2

Preparing the Sample

Whilst sample preparation may not be the most interesting aspect of NMR spectroscopy, it is nonetheless *extremely* important as it will have a huge bearing on the quality of the data obtained and therefore on your ability to make logical deductions about your compounds. This is particularly true when acquiring the most straightforward 1-D proton spectra. The most typical manifestation of sub-standard sample preparation is poor line shape. It is worth remembering that in terms of 1-D proton NMR, 'the devil' can be very much 'in the detail'. 'Detail', in this context, means 'fine structure' and fine structure is always the first casualty of poor sample preparation.

The reason for this can best be appreciated by considering just how small the differences in chemical shifts of signals really are – and indeed, just how small (but significant!) a long-range coupling can be. Consider for example, a 3-7 coupling in an indole. (Structure 2.1).

Being able to see this coupling is reassuring in that it ties the 3 and 7 protons together for us. It might seem a trifling matter, but observing it, even if it appears only as a slight but definite broadening, helps underpin the credentials of the molecule because we know it should be there. Such a five-bond coupling will be small – comparable in fact with the natural line width of a typical NMR signal. Let's say we are looking for a coupling of around 1 Hz, for the sake of argument. 1 Hz, on a 400 MHz spectrometer corresponds to only 1/400 of a part per million of the applied magnetic field (since 1 ppm = 400 Hz in a 400 MHz spectrometer). So in order to observe such a splitting, we will need resolution of better than 0.5 Hz, which corresponds to one part in $10^6/(0.5/400)$, or ideally, better than one part in 10^9! To achieve such resolution requires corresponding levels of magnetic field homogeneity through your sample but this can only be achieved in extremely clean solutions of sufficient depth. We will be dealing with this issue in detail later on. In real terms, establishing first class magnetic field homogeneity means that molecules of your compound will experience exactly the same field no matter where they are in the NMR tube – therefore, they will all resonate in unison – rather than in a fragmented fashion. Any factor which adversely effects field homogeneity will have a corresponding deleterious effect on line shape. We will see this more clearly later.

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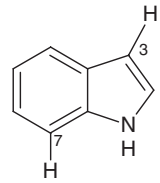


Structure 2.1 An indole with 3-7 coupling.

2.1 How Much Sample Do I Need?

This section might be alternatively titled, 'How long is a piece of string?' There is no simple answer to this question which we have been asked many, many times. What you need in solution is sufficient material to produce a spectrum of adequate signal/noise to yield the required information but this is no real answer as it will vary with numerous factors. How powerful is the magnet of the spectrometer you are using? What type of probe is installed in it? What nucleus are you observing? What type of NMR acquisition are you attempting? How pure is your sample? What is the molecular weight of your sample? Is it a single compound or is it a mixture of diastereoisomers? These are just some of the relevant questions that you should consider.

And there are others. If you are using a walk-up system, there will probably be some general guidelines posted on it. Assume that these are useful and adhere to them as far as possible. They will be by their very nature, no more than a guide, as every sample is unique in terms of its molecular weight and distribution of signal intensity. Also, a walk-up system is likely to be limited in terms of how much time (and therefore how many scans) it can spend on each sample.

If you are fortunate enough to be 'driving' the spectrometer yourself, you can of course compensate for lack of sample by increasing the number of scans you acquire on your sample – but this is not a licence to use vanishingly small amounts. It is worth remembering that in order to double the signal/noise ratio, you have to acquire four times the number of scans. Think about it. If your sample is still giving an unacceptably noisy spectrum after five minutes of acquisition, how long will you have to leave it acquiring in order for the signal/noise to become acceptable? Doubling the S/N is likely to do little. If you improve it by a factor of four (probably a worthwhile improvement) you will have to acquire for an hour and twenty minutes (16 × 5 minutes)! The law of diminishing returns operates here and makes its presence felt very quickly indeed.

All that having been said, we will attempt to draw up a few rough guidelines below.

If you are unfortunate enough to be struggling away with some old continuous-wave museum piece, then in all probability, you will only be looking at proton spectra. Even though the proton is THE most sensitive of all nuclei, you will still be needing *at least* 15 mg of compound, assuming a molecular weight of about 300 (if it's a higher molecular weight, you will need more material, lower and you may get away with a little less)

It's more likely these days that you will be using a 250 or 400 MHz Fourier transform instrument with multi-nuclei capability. If such an instrument is operating in 'walk up' mode so that it can acquire >60 samples in a working day, then it will probably be limited to about 32 scans per sample (a handy number – traditionally, the number of scans acquired has always been a multiple of eight but we won't go into the reasons here. If you want more information, take a look at the term 'phase cycling' in one of the excellent texts available on the more technical aspects of NMR). This means that for straightforward

Table 2.1 A rough guide to the amount of sample needed for NMR.

	Comfortable amount of material needed (mg)	
Field (MHz)	^{1}H	^{13}C
90	20	Lots!
250	5	30
400	2	10
600	1	5

1-D proton acquisition, you will need about 3 mg of compound as above, though you may get away with as little as 1 mg with a longer acquisition time, assuming a typical 5 mm probe. The same 3 mg solution (sticking with the approx. 300 mol. wt throughout) would also get you a reasonable fluorine spectrum, if available, since the ^{19}F nucleus is a 100 % abundant and is therefore, a relatively sensitive nucleus.

If you are looking for a ^{13}C spectrum, then you will probably find that they will only be available overnight. This is because the ^{13}C nucleus is extremely insensitive and acquisition will take hours rather than minutes (only 1.1 % natural abundance and relatively low gyromagnetic ratio – see Glossary). Whilst the signal to noise available for ^{13}C spectra will be highly dependant on the type of probe used (i.e., 'normal' geometry or 'inverse' geometry – see Glossary), about 10 mg of compound will be needed for a typical acquisition, which will probably entail about 3200 scans and run for about 2 h. Even then, the signal/noise for the least sensitive quaternary carbons may well prove marginal. (Note that the inherently low sensitivity of the ^{13}C nucleus can to some extent be addressed by acquiring various inverse-detected 2-D data such as HMQC/HSQC and HMBC, all of which we will discuss later).

Operating at 500 or 600 MHz and using a 3 mm probe should yield an approximate threefold improvement in signal/noise which can be traded for a corresponding reduction in sample requirement.

Various technologies do exist to give still greater sensitivities – perhaps even an order of magnitude greater, e.g., 'nano' probes, 1 mm probes and cryoprobes, but they are currently unusual in a 'routine' NMR environment. These tools tend to be the preserve of the NMR specialist.

Table 2.1 gives a very rough guide to the amount of sample you need, given all the previous provisos. Of course, if you are prepared to wait a long time and don't have a queue of people waiting to use the instrument, you can get away with less material. Generally, more is better (as long as the solution is not so gloopy that it broadens all the lines!).

2.2 Solvent Selection

The first task when running any liquid-phase NMR experiment is the selection of a suitable solvent. Obvious though this sounds, there are a number of factors worth careful consideration before committing precious sample to solvent. A brief glance at any NMR solvents catalogue will illustrate that you can purchase deuterated versions of just about any solvent you can think of but we have found that there is little point in using exotic solvents when the vast majority of compounds can be dealt with using one of four or five basic solvents.

Your primary concern when selecting a solvent should be the *complete* dissolution of your sample. Again, this might seem an unduly trivial observation, but if your sample is not in solution, then it

will remain 'invisible' to the spectrometer. Consider for a moment a hypothetical sample – a mixture of several components, only one of which being soluble in your chosen solvent. Under these circumstances, your spectrum may flatter you (your desired compound is preferentially soluble in solvent of choice), or alternatively, it may paint an unduly pessimistic view of your sample (one or more of the undesired components is preferentially soluble in solvent of choice). Either way, there are possibilities for being mislead here so the primary objective in selecting a solvent should be the total dissolution of your sample. In general, we advise adhering to the simple old rule that 'like dissolves like'. In other words, if your sample is nonpolar, then choose a nonpolar solvent and vice versa.

2.2.1 Deutero Chloroform ($CDCl_3$)

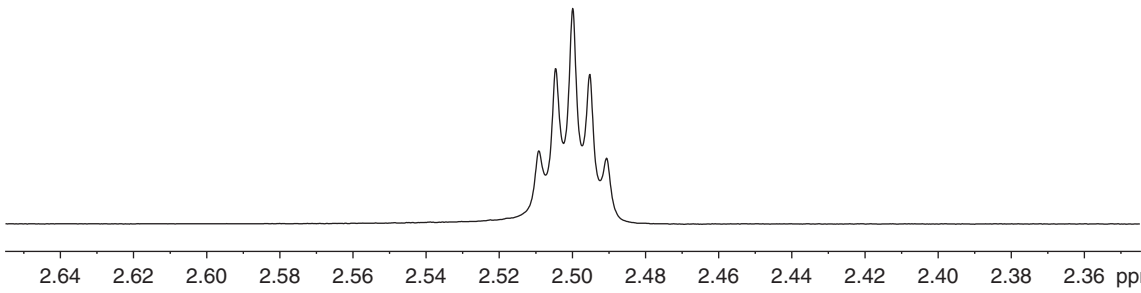
This is a most useful NMR solvent. It can dissolve compounds of reasonably varying polarity, from nonpolar to considerably polar, and the small residual $CHCl_3$ signal at 7.27 ppm seldom causes a problem. $CDCl_3$ can easily be removed by 'blowing off' should recovery of the sample be necessary. Should a compound prove only sparingly soluble in this solvent, deutero dimethyl sulfoxide can be added drop by drop to increase the polarity of the solvent – but see cautionary notes below! This may be preferable to running in neat D_6-DMSO due to the disadvantages of D_6-DMSO outlined below. It should be noted that D_6-DMSO causes the residual $CHCl_3$ signal to move downfield to as low as 8.38 ppm, its position providing a rough guide to the amount of D_6-DMSO added. The main disadvantage of using a mixed solvent system is the difficulty of getting reproducible results, unless you take the trouble of measuring the quantities of each solvent used!

It should also be noted that $CDCl_3$ is best avoided for running spectra of salts, even if they are soluble in this solvent. This is because deutero chloroform is an 'aprotic' solvent that does not facilitate fast transfer of exchangeable protons. For this reason, spectra of salts run in this solvent are likely to be broad and indistinct as the spectrometer 'sees' two distinct species of compound in solution; one with a proton attached and another with it detached. As the process of inter-conversion between these two forms is slow on the NMR timescale (i.e., the time taken for the whole process of acquiring a single scan to be completed in), this results in averaging of the chemical shifts and consequent broadening of signals – particularly those near the site of protonation.

2.2.2 Deutero Dimethyl Sulfoxide (D_6-DMSO)

Deutero dimethyl sulfoxide (D_6-DMSO) is undoubtedly very good at dissolving things. It can even dissolve relatively insoluble heterocyclic compounds and salts, but it does have its drawbacks. Firstly, it's relatively viscous, and this causes some degree of line-broadening. In cases of salts, where the acid is relatively weak (fumaric, oxalic, etc.), protonation of the basic centre may well be incomplete. Thus, salts of these weak acids may often look more like free bases! It is also a relatively mild oxidising agent, and has been known to react with some compounds, particularly when warming the sample to aid dissolving, as is often required with this solvent.

Problems associated with restricted rotation (discussed later) also seem to be worse in D_6-DMSO, and being relatively nonvolatile (it boils at 189 °C, though some chemical decomposition occurs approaching this temperature so it is always distilled at reduced pressure), it is difficult to remove from samples, should recovery be required. This nonvolatility however, makes it the first choice for high temperature work – it could be taken up to above 140 °C in theory, though few NMR probes are capable of operating



Spectrum 2.1 Residual solvent signal in DMSO.

at such high temperatures. At the other end of the temperature scale it is useless, freezing at 18.5 °C. In fact, if the heating in your NMR lab is turned off at night, you may well find this solvent frozen in the morning during the cold winter months!

The worst problem with DMSO, however, is its affinity for water, (and for this reason, we recommend the use of sealed 0.75 ml ampoules wherever possible) which makes it almost impossible to keep dry, even if it's stored over molecular sieve. This means that bench D_6-DMSO invariably has a large water peak, which varies in shape and position, from sharp and small at around 3.46 ppm, to very large and broad at around 4.06 ppm in wetter samples. This water signal can be depressed and broadened further by acidic samples! This can be annoying as the signals of most interest to you may well be obscured by it. One way of combating this, is to displace the water signal downfield by adding a few drops of D_2O, though this can also cause problems by bringing your sample crashing out of solution. If this happens, you've got a problem! You could try adding more D_6-DMSO to re-dissolve it. The residual CD_2HSOCD_3 signal occurs at 2.5 ppm, and is of characteristic appearance (caused by ^2H–^1H coupling). Note that the spin of deuterium is 1, which accounts for the complexity of the signal (see Spectrum 2.1). Even so-called 100 % isotopic D_6-DMSO has a small residual signal so you can't totally negate the problem by using it – just lessen it.

Extreme care should be taken when handling DMSO solutions, as one of its other characteristics is its ability to absorb through the skin taking your sample with it! This can obviously be a source of extreme hazard. Wash off any accidental spillages with plenty of water – immediately! (This goes for all other solvents too).

2.2.3 Deutero Methanol (CD_3OD)

This is a very polar solvent, suitable for salts and extremely polar compounds. Like DMSO it has a very high affinity for water and is almost impossible to keep dry. Its water peak is sharper and occurs more predictably at around 4.8 ppm. The residual CD_2HOD signal is of similar appearance to the D_6-DMSO residual signal and is observed at 3.3 ppm.

Its main disadvantage is that it will exchange ionisable protons in your sample for deuterons, and hence they will be lost from the spectrum, e.g., -OH, -NH and even -$CONH_2$, though these can often be relatively slow to exchange. Also, protons α to carbonyl groups may exchange through the enol mechanism. The importance of losing such information should not be underestimated. Solving a structural problem can often hinge on it!

2.2.4 Deutero Water (D_2O)

D_2O is even more polar than D_4-methanol and rather limited in its use for that reason – usually for salts only. Like deutero methanol, it exchanges all acidic protons readily and exhibits a strong HOD signal at about 4.9 ppm. Samples made up in D_2O often fail to dissolve cleanly and benefit from filtration through a tight cotton wool filter (cf. Section 2.4.1).

2.2.5 Deutero Benzene (C_6D_6)

D_6-Benzene is a rather specialised solvent and not normally used in 'routine' work. It is often added to $CDCl_3$ solutions, though it can of course be used neat, when it may reveal hidden couplings or signals by altering chemical shifts of your compound. It does this because it can form collision complexes with sample molecules by interactions of the pi electrons. This can bring about changes in the chemical shifts of the sample peaks because benzene is an anisotropic molecule, i.e., it has non-uniform magnetic properties (shielding above and below the plane of the ring, and deshielding in the plane of the ring). This is really an extreme example of a solvent shift. Whenever you change the solvent, expect a change in the spectrum! C_6D_6 shows a residual C_6D_5H signal at 7.27 ppm. *Cautionary note*: benzene is of course a well known carcinogen and due care should be taken when handling it – particularly if used in combination with DMSO!

With these five solvents at your disposal, you will be equipped to deal with virtually any compound that comes your way but it might be worth briefly mentioning two others.

2.2.6 Carbon Tetrachloride (CCl_4)

This would be an ideal proton NMR solvent, (since it is aprotic and cheap) were it better at dissolving things! Its use is now very limited in practise to very nonpolar compounds. Also, it lacks any deuterated signal that is required for locking modern Fourier transform spectrometers – (an external lock would be necessary making it inconvenient – see Section 2.3). Carbon tetrachloride is very hydrophobic, so any moisture in a sample dissolved in this solvent will yield a milky solution. This might impair homogeneity of the solution and therefore degrade resolution, so drying with anhydrous sodium sulfate can be a good idea. Carbon tetrachloride does have the advantage of being non-acidic, and so can be useful for certain acid-sensitive compounds. Take care when handling this solvent, as like benzene, it is known to be carcinogenic. Not recommended.

2.2.7 Trifluoroacetic Acid (CF_3COOH)

Something of a last resort this one! It seems to be capable of dissolving most things, but what sort of condition they're in afterwards is rather a matter of chance! It has been useful in the past for tackling extremely insoluble multicyclic heterocyclic compounds. If you have to use it, don't expect wonders. Spectra are sometimes broadened. It shows a very strong -COOH broad signal at about 11 ppm. Again, the lack of a deuterated signal in this solvent makes it less suitable for FT making an external lock necessary – see above. Not recommended unless no alternative available.

Well, that just about concludes our brief look at solvents. If you can't dissolve it in one of the common solvents, you've got problems. If in doubt, try a bit first, before committing your entire sample. Use nondeuterated solvents for solubility testing if possible, as they are much cheaper.

2.2.8 Using Mixed Solvents

Whilst it is perfectly possible to use a mixed solvent system ($CDCl_3$/DMSO is always a popular example as chemists have a tendency to opt for $CDCl_3$ out of habit or in the hope that it will dissolve their samples, only to find that solubility is not as good as expected), we advise against it, particularly if you are running your spectra on a 'walk up' automated system. Remember that the spectrometer uses the deuterated signal for frequency locking and if it has more than one to chose from, things can go wrong and you might find yourself the proud owner of a spectrum that has been offset by several ppm as the spectrometer locks onto the D_6-DMSO signal and sets about its business in the belief that it has in fact locked onto $CDCl_3$! Furthermore, it is very difficult to reproduce exact solvent conditions if you are required to re-make a compound. Using a suitable single solvent will prevent these issues ever troubling you.

2.3 Spectrum Referencing (Proton NMR)

NMR spectroscopy differs from other forms of spectroscopy in many respects, one of which is the need for our measurement to be referenced to a known standard. For example, considering infra red spectroscopy for a moment, if a carbonyl group stretches at 1730 cm^{-1}, then as long as we have a suitably calibrated spectrometer, we can measure this, confident in the knowledge that we are measuring an absolute value associated with that molecule.

In NMR spectroscopy, however, the chemical shift measurement we make takes place in an environment of our making that is both entirely artificial and arbitrary (i.e., the magnet!). For this reason, it is essential to reference our measurements to a known standard so that we can all 'speak the same language,' no matter what make or frequency of spectrometer we use.

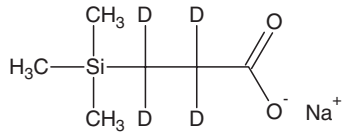
The standard is usually added directly to the NMR solvent and is thus referred to as an 'internal' standard, though it is possible to insert a small tube containing standard in solvent into the bulk of the sample so that the standard does not come into direct contact with the sample. This would be referred to as an 'external' standard. We recommend an internal standard wherever possible for reasons of convenience and arguably superior shimming.

Apart from some very early work in the field which was performed using water as a standard (It would be difficult to imagine a worse reference standard as the water signal moves all over the place in response to changing pH!) the historical reference standard of choice has always been TMS (Tetra Methyl Silane), as mentioned earlier. TMS has much to recommend it as a standard. It is chemically very inert and is volatile (b.p. 26–28 °C) and so can easily be removed from samples if required. Furthermore, only a tiny amount of it is needed as it gives a very strong twelve proton singlet in a region of the spectrum where other signals seldom occur.

TMS is not ideally suited for use in all solvents, however. As you can see from the structure, it is extremely nonpolar and so tends to evaporate from the more polar solvents (D_6-DMSO and D_4-MeOD). For this reason, a more polar derivative of TMS [3-(Trimethylsilyl) propionic-2,2,3,3-D_4 acid; TSP – see Structure 2.2] is often used with these solvents.

Note that the side chain is deuterated so that the only signal observed in the proton NMR spectrum is the trimethyl signal.

Deuterated solvents can be purchased with these standards already added if required and this would be our recommendation because so little standard is actually needed that it is very difficult to add



Structure 2.2 3-(Trimethylsilyl) propionic-2,2,3,3-D_4 acid, sodium salt.

little enough to a single sample without overdoing it! (An enormous standard peak, apart from looking amateurish, is to be avoided since it will limit signal/noise ratio as the spectrometer scales the build up of signals according to the most intense peak in a spectrum.) Of course, TMS and TSP do not have *exactly* the same chemical shifts so to be totally meticulous, you should really quote the standard you are using when recording data.

Of course, you don't have to use either of the above standards at all. In the case of samples run in deutero chloroform/methanol and dimethyl sulfoxide, it is perfectly acceptable, and arguably preferable, to reference your spectra to the residual solvent signal (e.g., CD_2HOH) which is unavoidable and always present in your spectrum (see Table 2.2). These signals are perfectly solid in terms of their shifts (in pure solvent systems) though the same cannot be said for the residual HOD signal in D_2O and for this reason, we would advise adhering to TSP for all samples run in D_2O.

We will discuss referencing issues with respect to other nuclei in later chapters.

2.4 Sample Preparation

Note that sample depth is important! When using a typical 5 mm probe, a sample depth of about 4 cm (approx. 0.6 ml) is necessary, though this varies slightly from instrument to instrument. There should be guidance available to you in this respect on each individual spectrometer. If you try to get away with less than this, magnetic field homogeneity, and therefore, shimming (see Section 3.8) will be compromised as the transmitter and receiver coils in the probe must be covered to a sufficient depth to avoid the problems of 'edge effects' (see Figure 2.1).

Of course, there is no point in overfilling your NMR tubes. This *can* make shimming more difficult (but certainly not impossible as in the case of too low a sample depth) but more importantly, it merely wastes materials and gives rise to unduly dilute samples giving reduced signal/noise. Any sample outside the receiver coils does not give rise to signal.

If your sample is reluctant to dissolve in the chosen solvent, avoid adding more solvent for the reasons outlined above. Instead, try warming the sample vial carefully on a hotplate or with a

Table 2.2 Residual solvent signals.

Solvent	Chemical shift of residual signal (ppm)
$CDCl_3$	7.27
CD_3SOCD_3	2.50
CD_3OD	3.30

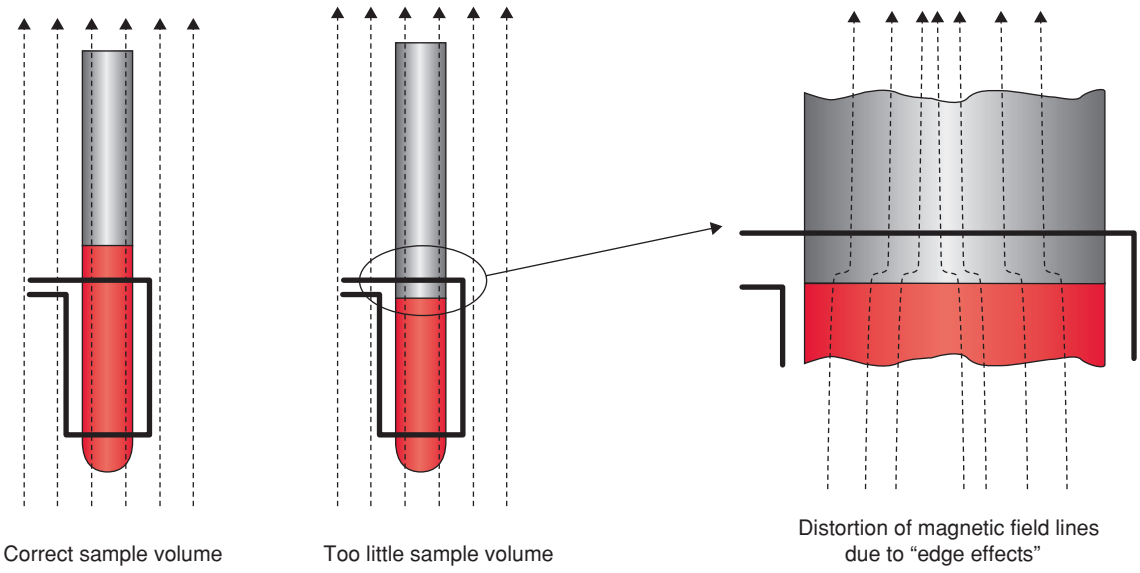


Figure 2.1 Sample depth and magnetic field homogeneity.

hairdryer – sometimes a bit of thermal agitation will be all that is required to assist the dissolving process. This is particularly true in the case of highly crystalline samples which can be slow to dissolve. Another useful approach is to use an ultrasonic bath. These provide very powerful agitation and are even more effective when used in combination with a heat source.

2.4.1 Filtration

Of course, there are always samples that refuse to dissolve completely even after ample exposure to heat and prolonged dunking in an ultrasonic bath. Samples that appear in any way cloudy when held up to the light, simply *must* be filtered. Any particulate matter held in suspension will severely compromise field homogeneity and thus line shape (Figure 2.2). Suspended material (of whatever origin) is *the* major cause of substandard line shape in NMR spectroscopy.

The whole filtration issue is perhaps a little confusing. Earlier in this section we were stressing the importance of dissolving the *whole* sample and yet here we are, now advocating filtration? On the face of it, there might seem to be an inherent contradiction in this – and perhaps there is. We can only say that in an ideal world, samples would dissolve seamlessly to give pristine clear solutions without even a microscopic trace of insoluble material in suspension. Samples in the real world are often not quite so obliging! Filtration is very much the lesser of the two evils. If you *know* that you have filtered something from your solution, you are at least aware of the fact that the spectrum is not entirely representative of the sample. But if you *don't* filter, the resultant spectrum may be so poor as to fail to yield any useful information at all – the choice is as simple as that.

Be warned that very small particle size material, that may even be invisible to the naked eye, is the worst in terms of ruining line shape. The big stuff quite often floats or sinks and therefore doesn't interfere much with the solution within the r.f. coils.

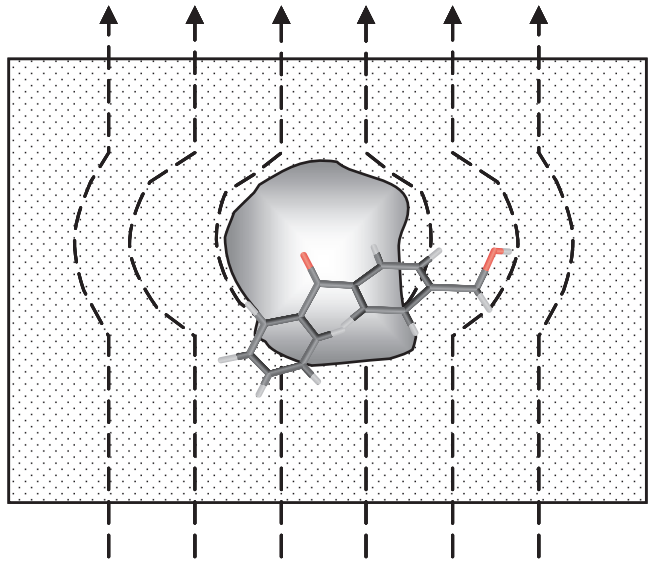


Figure 2.2 Undissolved material causing loss of magnetic field homogeneity.

A convenient method for the filtration of small volumes of liquids is shown in Figure 2.3.

The filter can in a sense be 'customised' as required. A tight plug of cotton wool (rammed hard into the neck of a pipette using a boiling stick) alone is often enough to remove fairly obvious debris from your solution but the addition of a layer of a similarly compacted 'hyflo' on top of the cotton wool makes for a very tight filter which will remove all but the most microscopic of particles. Note that using a pipette bulb to force the liquid through the filter is an excellent idea as it speeds the whole process considerably. Even so, if you are using D_6-DMSO as a solvent, be prepared for a long squeeze as the viscosity of this solvent makes it reluctant to pass through a tight filter. If you suspect that your sample is wet (usually, cloudy $CDCl_3$ solutions with no obvious particulate matter present), you can take this opportunity to dry it at this stage by introducing a layer of anhydrous sodium sulfate to the filter. This will remove most (but not all) of the water present.

A couple of final observations on line shape – just occasionally, we have encountered samples that give very broad lines even after the most stringent filtering. This can be caused by contamination by a tiny amount of paramagnetic material in solution. In one memorable case, a chemist had been stirring a sample around in an acidic solution with a nickel spatula. The tiny quantity of nickel leached from the spatula was sufficient to flatten the entire spectrum. The reason for this is that the ions of any of the transition (d-block) elements provide a *very* efficient relaxation pathway for excited state nuclei, enabling them to relax back to their ground state very quickly. Fast relaxation times give rise to broad lines and vice versa, so to summarise, keep NMR solutions well away from any source of metal ions! Should you find yourself in this situation, your only course of action is to run your sample down a suitable ion exchange column.

One other (very rarely encountered) situation is that of the stabilised free radical. It is possible for certain conjugated multi-ring heterocyclic compounds to support and stabilise a delocalised, free electron in their pi clouds. Such a free electron again provides an extremely efficient relaxation pathway for all

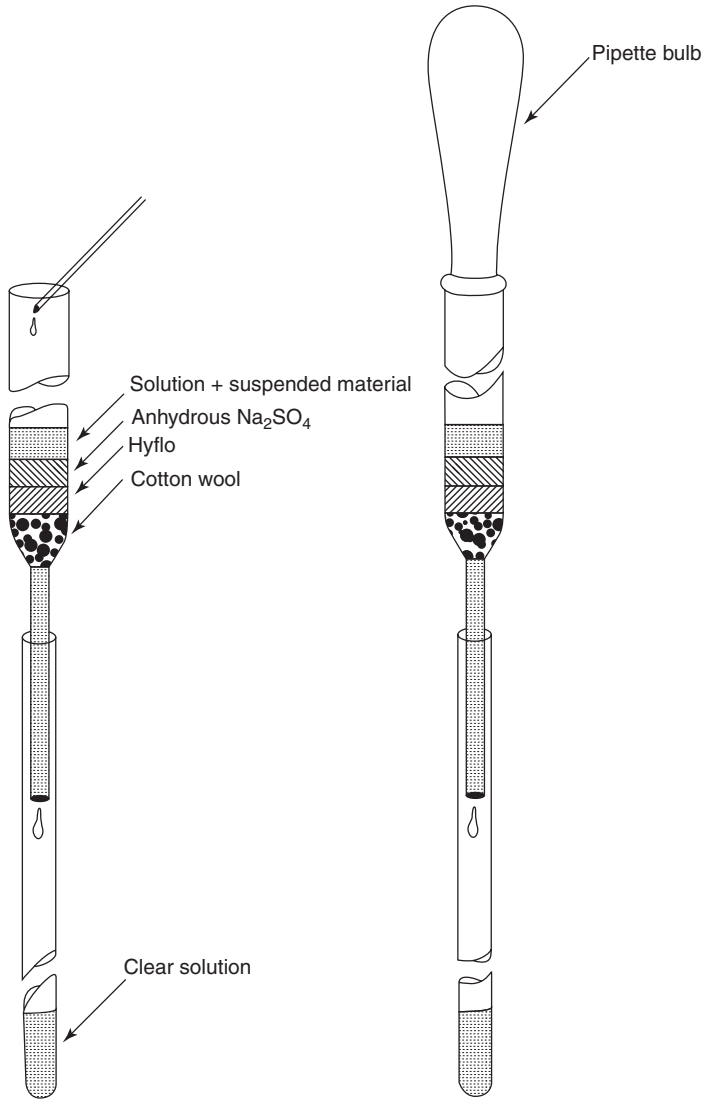


Figure 2.3 A convenient method of filtering NMR solutions.

nuclei in such a molecule and would give rise to an almost entirely flat spectrum. Such compounds usually give a clue to their nature by being intensely coloured (typically very dark blue) Filtration would do little to improve such a situation but running in the presence of a suitable radical scavenger such as dichloro, dicyano quinone can provide the solution. The scavenger mops up the lone electron and a spectrum can be obtained as normal.