us a great tool for quantification. This is because it offers a uniform response to the nucleus of interest (see caveats at the end of this chapter). We rely on this when we look at integrals in a proton NMR spectrum – a methyl group integrates for three protons, a methylene integrates for two protons, etc. As NMR spectroscopists, we get a little blasé about this – we just expect it. This is not true for all techniques though. For example, ultraviolet (UV) detection is often used on HPLC systems but its response depends on the degree of conjugation in the compound of interest. If we were to have a chromatogram with two different compounds in it, we would not be able to tell what their relative proportions were unless we knew their UV response at the wavelength (or wavelengths) being monitored. In NMR, this is not the case.

## 12.2 Relative Quantification

This is the easiest case for NMR (and other analytical techniques). What we are looking for is the relative proportion of compounds in a mixture. To do this, we identify a signal in one compound and a signal in the other. We then normalise these signals for the number of protons that they represent and perform a simple ratio calculation. This gives us the *molar* ratio of the two compounds. If we know the structure (or the molecular weight) of these compounds, then we can calculate their *mass* ratio.

Spectrum 12.2 shows a spectrum of salbutamol in $D_6$-DMSO with some TMS in it. As an exercise, we can easily quantify the TMS as follows. . .

The signal for TMS (0 ppm) is for 12 protons. The signals in the aromatic region are from salbutamol and represent one proton each. If we set the integral of the aromatic protons to equal 1.0 and assuming adequate relaxation time for the relevant protons of both salbutamol and TMS, then we see that the relative integral of the TMS is 0.32. Because this signal is for 12 protons we can calculate that we have $(0.32/12) \times 100 = 2.6$ mol% of TMS in the sample. The molecular weight of salbutamol is 239 and the molecular weight of TMS is 88 so their weight ratio is 0.36 which means that the weight ratio of TMS is $2.6 \times 0.36 = 0.96$ % w/w.

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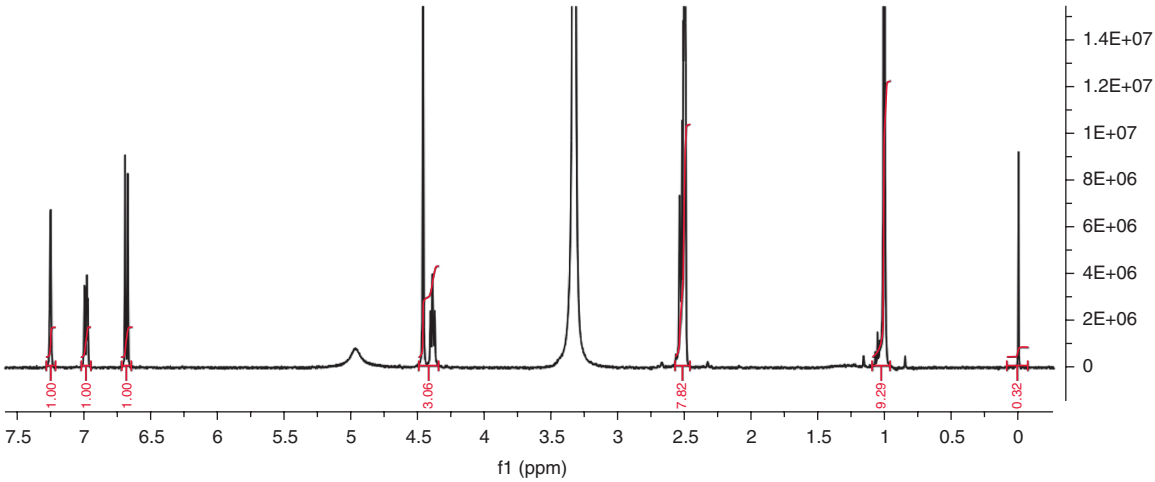



**Spectrum 12.1** Salbutamol with TMS.

## 12.3 Absolute Quantification

The example shown before is fine if all you want to know is the relative proportions of compounds in your solution. If you know the *absolute* concentration of one of the components, then you can work out the absolute concentration of the other as a result.

### 12.3.1 Internal Standards

If we add a known amount of a compound to our solution, we can use it to quantify the material of interest. This is great except that we may not want to contaminate our material with some other compound. A number of people have looked at using standards that are volatile so that they can be got rid of later (TMS is an example that we have seen published). The problem with this approach is that if the sample is volatile then you need to run it quickly before it disappears. TMS disappears really quickly from DMSO so it is probably not a good idea in this case. TMS also suffers from the fact that it has a long relaxation time so you have to be very careful with your experiment to ensure that you do not saturate the signal. The last major problem with TMS is that it comes at the same part of the spectrum as silicon grease which can be present in samples. Choosing a standard so that it has a short relaxation time, is volatile and comes in a part of the spectrum free of interference is really tricky. In fact, we wouldn't recommend it at all.

### 12.3.2 External Standards

So how do we quantify if we don't have an internal standard? One way is to use an external standard. This is done by inserting a capillary containing the standard into the NMR tube (Figure 12.1).

Of course, we still have the problem of selecting a compound that doesn't interfere with the spectrum and that has a suitable relaxation time but we don't need to worry about its volatility. What would be really good is a standard that doesn't interfere with the sample at all. Something that has no relaxation

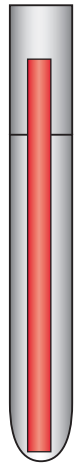


**Figure 12.1** External standard placed in the NMR tube.

time to worry about and something that you could put in the spectrum in an area where there were definitely no signals.

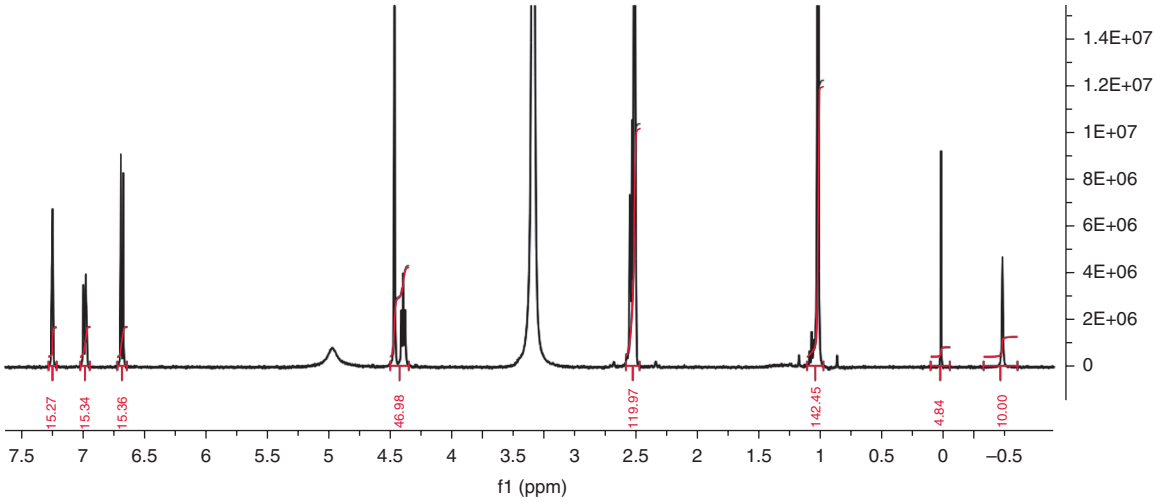
### 12.3.3 Electronic Reference

This problem was eventually solved in the magnetic resonance imaging world where they needed to be able to quantify things *in vivo*. The result was the use of an extra radio-frequency source during acquisition. It was called 'ERETIC' (***e***lectronic ***re***ference ***t***o access ***i****n vivo* ***c***oncentrations) and has been used extensively in recent years in the high-resolution liquid NMR areas. The great advantage of this approach is that you can make the signal as big or as small as you want and put it anywhere in the spectrum (–1.0 ppm is a favourite place). The way that you use it is to quantify the ERETIC signal against a sample of known concentration. Once this has been done, you can then insert the signal into your unknown concentration spectrum and integrate it against one of the signals in your compound.

There are some problems with the ERETIC approach. Firstly, it does not respond in the same way as the signals in your sample so if your probe tuning is not quite right, you will get an inaccurate answer. Secondly, it requires rewiring of your system so that you can introduce the signal (alternatively, you can rely on cross-talk in the system to let the signal bleed through – this too has some problems associated with it). Lastly, because the signal is generated in a different manner from those of the sample, it can suffer from phase-errors which give rise to inaccuracy when integrating the signal.

### 12.3.4 QUANTAS Technique

Given that the ERETIC approach has problems, why not introduce a defined intensity signal into your spectrum using software? This is the approach adopted in the QUANTAS technique (*quan*ification *t*hrough an *a*rtificial *s*ignal). No, not the Australian airline (which is QANTAS, by the way, short for *Q*ueensland and *N*orthern *T*erritory *A*erial *S*ervice). In this approach, a reference spectrum with a single signal is created using software. This is added to a spectrum of a sample of known concentration and a scaling factor is calculated to make the signal exactly the correct size for the concentration that it is to




**Spectrum 12.2** Spectrum with QUANTAS signal.

represent. If this spectrum is added to any sample spectrum, using the calculated scaling factor, it will be able to represent a defined concentration in the unknown concentration spectrum. This is shown in the salbutamol spectrum used before (Spectrum 12.2).

Unlike ERETIC, this approach does not track receiver gain or number of scans (the signal is a fixed intensity). This doesn't cause a problem though – you can choose to run under identical conditions to your reference, or you can compensate for differences in acquisition condition. For example, the signal builds directly proportionally to the number of scans (note, not the square root of the number of scans. It is the signal to noise that builds with the square root of the number of scans). On modern spectrometers, receivers are linear and it is possible to compensate for receiver gain differences linearly too. The current implementation of the QUANTAS method uses a small programme to automatically take into account any changes in receiver gain and number of scans so you just end up with the signal at the correct level.

This approach offers by far the most simple and flexible way of quantifying samples and is even better because it can be run retrospectively on any sample (as long as the spectrometer is performing similarly to when the signal was standardised). It turns out that for most modern spectrometers, the spectrometer is stable over many months or even years.

## 12.4 Things to Watch Out For

It all seems so simple when you look at this example. Unfortunately this is not necessarily the case. We need to be a little careful about how we acquire the data if we are going to use it for quantification.

The first thing to look out for is the relaxation time ($T_1$) of the protons that you are going to measure. In order to get an accurate integral, the protons must return to their rest state each time before you pulse them. The recommendation for a 90° pulse is to wait for 3–5 × $T_1$. Obviously this assumes that you know the $T_1$ of all of your protons. It is possible to measure them (and this is indeed the 'right' thing to do) but you need to decide how accurate you need the result to be. If you want a fairly accurate result, it is sufficient to 'guesstimate' your $T_1$ values just by looking at the chemical structure. Small molecules

tend to have long $T_1$s. Methyl groups tend have longer $T_1$s than methylenes. Methines may have long $T_1$s if they are isolated from any other protons. Symmetrical molecules have slightly longer $T_1$s than unsymmetrical molecules. If you use a 30° pulse (which is more normal) then you can probably get away with using a relaxation delay of about 5 s if your acquisition time is about 3 s (hence a total recycle time of about 8 s).

On older spectrometers, it is important that the signal that you are measuring is not at the edge of the spectrum. This is because older spectrometers used hardware frequency filters and these start to decrease signal intensity at the edge of the spectrum. More modern spectrometers use digital filters that are capable of very sharp cutoffs that will not affect the intensity at the edge of the spectrum. Be warned, even here you may get problems with distortions in the baseline at the edge of the spectrum (so-called 'smileys'). In general, try to avoid your signal of interest being at the edge of the spectrum.

All quantification relies on being able to standardise against a known concentration standard. This is not a trivial thing to do as it requires an accurately weighed amount of a known purity compound, made up accurately to a precise volume. If your standard is wrong, all your measurements will be wrong so it is worth spending some time getting it right!

Ultimately, you will be measuring and comparing integrals so you need to be very careful about how you get these. Your signals of interest must be perfectly phased, clear of other signals and on a good baseline. The integrals must also have good slope and bias (which they should do if everything else is correct). Any problems with any of these variables will seriously degrade the accuracy of your result. In our experience, the biggest single error with any NMR quantification approach is the error in measuring the integral.

## 12.5 Conclusion

If you do manage to get everything right, NMR offers excellent quantification results. What's more is that it is free if you have acquired a 1-D spectrum. Note that you can use this approach to quantify other nuclei – it works just as well for $^{19}$F. Note that it won't work very well for $^{13}$C because we normally acquire $^{13}$C data with NOE enhancement from the protons so the signals are not quantitative. (It is possible to collect carbon data in a quantitative way but it is not something that we would normally do. . .).