CHEMISTRY RESEARCH LABORATORY

A User Guide to Modern NMR Experiments

Barbara Odell and Tim Claridge

UNIVERSITY OF OXFORD

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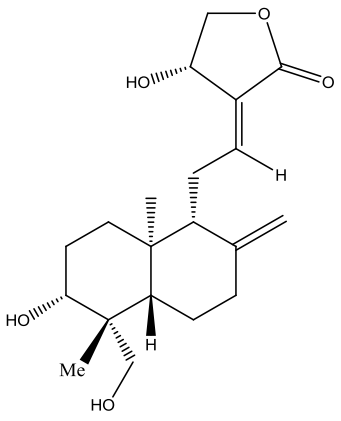
Purpose

The aim of this NMR guide is to familiarise NMR users with the principal experiments available to researchers so that they can make intelligent choices as to the type of experiments that will help solve their particular problem. It does not seek to explain in detail how the NMR methods work, but illustrates the type of information chemists can obtain from various experiments in a readily understood manner. Further details may be found in *High Resolution NMR Techniques in Organic Chemistry* (2nd Edn), TDW Claridge, Elsevier, 2009.

The experiments described are those routinely provided by the NMR Service using Bruker instruments and the command language used is specific to Bruker Topspin software.

Andrographolide in d6-DMSO is used as an example compound where applicable. It is a labdane diterpenoid that is the main bioactive component of the medicinal plant *Andrographis paniculata*. It has sharp distinct signals and a number of features well suited to illustrating the performance of a range of NMR experiments, including three OH groups, which are all well resolved in the DMSO proton NMR spectrum. Most experiments were run at 298K on a Bruker Avance 500 MHz instrument equipped with the cryoprobe (AVC500).



1. Standard ^{1}H NMR

1.1 What is it?

The aim of the standard ^{1}H NMR experiment is to record a routine proton NMR spectrum in order to obtain structure-related information for the protons of the sample.

1.2 What does it tell me?

The information available includes three elements:

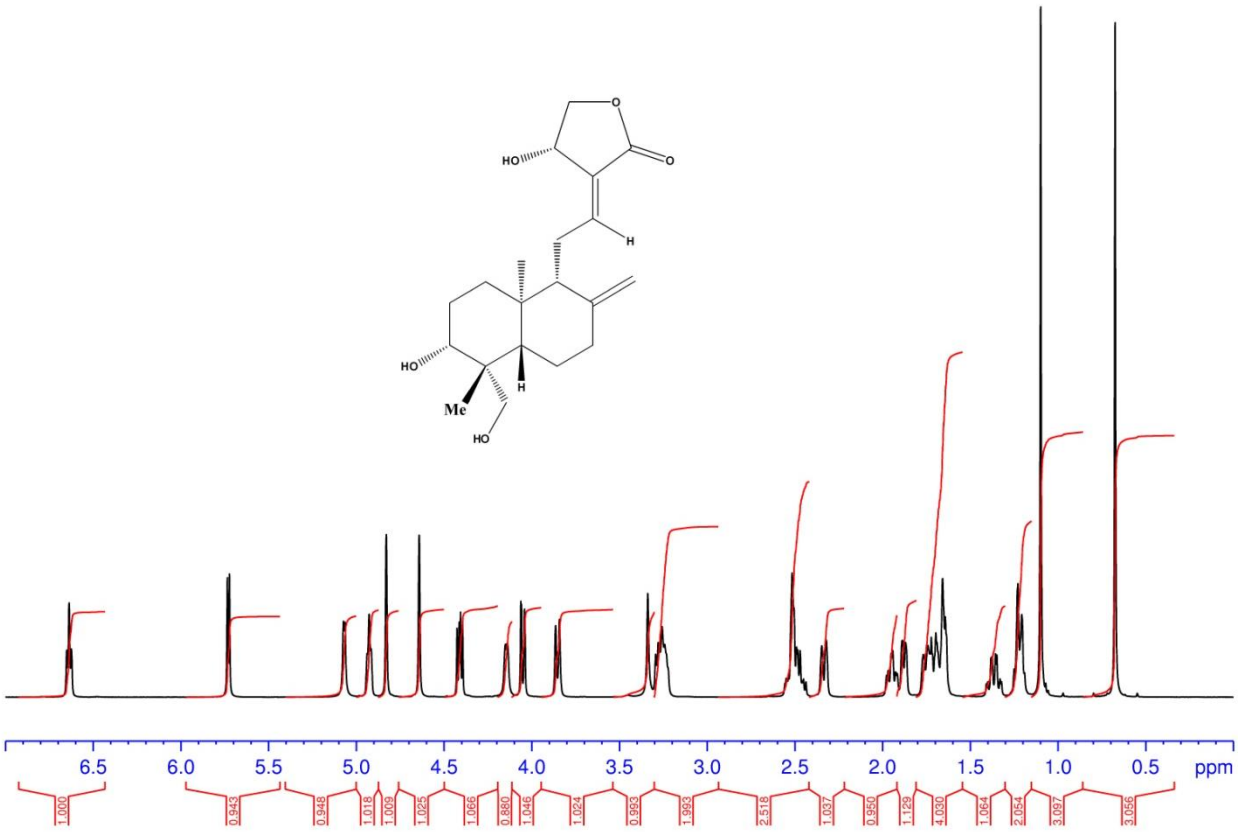
a) **Chemical shifts** (δ) measured in ppm for individual resonances, reflecting the local chemical environment of the proton.

b) **Spin-spin coupling constants** (J) measured in Hz represent the interaction of one proton with others in the molecule solely through chemical bonds (so-called scalar coupling).

c) **Resonance intensities** (integrals) reflecting the *relative* number of protons giving rise to each resonance.

1.3 What does it look like?

The figure below shows a 500MHz ^{1}H NMR spectrum of Andrographolide. The chemical shift region 6.6 to 0 ppm is displayed including integrals for each multiplet. Noteworthy points are that water peak at ~3.4 ppm in DMSO-d6 does not share integral value with the other protons in the molecule and the residual protonated DMSO signal at 2.5ppm is interspersed with the methylene multiplets of the compound enhancing its integral value.



1.4 Further comments

Most samples run adequately with our standard default parameter sets. Salient parameters are; time domain ***td***, the number of points which define the fid, spectral width ***sw***, which determines the total ppm window e.g 20 ppm. The settings of ***sw*** and ***td*** determine the fid acquisition time ***aq*** and hence the spectrum digital resolution (the number of hertz per data point, the smaller this number, the higher the digital resolution). The middle of the spectrum is ***o1p*** in ppm, the frequency offset of the rf pulse at the centre of the spectrum. Two other **important** parameters are the relaxation delay, ***d1*** (usually set at 1.0 sec) to establish thermal equilibrium of the spin system before the excitation pulse is applied and ***ns***, the number of *scans* or *transients* (usually 16) which, in part, determines the signal-to-noise ratio in the final spectrum.

According to *special requirements* of your particular sample you may need to change;

- ***sw*** **-** changes spectral width in ppm (unusual ^{1}H shifts)
- ***o1p*** **-** changes middle of spectrum in ppm
- ***td*** **-** changes digital resolution; usually 64K (digital resolution = 2x sw/td Hz/pt)
- ***ns*** **-** more transients increases signal : noise; however, four times the number of transients will be required to double the signal : noise ratio.
- ***d1*** **–** this may need to increase if the relaxation time constants (T_1 values) of some of your protons are particularly long, causing them to have reduced integrated intensity.

Note that for most organic compounds, the default values are perfectly adequate.

Processing

Having obtained your spectrum there are several processing aids;

a) Zero filling: The default size of the processed data file is usually 32K points (si = td/2) but this can be increased to 64K so that si = td to improve digital resolution and enhance peak definition.

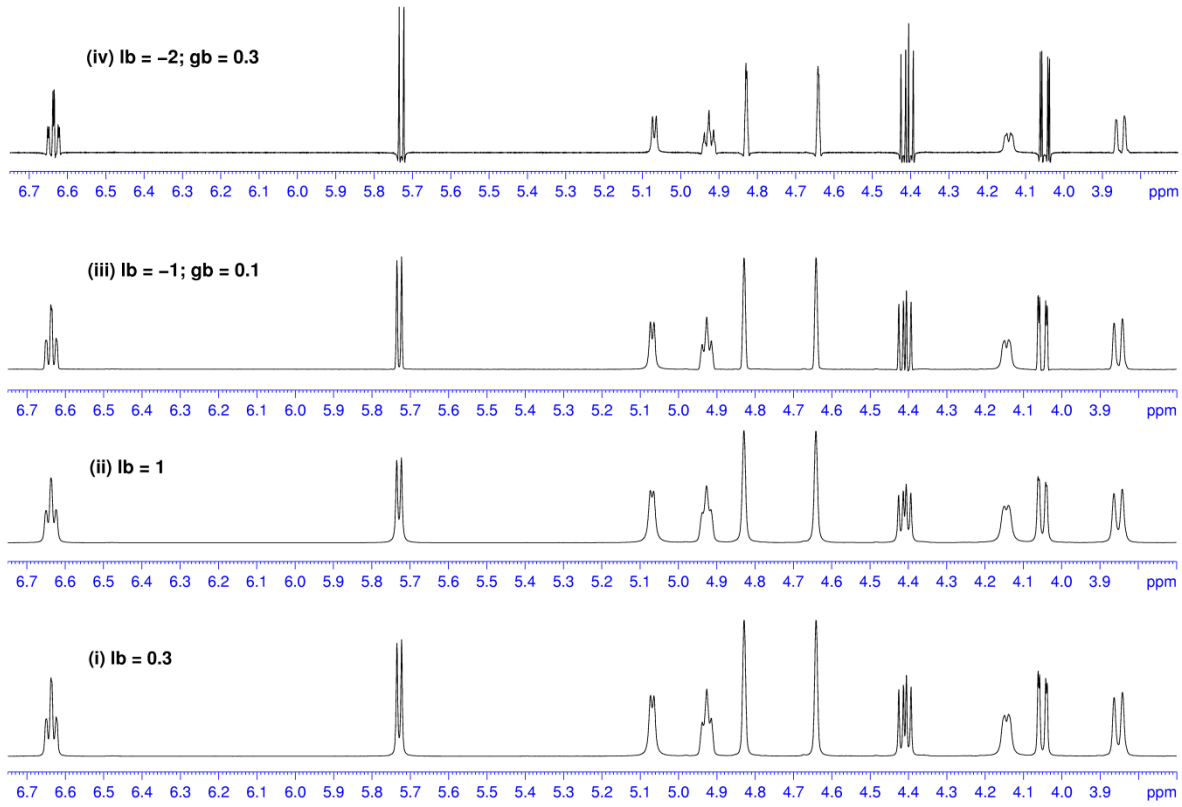
b) Apodization: application of window functions to modify the FID and enhance data content:

Exponential multiplication (***em***): can improve signal : noise. The default ***lb*** is 0.3 Hz; employing a higher line broadening value can yield better signal : noise, but at the expense of resolution.

Gaussian multiplication (***gm***): used for resolution enhancement using ***lb*** as a negative value e.g. -1, -2 Hz and ***gb*** (Gaussian broadening; 0 to 1); typically 0.2-0.3.

This reduces linewidths, making multiplets clearer, but at the expense of signal : noise and may also lead to peak-shape artefacts.

Examples are shown below:



Problems

The most common problems seen in ^{1}H NMR spectra are

i) Signal overlap hiding multiplets. Changes of solvent can be very effective in such cases and may alter shifts quite dramatically, one useful solvent is C_6D_6.

ii) Broad signals which can be due to dynamic exchange behaviour of your molecule e.g. rotamers, proton exchange etc. In such cases you may need variable temperature experiments or a different solvent can reduce proton exchange (note $CDCl_3$ contains traces of DCl/HCl).

iii) Integrals values which are inconsistent with the number of expected protons, due to slow spin relaxation, which may require acquiring the spectrum with a longer relaxation delay, ***d1***.

2. Standard ^{13}C NMR

2.1 What is it?

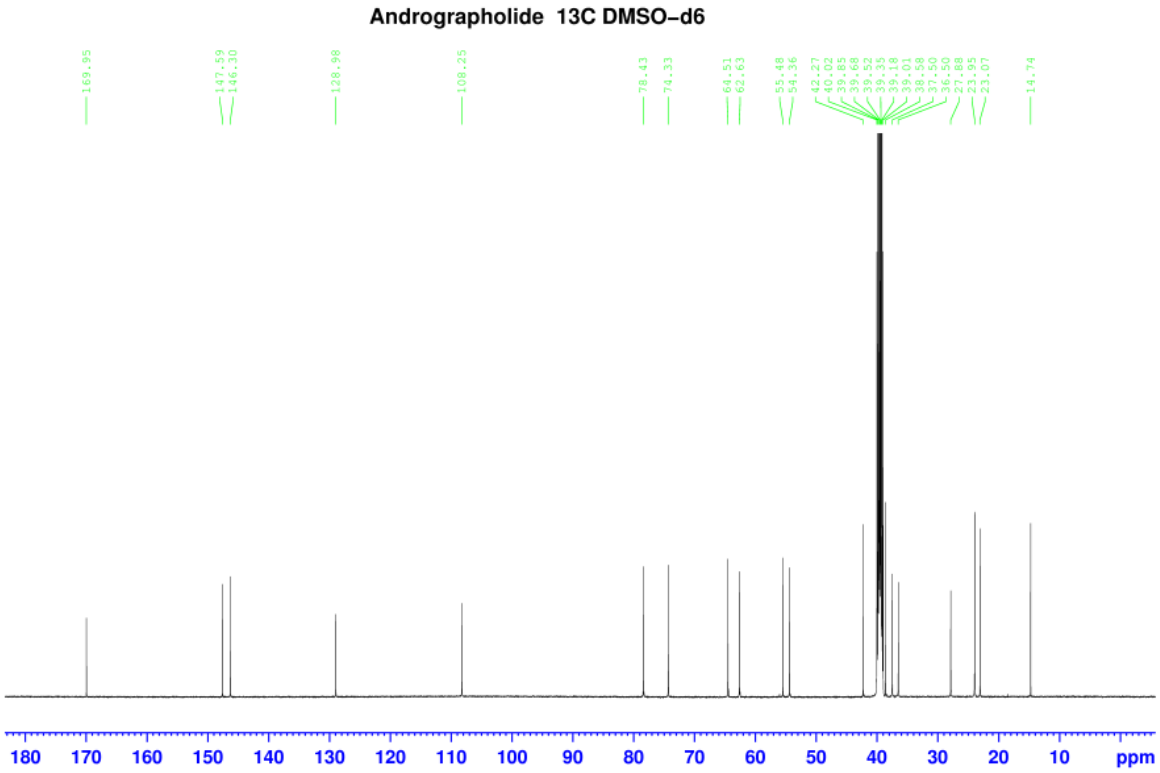
The standard ^{13}C NMR experiment records a 1D ^{13}C NMR spectrum with broadband proton decoupling (meaning all J- couplings to protons in the molecule are removed, but couplings to other NMR active nuclei may remain).

2.2 What does it tell me?

The principle information one obtains from this experiment is the number of unique carbon environments in the molecule (usually represented by the number of resonances in the spectrum, since peak overlap is rare, although not impossible) and the chemical shifts, reflecting the local carbon chemical environments. Multiplet structures are not usually seen, since all couplings to protons are removed, and integral data are not relevant. This is because under standard ^{13}C acquisition conditions, peak intensities do not accurately reflect carbon numbers; see below for explanation.

2.3 What does it look like?

The figure below shows a 125 MHz ^{1}H decoupled ^{13}C NMR spectrum of Andrographolide. The chemical shift region 200-0ppm is displayed with peak picking of each carbon signal. The intense signal centred at ~40 ppm is from the solvent (DMSO) and its multiplet structure arises from one-bond coupling to deuterium ($^{1}J_{CD}$=21Hz, seven lines due to three spin-1 deuterium nuclei coupled to carbon-13).



2.4 Further comments

Most samples are run adequately with the standard default parameter sets; ***sw*** for ^{13}C of 250ppm, the ***o1p*** of 100ppm and ***ns*** of 256 which is sufficient for a 5 mg sample of molecular weight approximately 500 when

recorded with the cryogenic probe. It is thus important to weight your sample, before submission as 1mg samples would require many more scans to give adequate signal/noise.

Note that no integration is performed, since under routine conditions the signals are not necessarily proportional to the number of carbon nuclei. This is due to a number of factors. Firstly, the nuclear Overhauser effect (nOe) enhances the intensity of carbon resonances which are directly attached to protons to varying degrees (the nOes arise from the application of proton decoupling). The intensity of quaternary carbon signals are not enhanced due to the absence of directly attached protons. Furthermore, non-protonated carbons relax rather more slowly than those bearing protons and therefore have reduced intensity. Finally, the digital resolution of carbon spectra is usually low as no fine structure needs to be recorded, meaning there are insufficient data points to define the peak shapes and intensities accurately.

The influence of protons can be seen in the above spectrum; see especially the quaternary carbon intensities which have no attached protons e.g. carbonyl carbon at 169.96 ppm is reduced in intensity compared to the methyl signals at 14.7 and 23.9 ppm. On the rare occasions that you require accurate quantitative ^{13}C NMR, for example to determine mixture ratios, please refer to section below.

Apodization (a window function) is critically important for the processing of heteronuclear spectra such as ^{13}C. Unlike ^{1}H NMR spectra, signal : noise is more important than resolution and some sensitivity enhancement is usually applied; the ***lb*** parameter is typically 1-2 Hz. Resolution enhancement is used only rarely in cases of severe carbon peak overlap.

According to *special requirements* of your particular sample you may want to change;

- ***sw* , -** changes spectral width in ppm
- ***o1p* -** changes middle of spectrum in ppm
- ***td* -** changes resolution usually 64K (digital resolution = 2x sw/td Hz/pt)
- ***ns* -** increases signal : noise
- ***d1* –** this may need to increase if the T_1 values of some of your carbon(s) are particularly long.

Note that for most organic compounds, the default values are perfectly adequate.

3. Quantitative ^{13}C NMR

3.1 What is it?

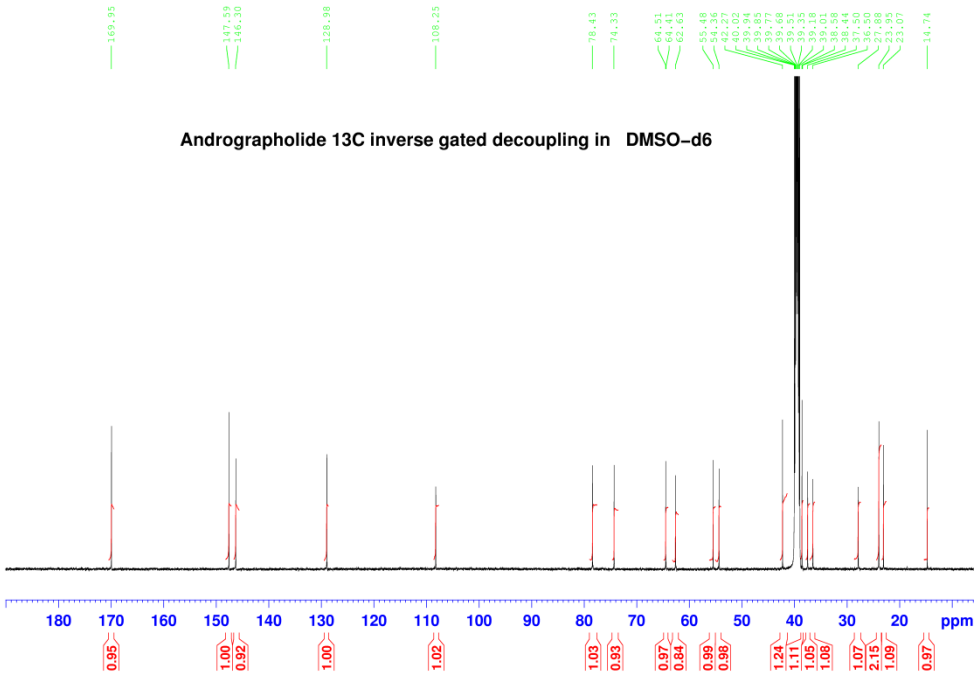
This experiment utilises "inverse gated decoupling" of protons to yield a ^{1}H-decoupled ^{13}C NMR spectrum **without** signal enhancement by the nuclear Overhauser effect (nOe). Its appearance is similar to the standard ^{13}C experiment, but it may be integrated if appropriate parameters have been used, and is therefore appropriate for quantitative ^{13}C NMR. The experiment takes much longer than a standard carbon-13 due to the longer relaxation delay (***d1***) that is required (see section 3.4).

3.2 What does it tell me?

The principle use of this experiment is to obtain quantitative ^{13}C spectra which can be subject to accurate integration. Normally, nOes from protons onto their directly attached carbons (CH, CH_2, CH_3 groups) will enhance carbon signal intensities to varying degrees, thus rendering carbon experiments unreliable with regard to integration. This experiment operates to suppress the Overhauser effect and employs longer periods for signal relaxation so that all ^{13}C signals may be quantified. It can be particularly useful in the determination of isomer ratios when ^{1}H NMR spectroscopy fails.

3.3 What does look like?

The figure below shows an inverse gated ^{1}H decoupled ^{13}C NMR spectrum of Andrographolide. All carbon signals have now been integrated and the signals of the protonated carbon atoms all have nearly the same height.



3.4 Further comments

In this experiment the ^{1}H broadband decoupling is applied only during the carbon acquisition period (and "gated off" or "switched off" at all other times), so that nOes have insufficient time to build-up but allowing a ^{1}H decoupled spectrum to be collected. The relaxation delay is also set to be, ideally, five times the longest spin relaxation time constant (T_1), to allow all carbon signals to relax back to thermal

equilibrium; in the case above ***d1*** was 30 sec. Obviously, due to the long relaxation delays this experiment can take **appreciably longer** that a standard ^{13}C spectrum. Furthermore, data acquisition times ***td*** are typically increased by a factor of 2-3 to improve digital resolution and thus peak definition. Processed data sizes are likewise increased but other considerations are as for the standard ^{13}C experiment above.

4 Edited ^{13}C Experiments

4.1 What are they?

The DEPT (Distorsionless Enhancement by Polarisation Transfer) family of experiments provide a means of a) improving the sensitivity of heteronuclear spectra and b) manipulating the spectra to help distinguish peak multiplicity eg CH, CH_2, and CH_3, a process known as *spectrum editing*. All DEPT experiments utilise polarisation transfer from protons onto a heteronucleus such as ^{13}C to increase signal strength compared to the normal ^{13}C spectrum. In typical use, the most valuable feature is spectral editing. The key to this lies in the fact that the sign (and intensity) of carbon resonances depends on the final proton pulse tip angle θ in the sequence; the value of this parameter gives rise to the name e.g. DEPT-135 correspond to an editing pulse tip angle of 135^0.

In this guide we illustrate three of the most useful DEPT experiments, which if used together can distinguish all carbon groups in your molecule as shown below.

4.2 What does it tell me?

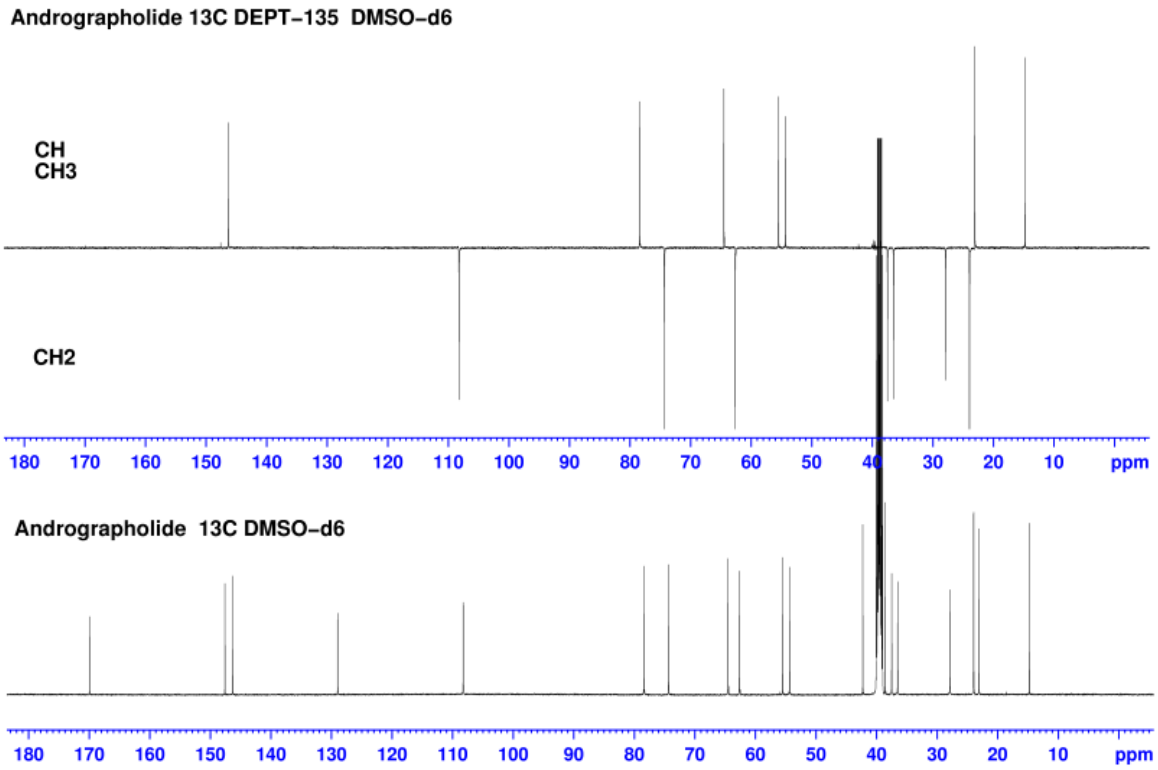
In standard ^{13}C spectra all multiplicity information is lost through the use of proton decoupling, meaning one cannot readily distinguish a CH from a CH_2 resonance (in the presence of proton coupling, $^1J_{CH}$ these would appear with characteristic doublet and triplet multiplicities, respectively). DEPT experiments seek to recover and encode this information in the signs of the ^{13}C resonances, as summarised in the table below.

Sign of Carbon signals

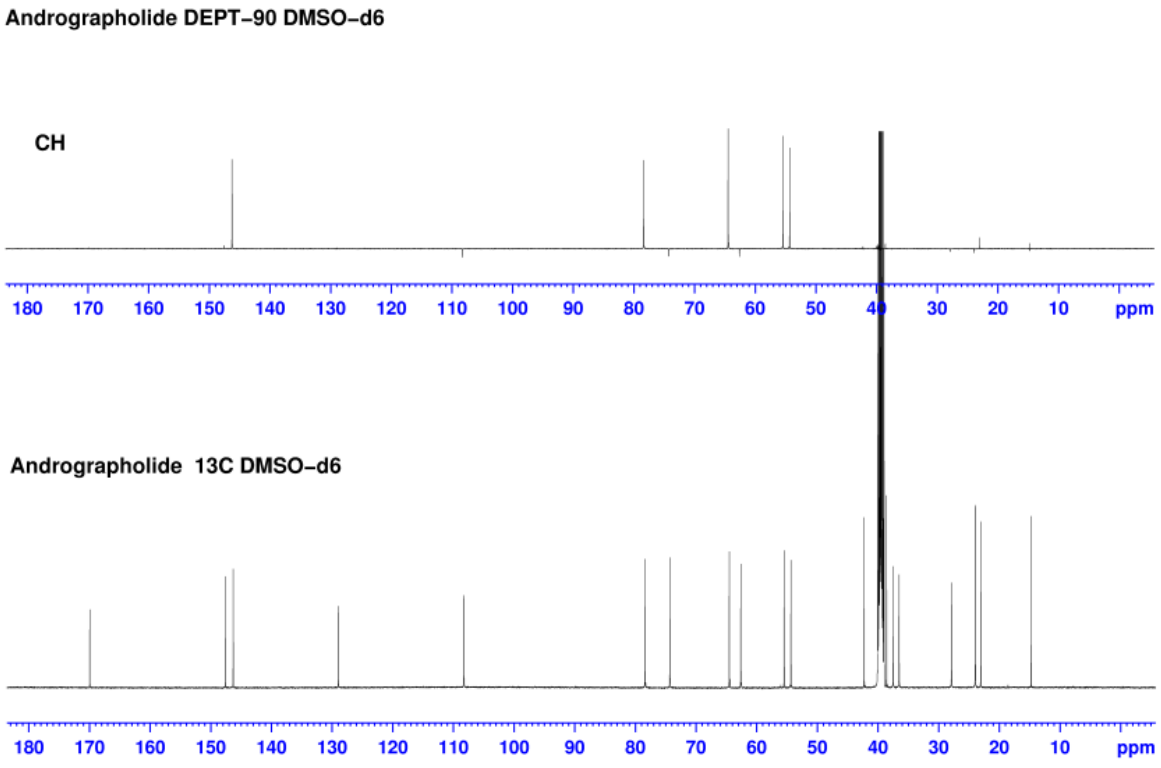
Carbon	DEPT-90	DEPT-135	DEPT-Q
C	nothing	nothing	-
CH	+	+	+
CH_2	nothing	-	-
CH_3	nothing	+	+

4.3 What does it look like

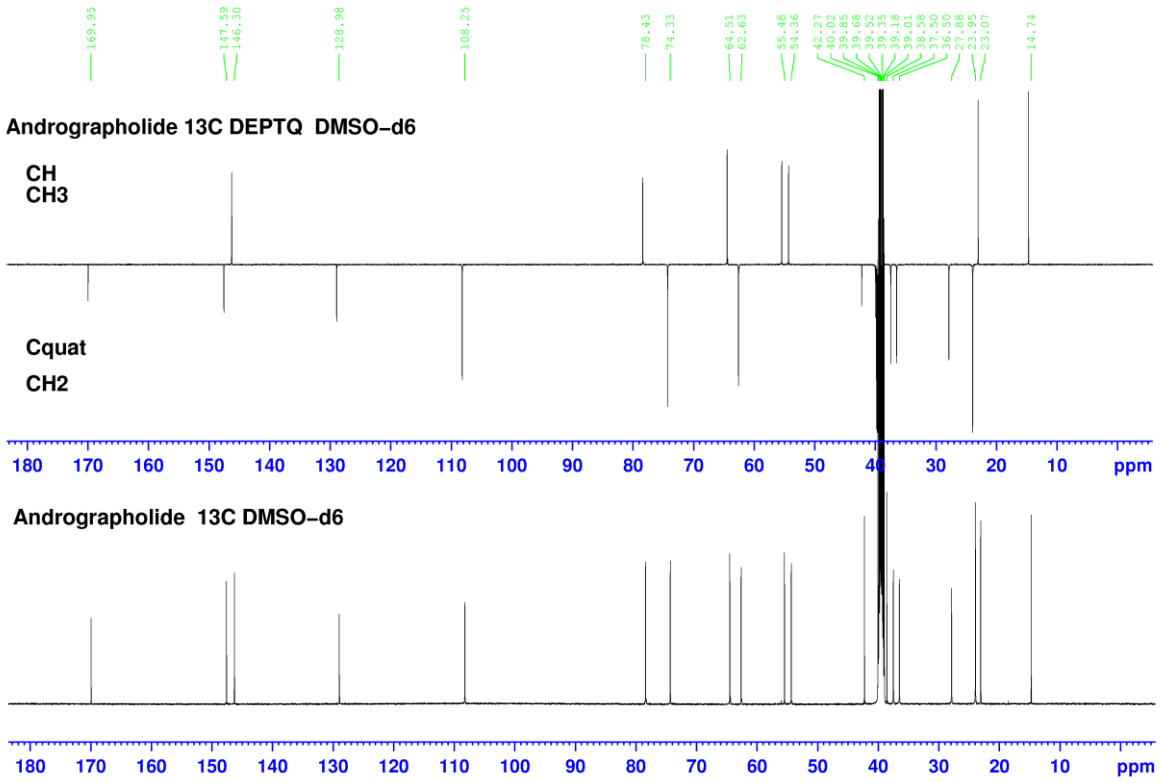
DEPT-135 is a very powerful experiment because it shows all CH, CH_2, and CH_3 resonances (but excludes all quaternary carbons) and also distinguishes CH and CH_3 groups from CH_2 groups by their sign differences + or -. Traditionally, CH_2 groups are shown as inverted peaks, but in some instances this may be reversed, it is the relative sign differences of the carbon signals that are important. By comparing the normal ^{13}C spectrum with that of DEPT-135, quaternary carbons can also be identified by their absence in DEPT-135, as shown below. Another useful feature is the loss of the deuterated solvent multiplet signals, which can itself be useful if a carbon signal of interest is overlapping with that of the solvent.



DEPT-90 can be a useful experiment, in that it only retains methine CHs in molecules. If your compound has a mixture of CHs and CH_3s which cannot be distinguished in DEPT-135, then this is one of the only techniques that can distinguish CHs from CH_3s. As shown below Andrographolide only contains 5 CHs which can be easily observed in DEPT-90.



DEPT-Q (DEPT retaining quaternary centres) – this is an experiment that retains all carbon signals **including** quaternary centres and operates in a similar manner to the DEPT-135. Thus, it maintains all the sensitivity benefits other DEPT experiments by using polarisation transfer, but also retains quaternary carbons which are phased the same way as CH_2s. It does not seem to be as popular at DEPT-135, probably due to unfamiliarity. In practice the technique suffers from low sensitivity of the quaternary carbons, since they are marginally weaker than the simple proton decoupled carbon experiment.



4.4 Further comments

DEPT is a relatively old NMR technique and has now been largely superseded by the 2D edited-HSQC experiments (see Section 6.2), which is even more informative and has greater sensitivity. To save time, the edited-HSQC is often used instead of DEPT-135 to provide equivalent spectral editing information. The recent DEPT-Q experiment also has greater information content e.g. all carbons signals and is often used now in preference to DEPT-135.

5. Homonuclear Correlations

5.1 ^{1}H-^{1}H COSY- Correlation Spectroscopy

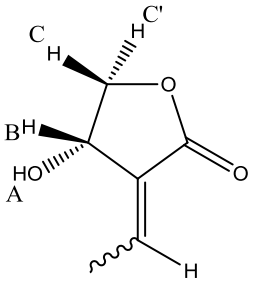
5.1.1 What is it?

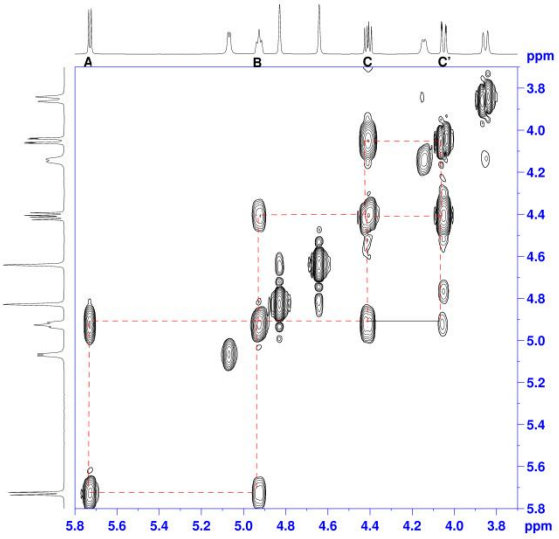
COSY is a 2-D NMR experiment which provides a means of identifying mutually coupled protons and is the most widely used 2D experiment. The name 2-D NMR refers to the fact that the experiment generates 2 frequency axes F2 (x-axis) and F1 (y-axis). The COSY experiment is the most efficient way of establishing connectivities when a large number of coupling networks need to be identified, as it maps **all correlations** within a single experiment and is now used routinely. It has superseded the older 1-D homonuclear decoupling experiment, which is very seldomly used now.

5.1.2 What does it look like?

The experiment, like all 2-D NMR experiments, presents a two-dimensional contour map. In COSY, each dimension represents proton chemical shifts and the contours represent signal intensity (just as contours are used to map mountains heights). The diagonal (running top right to bottom left) shows peaks that correspond with those in the usual 1D spectrum, and contain no new information. The peaks of relevance are the off-diagonal or *crosspeaks*. Each of these represents J-coupling between the protons that are correlated by the crosspeak. The spectrum is symmetrical about the diagonal as a coupling from proton A to B will always be matched with one from B to A.

A portion of the COSY spectrum is shown below and the coupling pathway from the hydroxyl proton (which is slow to exchange in DMSO-D6) to neighbouring protons is illustrated. This experiment allows one to identify possible structural fragments, such as OH-CH-CH$_2$ in **Andrographolide.**

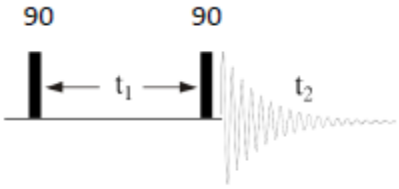




*A section of the ^{1}H-^{1}H COSY spectrum of **Andrographolide**, correlations between coupled protons are mapped out in stepwise as shown for the fragment.*

5.1.3 Different types of COSY Experiments – Which one do I use?

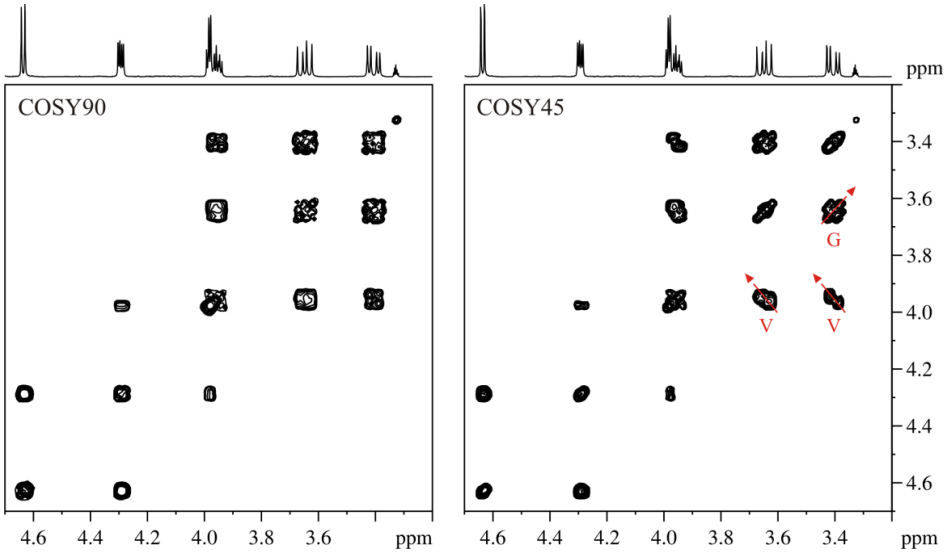
The default COSY experiments set up for automation are variants of magnitude mode (no phasing is required during processing) β-COSY, where β is a variable flip angle of the second pulse. The commonly used variants are COSY-45 and COSY-90, the main difference between the two lies in the flip angle of the second pulse- 45 degrees or 90 degrees respectively; see pulse sequence below;



During the incremented time period t_1, the chemical shifts and J-couplings evolve and it is the second pulse in which 'coherence transfer' occurs between J-coupled spins giving rise to the expected correlated cross peaks.

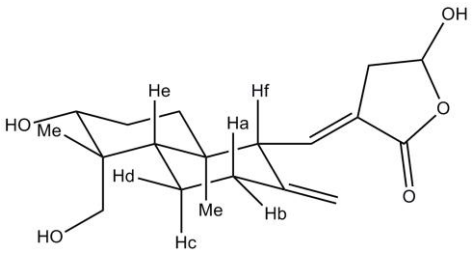
Both experiments can be acquired in a matter of minutes for strong samples, although the signal to noise ratio is theoretically higher for COSY-90, COSY-45 is usually the preferred experiment because the diagonal signals are smaller and less intense, allowing correlations between closely overlapping resonances to be resolved more easily. Another advantage of COSY-45 is the cross peaks have a tilted appearance, such that positive $^{3}J(^{1}H\text{-}^{1}H)$ vicinal couplings display a negative slope with respect to the diagonal, whereas as negative $^{2}J(^{1}H\text{-}^{1}H)$ geminal couplings which are usually negative in sign, possess the positive slope with respect to the diagonal. Where there are additional couplings within multiplets, tilting may not be so clearly defined. Due to the clarity of the cross peak versus the diagonal, COSY-45 is usually the method of choice.

Comparison of COSY-90 and COSY-45 illustrating sloping vicinal and geminal coupled cross peaks.



5.2 DQF-COSY – Double-quantum filtered COSY

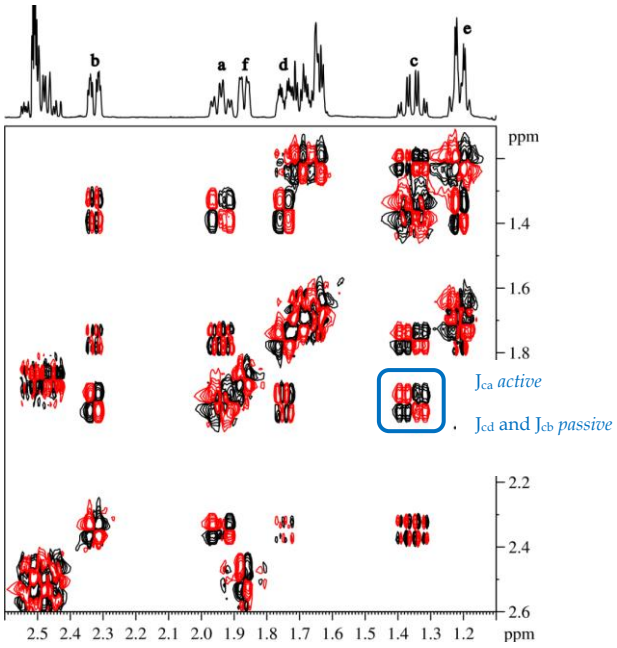
The basic COSY experiments above suffer from the drawback that the diagonal peaks possess dispersive tails from singlets which can mask relevant cross peak correlations that fall close to the diagonal, so that important correlations can be lost or are difficult to decipher. This limitation may be overcome with DQF-COSY (double-quantum filtered COSY), which is a phase sensitive experiment (spectrum requires interactive phasing during processing for +/- red/black contours) and is the method of choice for very crowded and overlapping multiplets, yielding higher resolution cross peak structure. The DQ filter thus only retains the relevant J-coupled cross peaks and excludes nuisance tailing singlets. Another advantage of DQF-COSY is that it is possible with care to extract larger J coupling constant values, once *'active'* and *'passive'* spins have been assigned.



In-phase red-red or black-black show *passive* J-couplings to other protons

Anti-phase red-black show *active* couplings between the correlated protons

Note - *J couplings are measured in Hz between centres of contours. Measurements of smaller J values may be inaccurate due the low digital resolution and hence insufficient data points to define J accurately.*



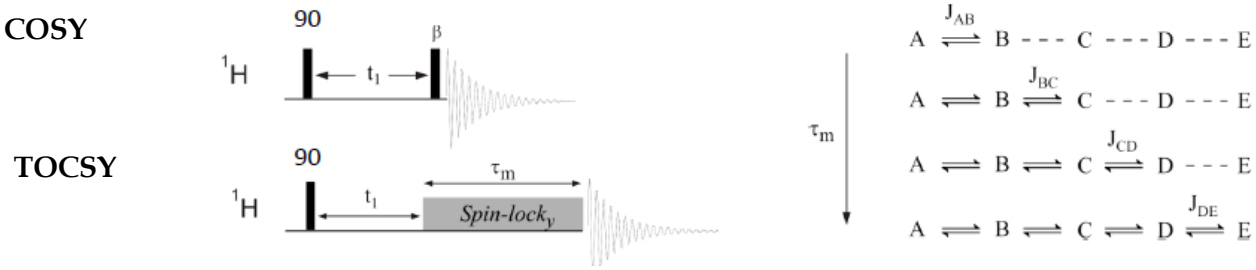
For a more detailed explanation of active and passive coupling please read '*High Resolution NMR Techniques in Organic Chemistry*' by TDW Claridge.

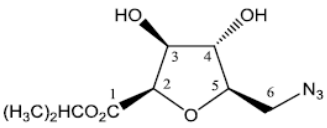
5.3. TOCSY – Total Correlation Spectroscopy

5.3.1 What is it?

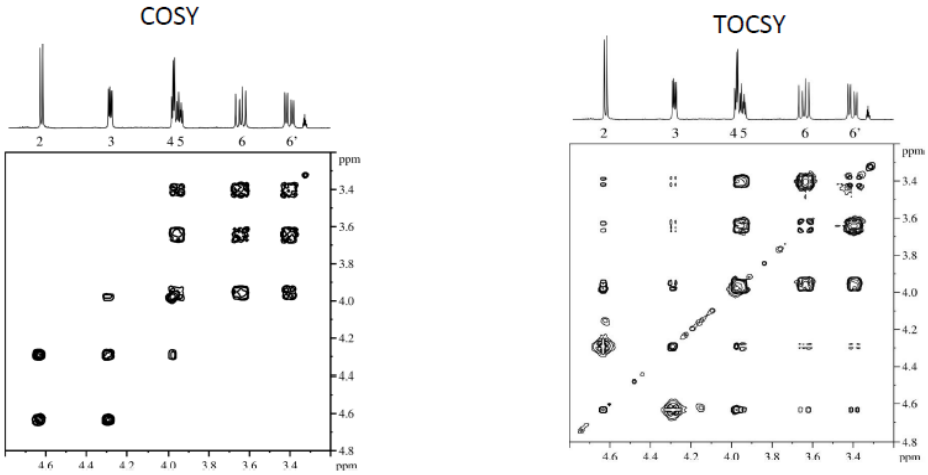
TOCSY is a very powerful alternative to COSY and is the experiment you do **after** careful examination of COSY, if you have an extended continuous chain of spin-spin coupled protons, typically for rings systems or long chain spin systems (e.g. sugars, peptides), particularly where there are overlapping multiplets. You can trace the complete spin system associated within the network of coupled spins of interest. TOCSY not only yields neighbouring homonuclear ^{3}J-coupling proton-proton correlations like COSY, but also provides remote correlations to distant spins as long as there is a J-coupling network from one proton to the next.

The main difference between COSY and TOCSY pulse sequences is the replacement of the COSY β (90^0 or 45^0) pulse with a *spin-lock* (which constitutes a train of closely spaced pulses given names like MLEV-16 or DIPSI-2) applied for a mixing time τ_m of between 20 and 200 ms. A short spin-lock makes the TOCSY more COSY-like in that more distant correlations will usually appear weaker than short-range ones whereas a longer spin-lock allows correlations over large coupling networks (illustrated schematically below). The length of the spin-lock is roughly related to the distance through the coupling network that correlations are seen. However, there is a limit to τ_m, too long a spin-lock will heat the sample causing signal distortion and may also damage the electronics of the probehead. TOCSY experiments typically take slightly longer than COSY, due to the fact that magnetisation is distributed across many more spins, thus reducing peak intensities.



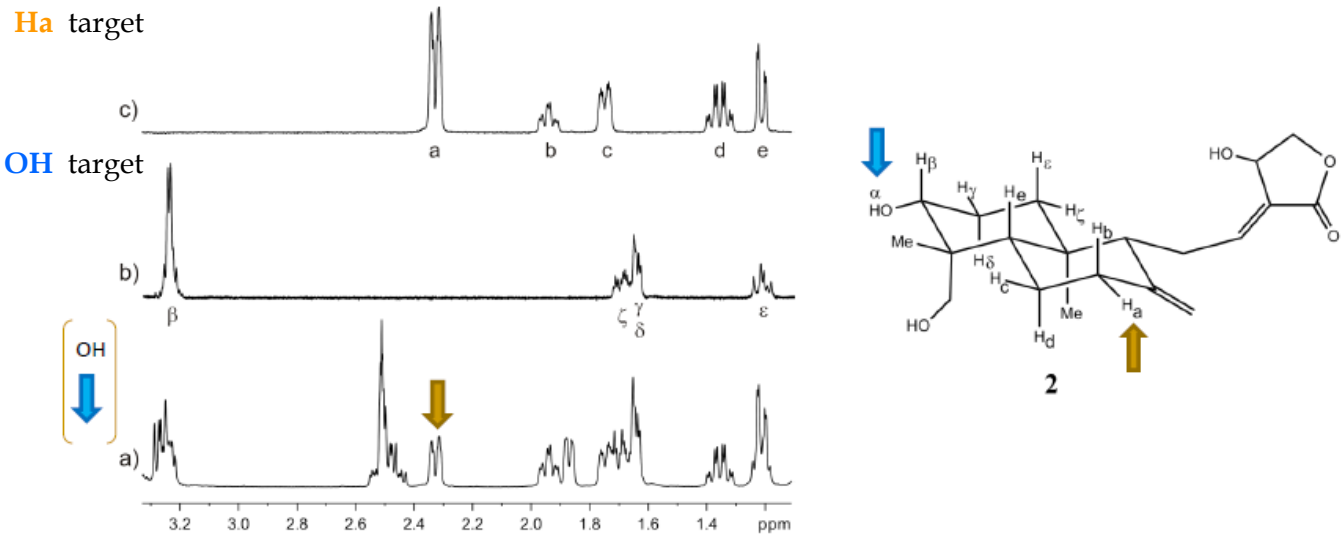


Examples of COSY and TOCSY showing the extended chain of coupled proton cross peaks in TOCSY



5.3.2 1-D Selective TOCSY

A useful alternative to the 2-D TOCSY above, is the 1-D TOCSY analogue, which can give clearer results particularly for over-crowded spectra or mixtures (e.g. diastereoisomers), where a pure single component spectrum can be edited out from the overlapping multiplet mixture. These are also often quicker to acquire and are recorded under higher resolution in the 1-D experiment. What is required, however, is a single isolated (in chemical shift) target peak selected in the initial set up of the experiment and to be used as the magnetisation transfer source. The resultant 1-D spectra reveal 'pure' multiplet structures of discrete spin systems within the molecule that were otherwise overlapped or buried. Note the approach is also often applicable to selectively targeting amide protons in polypeptides and anomeric protons in oligosaccharides.



1-D TOCSY spectra where α-OH and Ha protons were selectively excited sources for TOCSY transfer.

6. Heteronuclear shift correlations

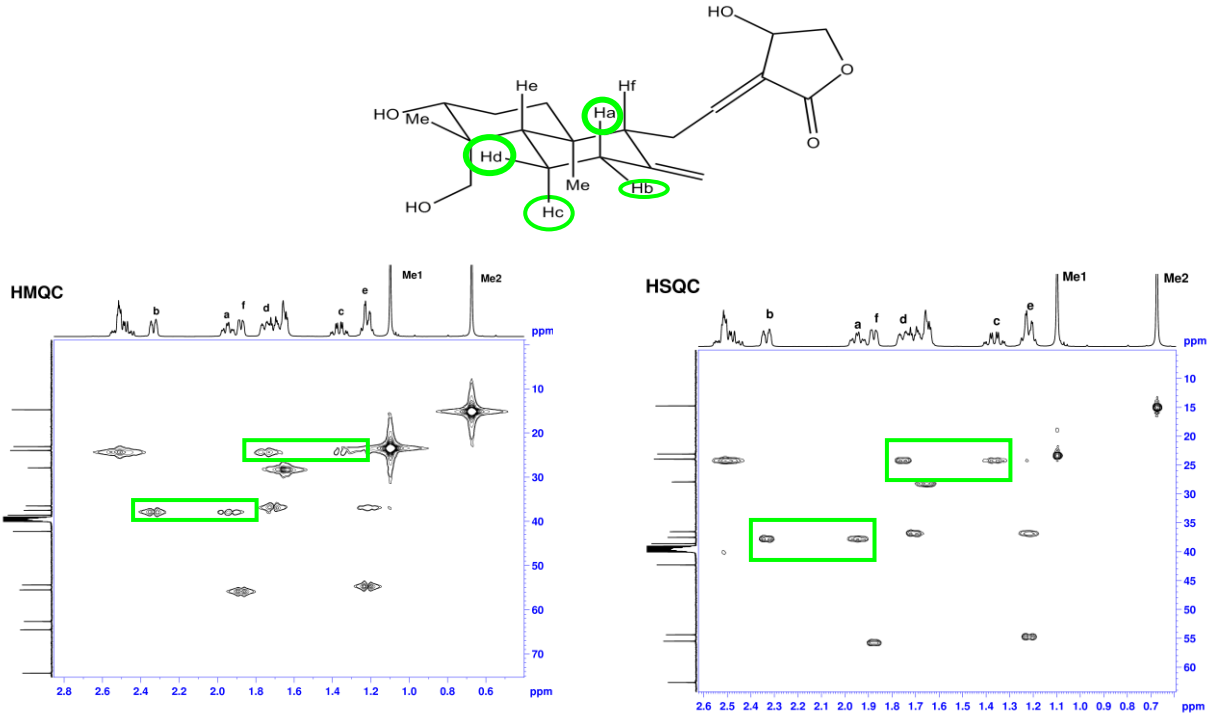
6.1 HMQC and HSQC

6.1.1 What are they?

Heteronuclear shift correlations generate 2-D correlation spectra between two different nuclei, typically one is ^{1}H and the other a less sensitive nucleus e.g. ^{13}C , ^{31}P, ^{15}N etc. For organic chemists this section will be directed at ^{1}H-^{13}C correlations, but similar principles apply to other nuclei. These useful techniques directly correlate the chemical shifts of protons and their directly attached carbons, utilising $^{1}J_{CH}$ couplings (and are typically optimised for the 'average' $^{1}J_{CH}$ ~ 145Hz). These can enable proton assignments to be mapped directly onto their bonded carbons and may be used to spread the resonances of a complex proton spectrum according to the greater dispersion of carbon resonances to which these protons are attached. HMQC (heteronuclear multiple quantum correlation) and HSQC (heteronuclear single quantum correlation) use '*inverse*' proton detection for maximum sensitivity. HMQC tends to be more robust to experimental imperfections than HSQC (so is sometimes preferred on older spectrometers), but the correlations are not as well resolved along the ^{13}C dimension in the former experiment, so HSQC tends to dominate nowadays.

6.1.2 What do they look like?

The 2-D spectra provide a clear map of connectivities where the cross peaks correlate each proton signal to its attached carbon-13 neighbour. The spectrum indirectly provides ^{13}C chemical shifts and has greater sensitivity than directly observed 1-D ^{13}C experiments. Where there is complexity due to overlapping proton multiplets, the structural assignments of diastereotopic geminal proton pairs can be easily discerned, as two dispersed proton resonances correlate with a single carbon shift; see example spectra below for Andrographolide (green).

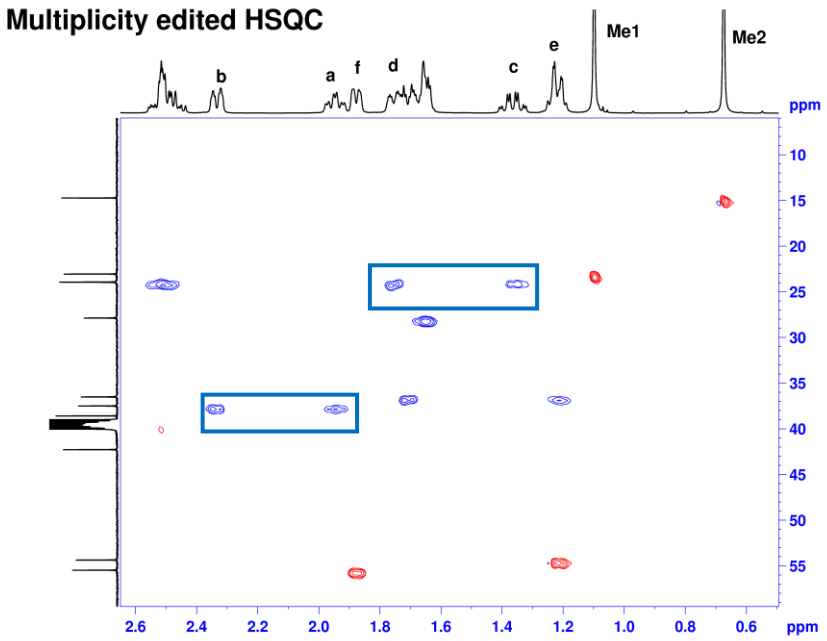


Comparison between HMQC and HSQC showing greater clarity of cross peak in HSQC.
The boxed regions show correlations of diastereotopic geminal protons.

6.2 Multiplicity Edited-HSQC

6.2.1 What is it?

This is a variant of HSQC which combines both spectral editing and HSQC into one experiment and thereby 'kills two birds with one stone', and has now become one of the **most useful** NMR experiment for spectral assignments. The correlation cross peaks are conveniently colour coded according to DEPT-135 multiplicity e.g. CH and CH_3 are the same colour (sign +, red), whereas CH_2s are a different colour (sign -, blue); the colour choice is arbitrary. As shown below, the CHs and CH_3s are instantly distinguishable from CH_2s even though there is appreciable overlap in the proton dimension.



Edited-HSQC spectrum showing colour coding of CH_2s (blue) and CHs and CH_3 (red)

6.2.2 Which one do I use?

As shown both HMQC and HSQC provide equivalent information, but HSQC is often the experiment of choice due to its superior resolution in the ^{13}C dimension, particularly for crowded spectra. The clarity of the contours in HSQC is due to the absence of ^{1}H-^{1}H couplings which contaminate the ^{13}C dimension in HMQC. The disadvantage of HSQC over HMQC is a loss of signal intensity due to the greater number of pulses in the former pulse sequence and its susceptibility to pulse miscalibrations which might be a feature on automated instruments, but is now less problematic on modern instruments. Due to its superior resolution, and the powerful CH/CH_3 and CH_2 spectral editing ability of edited-HSQC, this experiment is now mostly routinely used for data collection.

Warnings !

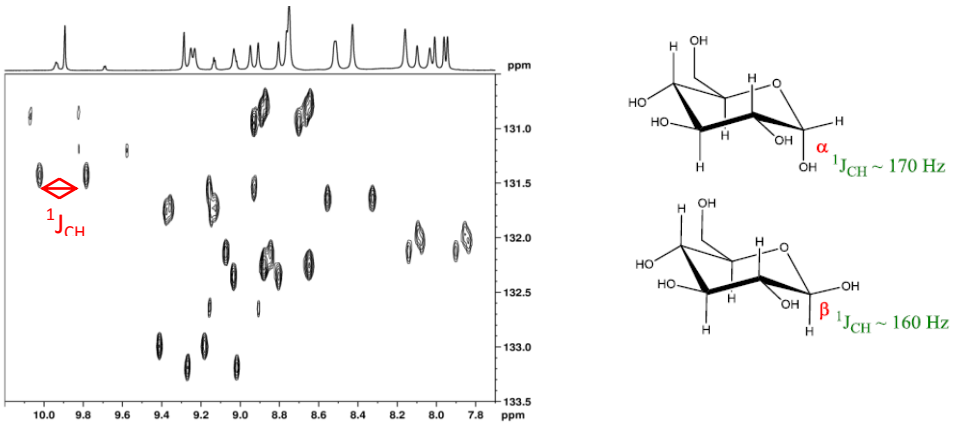
The pulse sequences of HMQC, HSQC and edited-HSQC all involve delay periods Δ which depends upon $^{1}J_{C\text{-}H}$, usually taken as 145Hz being an average value for one bond $^{1}J_{C\text{-}H}$ values; see table below for the range of ^{1}J(C-H) values. In exceptional circumstances you may not observe an expected correlation, typically for alkynes with $^{1}J_{C\text{-}H}$ 240-250Hz, which depart from the nominated average $^{1}J_{C\text{-}H}$ of 145Hz. In rare cases longer range correlations could appear if their coupling constants approach this average value e.g. 2-bond couplings can appear for alkynes with $^{2}J_{CH}$ ~50Hz. So care is needed for data interpretation, with a view to discussing this limitation with an NMR expert who will then adapt the experiment to the needs of your compound.

Proton environment	Typical $^{1}J_{CH}$ range/ Hz
Aliphatic, CH_n-	125-135
Aliphatic, CH_nX (X= N, O, S)	135-155
Alkene	155-170
Alkyne	240-250
Aromatic	155-165

6.3 Coupled-HSQC - for measuring $^{1}J_{CH}$

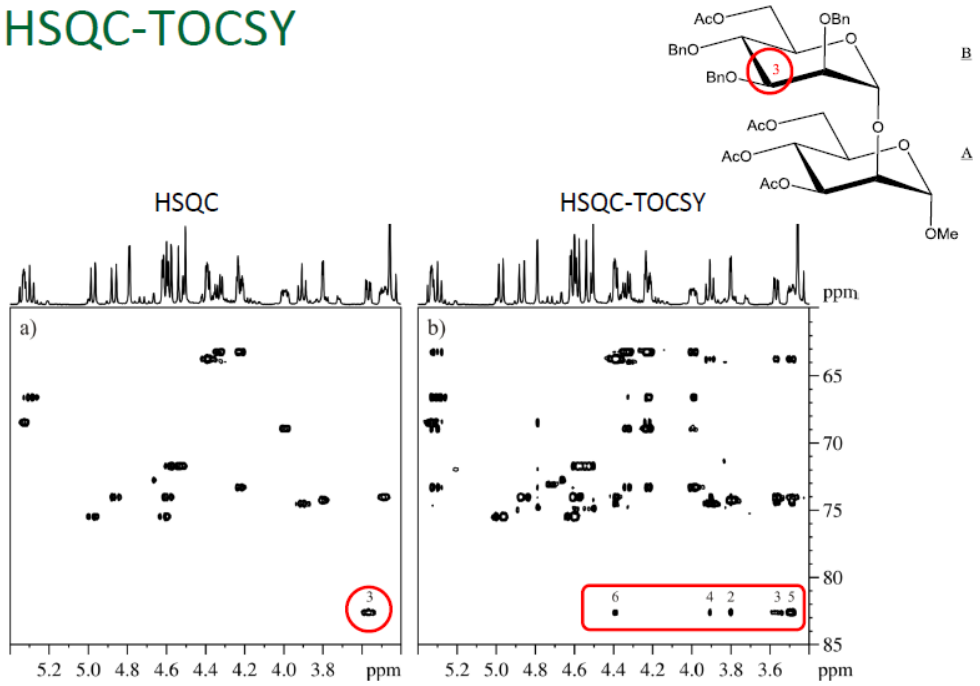
HSQC detects ^{13}C satellites as doublets, but these are normally ^{13}C decoupled so they appear as single cross peaks. In the coupled-HSQC experiment this coupling is retained and provides a means for measuring $^{1}J_{CH}$ values with high detection sensitivity relative to a proton-coupled ^{13}C spectrum. The application of coupled–HSQC can be readily appreciated in situations where the coupled signals are isolated in chemical shift and where the size of the coupling constant can be definitive for distinguishing stereochemistry e.g. the anomeric α or β centres in sugars. Here the ^{13}C satellite doublet structure remains in the proton dimension and $^{1}J_{CH}$ can be measured from the spectrum directly, see below.

Coupled HSQC for measuring $^{1}J_{CH}$ values



6.4 HSQC-TOCSY – A tool for over-crowded ^{1}H spectra

This experiment can be used for very crowded proton spectra, for which COSY and HSQC have failed to provide unambiguous assignments. HSQC-TOCSY is a hybrid NMR experiment which combines the two pulse sequences HSQC and TOCSY in order to relay the magnetisation along proton network, using the greater dispersion of the carbon chemical shifts to elucidate assignments. Each row for a particular carbon chemical shift contains a proton sub-spectrum of the molecular fragment. In the example shown below for the disaccharide, C3 contains many correlations compared to the normal HSQC spectrum, allowing assignments of the complete sugar B ring system.

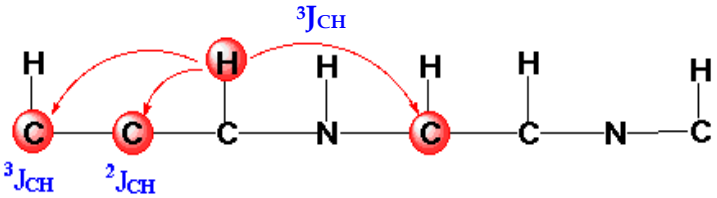


At the row representing C3, the observable proton coupling pathways of the B ring are mapped out, with the exception of H1 which is not observed due to the $^{3}J_{(H1H2)}$ ~ 0 Hz. The simplification of crowded proton spectra using HSQC-TOCSY makes this a very powerful tool.

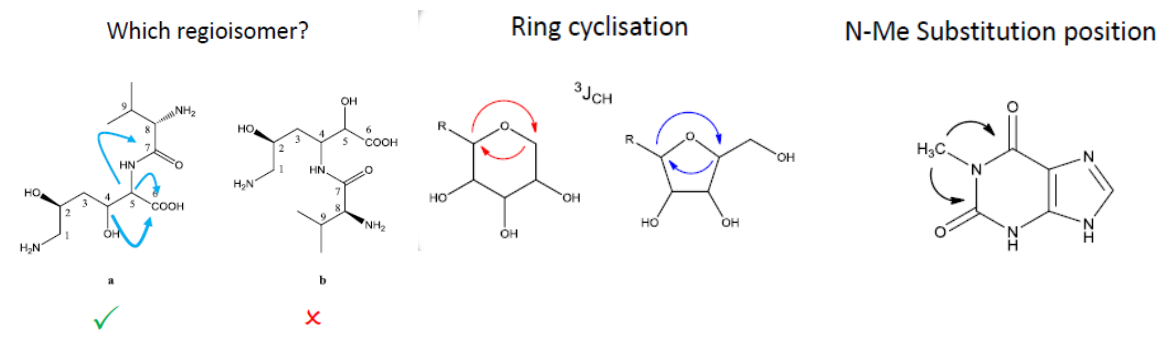
6.5. HMBC- Heteronuclear Multiple-Bond Correlation

6.5.1 What is it?

HMBC is similar to the HMQC/HSQC experiments in that they are all '*inverse*' proton detected proton-carbon correlation experiments. Whereas HSQC detects correlations due to $^{1}J_{CH}$ couplings, HMBC detects the smaller $^{2/3}J_{CH}$ couplings that operate across multiple bonds. It therefore has the ability to identify ^{1}H-^{13}C correlations across multiple C-C and C-X linkages providing one of the **most powerful** tools in NMR, enabling connectivity of molecular skeletons to be defined.



Decisions as to a particular regio-isomer may be made using HMBC, for example proving five versus six membered rings or identifying a substitution position. Correlations to quaternary carbons (which do not appear in HSQC) may also be observed, thus revealing their chemical shifts.



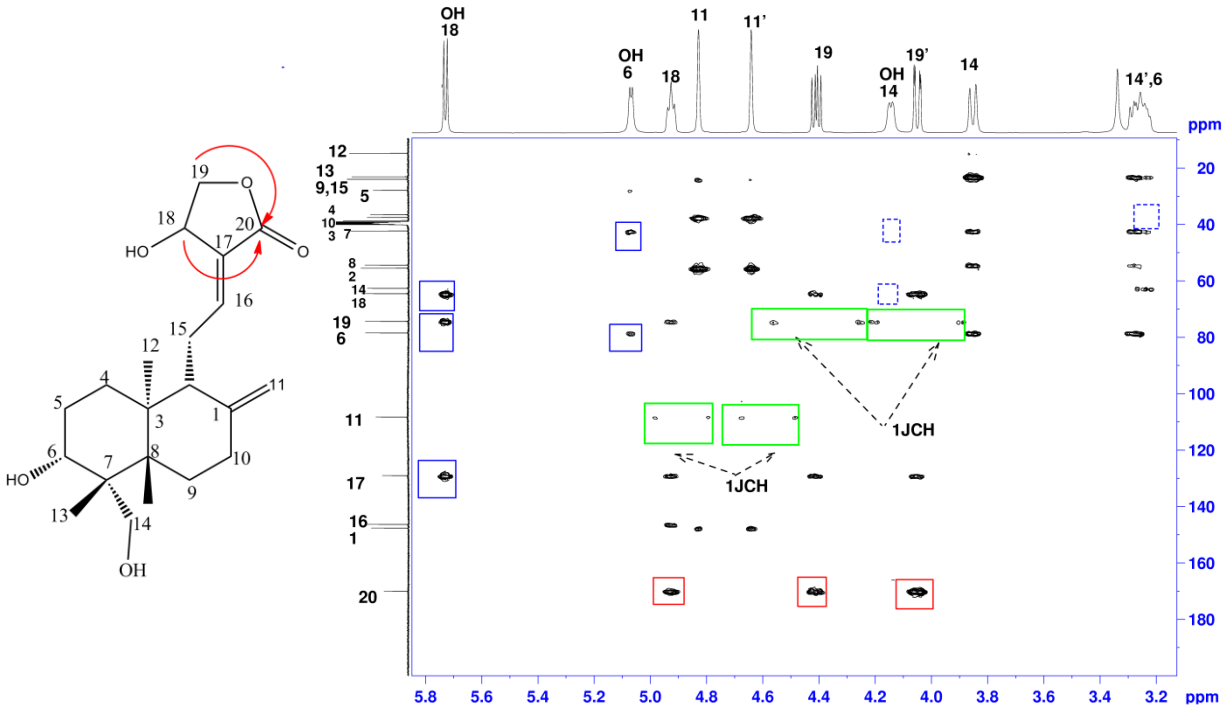
Thus **HMBC** is a long range or multiple bond correlation technique yielding connectivities for $^{n}J_{CH}$ where n= 2, 3, possibly 4 for extended conjugated systems); see the table below for the example $^{n}J_{CH}$ values. The preparation period, $\Delta_{LR} = 1/2\ ^{n}J_{CH}$, is a parameter of the pulse sequence used to select $^{n}J_{CH}$ couplings between 1-20 Hz and is typically optimised for ~ 8 Hz.

$^{2}J_{CH}$	Coupling pathway	$^{3}J_{CH}$	Coupling pathway	$^{4}J_{CH}$	Coupling pathway
(±) ≤ 5	**H—C—C**	≤ 5	**H—C—C—C**	(±) ≤ 1	**H—C=C—C=C**
≤10	**H—C=C**	≤15	**H—C=C—C**	≤ 1	**H—C—C—C—C**
40-60	**H-C≡C-C**	≤ 5	**H—C≡C—C**		
20-25	**H—C(=O)—C**				

The intensities of the cross peaks depend on the size of $^{n}J_{CH}$ and are very much weaker for longer 4 or even 5 bond correlations. ***It is often difficult to tell the difference between 2 and 3 bond correlations due to their coupling constants having similar magnitude, and this must be born in mind during data interpretation.***

6.5.2 What does it look like?

The HMBC spectrum closely resembles that of HMQC and HSQC in appearance, but with many more long range correlations associated with each proton chemical shift.



As shown, although $^{2}J_{CH}$ and $^{3}J_{CH}$ correlations are clearly observed, they cannot be differentiated in this experiment. 3-Bond correlations across heteroatoms and correlations to quaternary centres (e.g. carbonyl carbons, see red squares) are often very informative. Most $^{1}J_{CH}$ couplings are filtered out, but at low contour levels breakthrough can occur and they appear as equidistant dots (these doublets are $^{1}J_{CH}$ satellites and are apparent as no ^{13}C decoupling is used; see green rectangles).

Warnings!

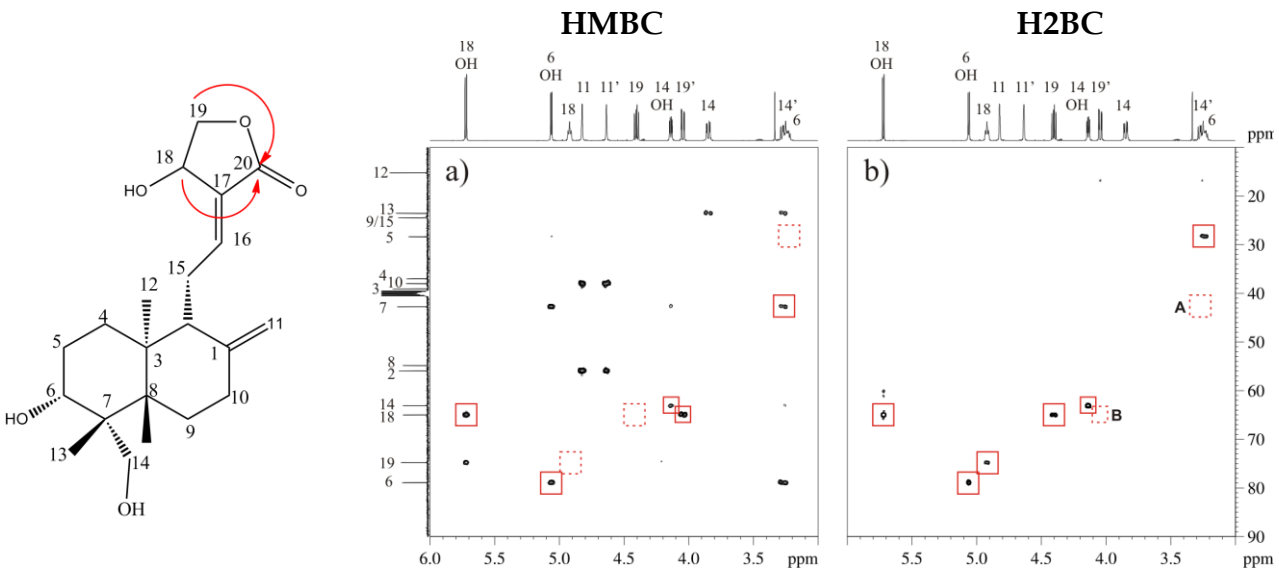
One of the disadvantages of HMBC is that some expected correlations may be '*missing*' (see blue dotted squares). There may be two reasons as to why correlations may be absent;

i) Very small $^{2}J_{CH}$ and $^{3}J_{CH}$ couplings <1.5Hz may negate the expected correlation; for vicinal couplings this may imply near 90^{0} dihedral angles between proton and carbon atoms.

ii) Broad lines lead to peak loss; broadening may be due to dynamic exchange e.g. OHs and NHs which exchange with water exhibit no correlations as shown for OH_{14} which appears to exchange faster with water than the other hydroxyls, OH_{6} or OH_{18}.

6.6. H2BC- Identifying 2-bond H-C correlations

One problem with HMBC is its inability to differentiate $^{2}J_{CH}$ from $^{3}J_{CH}$ correlations due to their similar magnitudes. One way of selecting two-bond H-C correlations without interference from three-bond correlations is the recent H2BC experiment. This in fact exploits two transfer pathways $^{1}J_{CH}$ followed by $^{3}J_{HH}$ and is essentially an HMQC-COSY experiment. It does not depend on the presence of $^{2}J_{CH}$ couplings BUT does require both carbons to bear protons which share a measurable $^{3}J_{HH}$ coupling constant.



A comparison of the HMBC and H2BC experiments.

However, the limitations of H2BC as shown by the missing contours (dotted red) are due to;

i) Box A - absent correlations to non-protonated quaternary carbons (e.g. H14-C7)
ii) Box B -absent due to small $^{3}J_{HH}$ <2Hz (e.g. H19'-H18).

7. Nuclear Overhauser Effect (NOE) Experiments

7.1 What are they?

Unlike the proceeding sections where the experiments utilise **through bond** *scalar (J) couplings*, nOes utilise direct **through space** magnetic interactions between nuclear spins known as *dipolar couplings*, which do not give rise to multiplet structure in solution state spectra. nOes are of interest to chemists because they can be related to inter-proton distances and may therefore provide information on 3D molecular shapes. nOes are changes in resonance intensities that arise due to close spatial proximities between nuclei, typically protons. They arise when one resonance in a spectrum is perturbed by either ***saturation*** or ***inversion*** (***saturation*** = equalising populations of spin states, ***inversion*** = inverting populations of spin states) brought about the application of radiofrequency energy. The proton resonance which is perturbed produces changes in the intensities of other resonances belonging to protons that are spatially close. The nOe enhancement may be quantified as η, the fractional enhancement of resonance intensity after either saturation or inversion:

$$\eta = \frac{I - I_0}{I_0} \quad (\times 100\ \%)$$

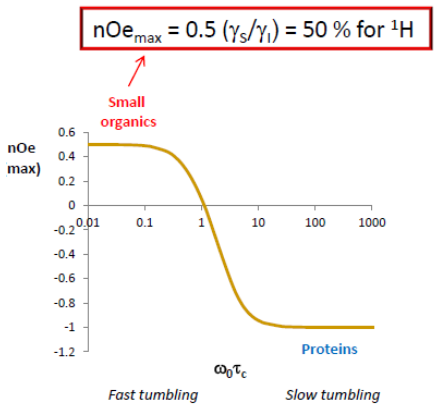
where I_0 = peak intensity of resonance in the absence of nOe and I = peak intensity of resonance in the presence of nOe.

The nOe is a rather weak effect (η << 10%) and experiments can take many hours of instrument time. Before embarking on nOe experiments, a full unambiguous assignment of the proton spectrum is a pre-requisite as wrong assignments can result in considerable time wasting. More than any other NMR experiment, nOes represent the most stringent tests for spectrometers both for short and long term stability, in particular, probe temperature stability, and use of accurate pulse calibrations.

Once an nOe experiment is deemed necessary, an important consideration which is often overlooked is the actual size or mass of the molecule and the solvent. As shown in the figure below, for small molecules of molecular mass < ~500 in a low viscosity solvent (e.g. $CDCl_3$) their tumbling rates are fast (τ_c the tumbling time in solution is short for small molecules; this is called extreme narrowing limit), the nOe is ***positive***, corresponding to an ***increase*** in signal intensity, and has a maximum theoretical value of 50%. For molecules that have molecular masses ~800-1000 (intermediate tumbling), the nOes diminish and tend toward zero. In fact, molecules of mass ~700 in viscous solvents (e.g. DMSO-d6) can also exhibit the same zero nOe effect, as can solutions of molecules of mass <500 in viscous solvents at low temperatures. This may come as an unexpected surprise when nOes are anticipated! Molecules that tumble very slowly such as polymers or proteins with masses >2,000 experience negative nOes, corresponding to a *decrease* in signal intensity, up to a maximum -100%.

The dependence of nOe signal intensity as a function of the molecular correlation time (a measure of how rapidly molecules tumble in solution). Smaller molecules tumble rapidly and produce weak positive nOes whereas large molecules tumble slowly and produce stronger negative nOes.

This represents theoretical maximum effects; experimental enhancements are often less than this.



ϖ_0= spectrometer frequency
τ_c = correlation time: a measure of how long it takes a molecule to rotate in solution

Another consideration is the strength of the magnetic field, as the size of the nOes are also field dependent. You may think that for sensitivity reasons your nOes may be clearer and better resolved at 700 MHz rather than at 500 MHz, but with a higher field strength the relatively 'small' molecule may fall within the intermediate regime at this field strength (due to the $\omega_0\tau_c$ dependence shown above, where ω_0 is the spectrometer frequency), and its nOes may then become negligible or even negative.

Lastly, sample preparation is of prime concern for nOes. Target resonances for selective 1-D nOes need to be isolated from neighbouring peaks. If they are overlapped, changing solvents e.g. from chloroform to benzene can be sometimes beneficial for separating resonances. Relevant signals should be sharp as broad signals such as OHs or NHs may be undergoing exchange with residual water or other exchangable signals and will lead to confusing saturation transfer (exchange) effects. In this case carefully drying solvents and solutes may prove helpful. Broad signals from CHs or CH_2s may indicate conformational exchange so low or high temperature nOes may prove more informative. Traces of metal ions e.g. from a nickel spatula can completely negate nOes due to paramagnetic relaxation. It may pay you to de-gas your sample by bubbling nitrogen or argon into the sample because traces of oxygen can also act as a paramagnetic relaxation source and negate nOes; this can be especially useful for the study of very small molecules.

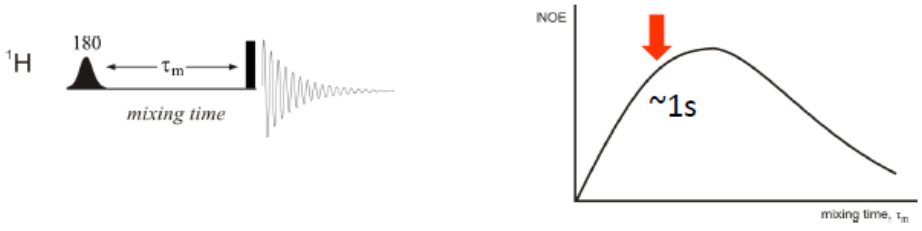
What choice of nOe experiment do I have, what do they look like and what type of nOe experiment do I use for my sample?

There are several choices of 1D and 2D nOe experiments; selection depends on the compound under investigation, the size of molecule, what type of information is required, whether resonance overlap exists or whether qualitative or quantitative information is needed. The following sections describe the individual experiments and their application. Nowadays most 1D experiments employ gradient selected transient nOe experiments which have largely superseded the original 1D steady state (or equilibrium) measurements; as such only the modern methods will be described herein.

7.2. 1D-Transient nOes – The gradient-selected nOe Experiment

The recent development of 1-D gradient selected nOes (also known as single or double pulsed field gradient spin echo experiments- SPFGSE or DPFGSE) have revolutionised 1-D nOe experiments because they provide very clean spectra with few artefacts and are now the routine method of choice for small molecules. They have the added advantage in that they are reasonably quick and are ideal for providing qualitative stereochemical and regiochemical answers to structural problems in organic chemistry.

In this experiment a target resonance is selectively inverted with a shaped 180^0 pulse from which the nOes ultimately develop during a time interval know as the 'mixing time', τ_m. The choice of mixing time is dictated largely by the spin relaxation rates of the protons which define the initial build-up rates of the nOes themselves. Too short a mixing time e.g. <100 msec would yield very small enhancements as no substantial nOe will have developed, whereas very long times would give similarly weak signals due to decay of the nOe (see figure below). For most small molecule work, τ_m is usually 500-800 msec.



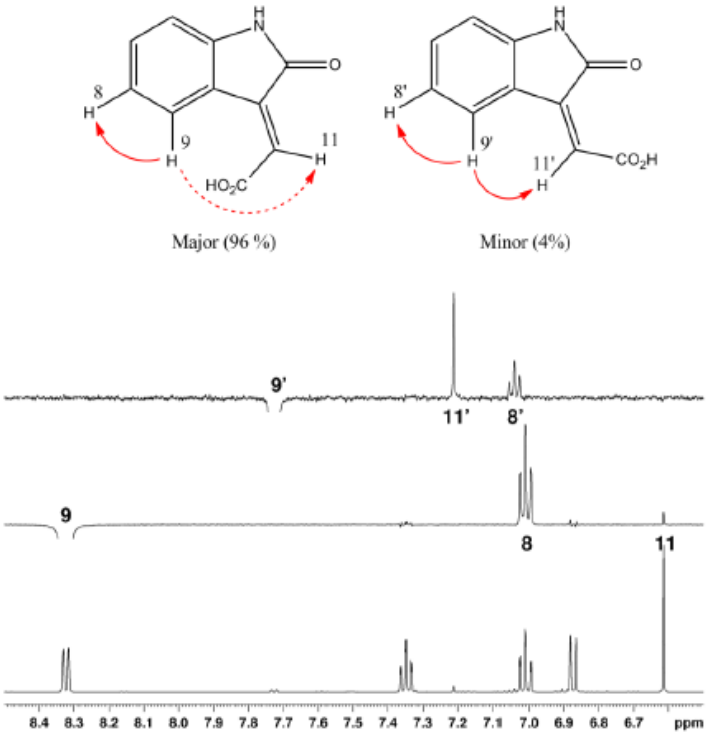
Schematic Pulse sequence for the 1-D Selective Transient nOe experiment and graph showing the evolution of the transient nOe.

The nOes are termed transient because they are not driven towards a steady state experiment but initially build and later decay through relaxation processes operating after the initial perturbation and are typically

sampled at a τ_m chosen during the linear part of the build-up curve. As a general rule, smaller molecules require longer mixing times and vice versa. Occasionally, if longer inter-proton distances are sought, τ_m is extended to 1-1.5sec.

The clear advantages of this modern gradient selected nOe experiment can be seen in the example below for the major and minor isomers of the indol carboxylic acid, where even the nOes of the minor isomer at 4% can be detected without interference from the major form.

Selected transient 1-D gradient nOe experiment on a mixture of isomers of indol carboxylic acids recorded with a mixing time of 800msec showing how the nOes of the two compounds can be clearly separated.



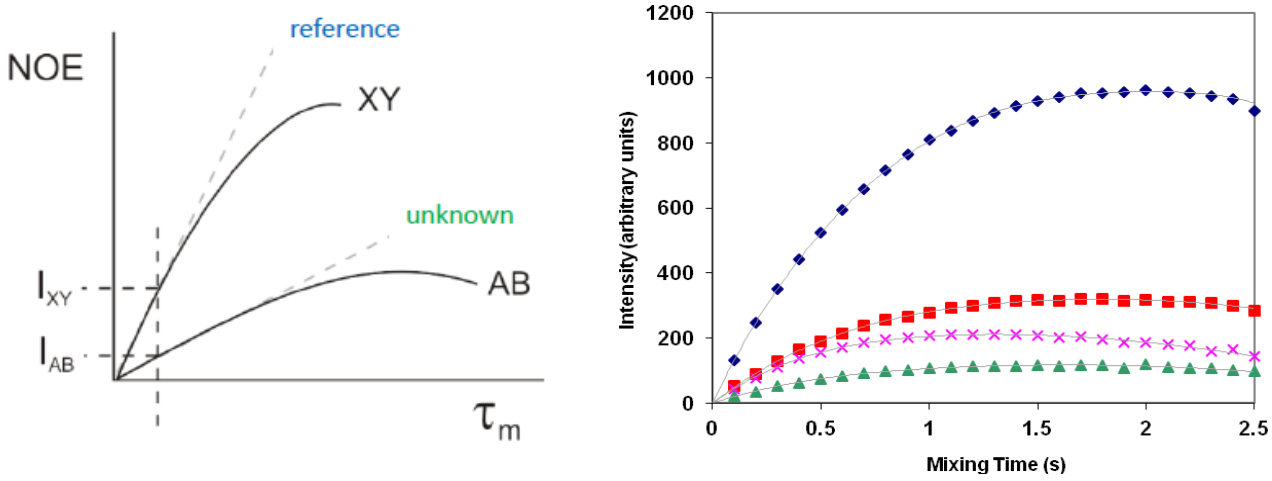
Due to the time dependence of transient nOes and complexities associated with quantifying their absolute intensities, these 1-D gradient nOes are rarely defined as percentage enhancements in routine measurements. Instead transient nOes are customarily reported semi-quantitatively, as small, medium and large (the range of inter-proton distances typically being 0.25-0.5 nm). Each nOe then provides a relative distance relationship between the target spin and those close to it in space.

7.2.1 Estimating interproton distances

Despite the comments above, one advantage of gradient nOe experiments, in addition to producing 'clean' nOe spectra, is the ability to measure inter-proton distances with appropriate experimental procedures. Careful thought needs to be given to this activity as it is tremendously time consuming both for the instrument and the experimenter! However, by systematically recording several (>8) measurements with different mixing times, τ_m, the initial build-up curve for proton pairs be plotted (for which typically τ_m < 500 ms); see schematic graph and actual curves below. The initial nOe build-up rates scale linearly with $1/r^6$, meaning the nOe intensities build slowly with increasing inter-proton distances. The procedure to determine these is to select a known reference distance in the molecule from which the unknown distance can be estimated using the relationship below;

$$\frac{I_A\{B\}}{I_X\{Y\}} = \frac{r_{AB}^{-6}}{r_{XY}^{-6}}$$

Most often the reference distance is taken to be diastereotopic geminal pairs (r_{HH} ~0.175 nm) or ortho aromatic protons (r_{HH} ~0.28 nm)



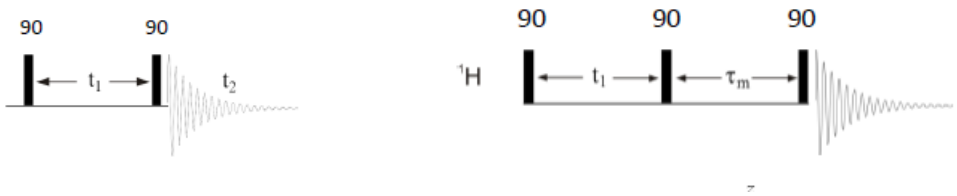
Left: Schematic representation for estimating inter-proton distances from 1-D transient nOes in which a reference pair of protons XY provides the 'known' calibration distance r_{XY} from which the unknown distance for AB, r_{AB} can be estimated, by taking ratio of the initial slopes I_{XY} and I_{AB}. Right: Example of real experimental nOe build-up data.

7.2.2 Disadavantages of 1-D gradient nOe experiments

For small molecules the only notable disadvantage is the inability to readily quantify and report percentage enhancements, meaning nOes are more often listed in a semi-quantitative manner (weak, medium, strong). However, nOe spectra free of artefacts that are relatively quick to acquire outweigh the lack of percentage nOe data.

7.3. 2D NOE Experiments (NOESY) - Experiments for small and large molecules

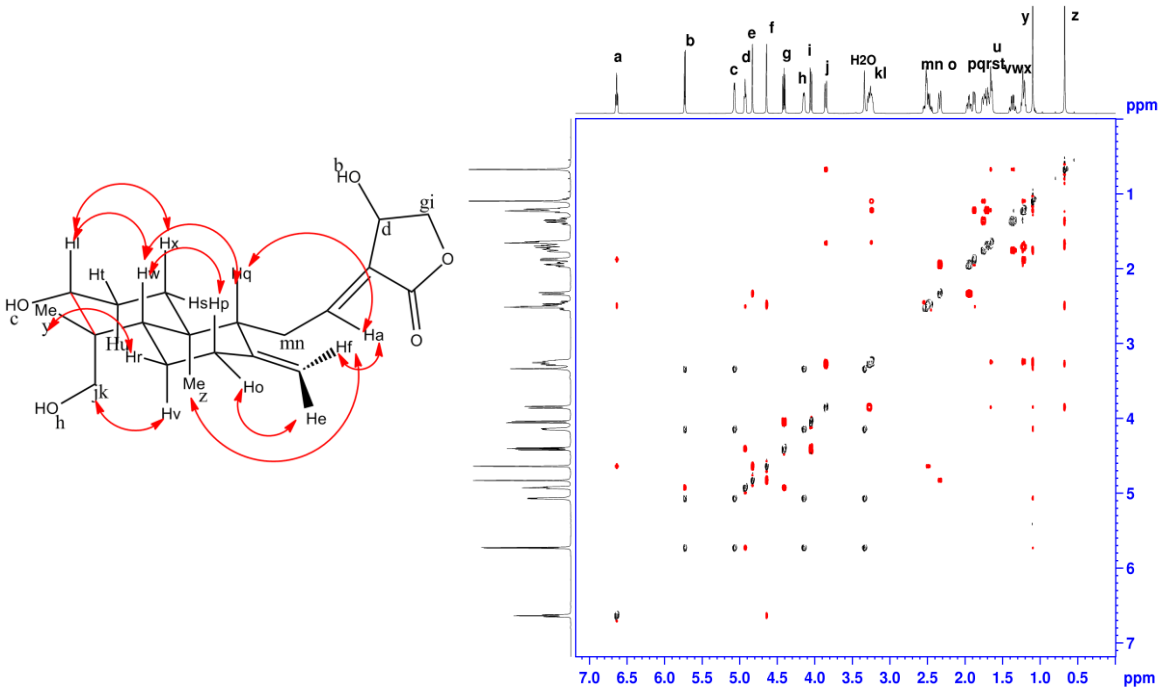
The 2-D analogue of the 1-D transient nOe experiment described above is 2-D NOESY, analogous to the 2D COSY experiment, but instead of mapping **all** the J-couplings in the molecule, NOESY maps **all** the nOes within the one experiment. The basic pulse sequences differ by the introduction of a mixing time τ_m in which the nOe develops and a further 90° read pulse for the NOESY sequence.



Pulse sequences for COSY (left) and NOESY (right)

2-D NOESY is a transient phase-sensitive 2-D nOe experiment that can be used for both small and large molecules, but is unsuitable for medium sized compounds approaching 1000-2000 Da molecular mass. Being phase-sensitive means phase manipulation is required during processing of the spectra. NOes appear as symmetrical off-diagonal contours and are strong and of the same phase (colour) as the diagonal for large molecules (negative nOes), but are weaker and the opposite phase to the diagonal (different colour) for small molecules (positive nOes). The NOESY spectrum for andrographolide (Mr =350) in DMSO-d6 is

shown below using a τ_m of 800 msec and depicts a complete map of all the nOes (red) illustrated on the structure below:



2-D NOESY spectrum of Andrographolide in DMSO-d6 800 ms mixing time, recovery delay of 1.5s, ns =16. nOes are red, hydroxyl OH and water exchange peaks are black, and the nOes are depicted as red double arrows on the structure.

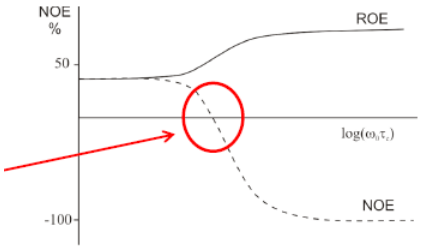
Note, there are some very intense cross peaks (3-6 ppm in black) that share the same phase as the diagonal and are attributed to OHs that arise from chemical exchange with H_2O. In fact this same experiment can be used to investigate dynamic exchange processes, wherein it is termed EXSY (exchange spectroscopy). Like heteronuclear correlations, broad peaks may not show cross peaks due to their short relaxation times or they may indicate underlying exchange processes. The use of phase sensitive representation allows the discrimination of nOes (red) from exchange peaks (black). The sign (colour) of the nOes with respect to the diagonal, is also indicative of the size and motional behaviour of the molecule, with large molecules having the same sign (colour, black) as the diagonal.

Unlike 1-D nOe experiments, 2-D NOESY experiments may be readily set up in automation, but do demand significant time relative to most other common experiments (remember nOes are rather weak effects). It is therefore best to perform a full spectral assignment before considering NOESY. The default mixing time τ_m in automation in 800msec for small molecules, but the appropriate choice of τ_m is critical and should be tailored to suit the molecular system under study. The optimum τ_m will be where nOes for the particular molecule have their maximum intensity. Large molecules require much shorter τ_m of < 400 msec, since too long a τ_m would result in a relay process known as *spin diffusion*, whereby nOes are indiscriminately propagated throughout the molecule giving no structural information of any value. As discussed previously, 1D or 2D NOESY is not suitable for medium sized molecules (M_R ~ 1000 Da) or smaller molecules in viscous solvents as the nOes may then become negligible. For these molecules, the method of choice is the ROESY experiment discussed below.

7.4 Rotating Frame nOes – ROESY and Tr-ROESY

As eluded to previously, medium-sized molecules (M_r ~800-2000 Da) are problematic because conventional 1-D nOe and 2-D NOESY experiments show weak, if any, nOes. For this category of molecules, the nOes tend towards zero intensity, called the 'zero-crossing point';

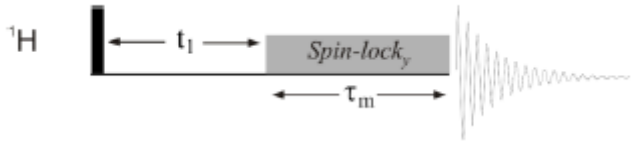
'zero-crossing point' where $\omega\tau_c \sim 1$



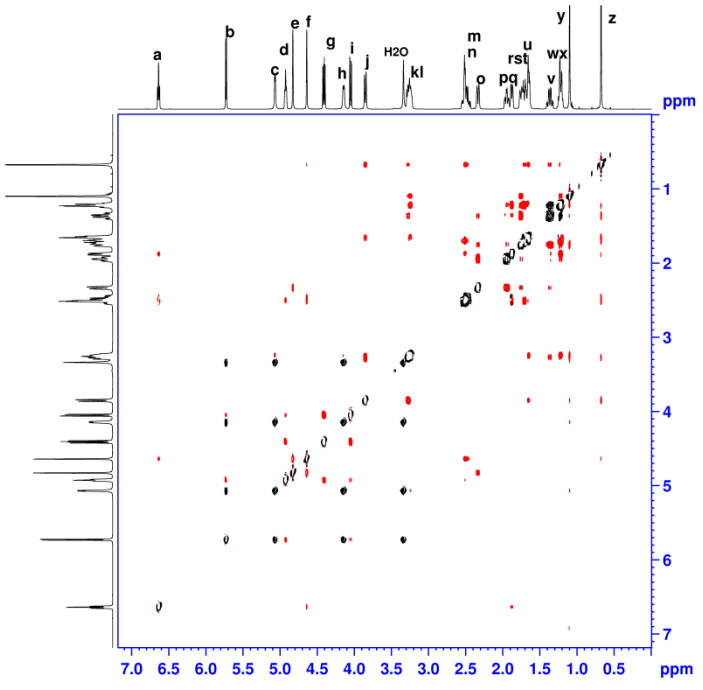
Schematic representation of nOes versus tumbling rates of molecules, showing the zero-crossing point for mid-sized molecules of $M_r \sim 1000$.

The advantage of rotating frame nOes, called ROEs is that they are ***all positive*** for all molecular sizes, so this limitation of the 'zero-crossing' is overcome. The 2-ROESY experiment also gives transient enhancements and therefore parallels NOESY. The difference between NOESY and ROESY pulse sequences is that the mixing time τ_m (usually ~600 msec for small molecules) comprises a continuous low power radio frequency pulse (known as a *spin-lock*) during which the ROE develops.

Schematic diagram of the 2-D ROESY pulse sequence



2D-ROESY spectra are like 2-D NOESY, and map all the through-space interactions with crosspeaks that have opposite sign to the diagonal (red for ROE), the exchange peaks have the same phase as the diagonal (black for exchange). The standard ROESY experiment exhibits unwanted cross peaks that are not ROEs, but are due to complications arising from the spin lock. These are COSY and TOCSY transfer cross peaks between J-coupled spins and other artefacts due to off-resonance radiofrequency effects, which become more significant at higher fields. The presence of these can lead to misinterpretation of 'real' ROEs meaning greater caution is required in their analysis. A variation of the ROESY experiment called Tr-ROESY (transverse ROESY) can help reduce these unwanted contributions, although one must remain aware of potential coupling artefacts. Tr-ROESY is now the default experiment on all our high field spectrometers.



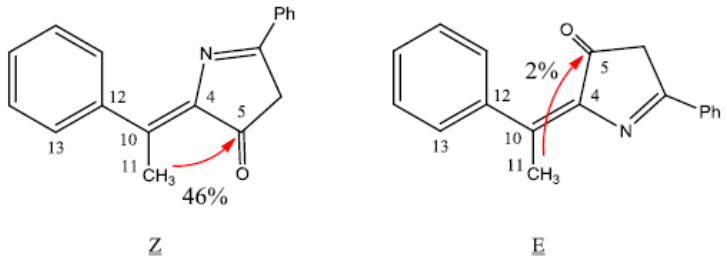
2-D Tr-ROESY spectrum of Andrographolide showing nOes (red) and exchange (black) cross peaks

7.5 Heteronuclear nOes – HOESY

Heteronuclear nOes are those arising between differing nuclides, most a frequently proton and one other nucleus. Whilst not often considered, they play an important role in routine carbon-13 NMR experiments whereby decoupling (irradiation) of protons during the experiment leads to significant enhancement of the ^{13}C signal intensity by virtue of the proton to carbon nOe. Beyond this, heteronuclear nOes also have a role to play in structure assignment problems, for which the various techniques as referred to as HOESY experiments. It is some of these methods that are considered in this section.

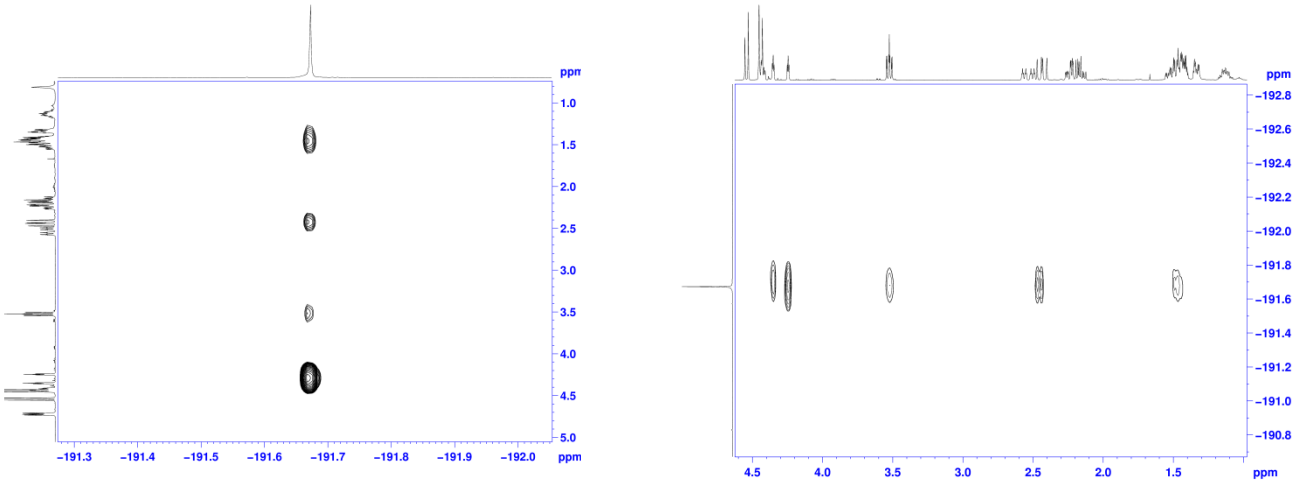
7.5.1 What are HOESY spectra?

As noted above, heteronuclear nOes may be observed between protons and another spin ½ nucleus; ^{13}C and ^{19}F are the most common nuclei for which pulse sequences have been optimised. In the case of ^{13}C, structurally informative nOes between ^{1}H and ^{13}C are only observed for non-protonated carbon signals e.g. carbonyls since nOes for protonated carbon centres are dominated by the attached proton(s). An example is shown below for resolving E/Z stereochemical assignments from a 1-D heteronuclear nOe experiment, where the nOe from CH_3(11) protons is stronger in the Z isomer than the E isomer (compare 46% with 2%). Due to the lack of protons in the molecule, differentiating the isomers any other way is near impossible!



7.5.2 What do HOESY spectra look like?

A more sensitive and powerful HOESY experiment that has largely been investigated here in Oxford is the 2-D and 1D ^{19}F-^{1}H HOESY experiments. The 2-D HOESY experiment can observe either (i) $^{19}F\{^{1}H\}$ - ^{19}F (along the x axis) and indirectly detect ^{1}H (along the y axis) or visa-versa (ii) $^{1}H\{^{19}F\}$ - called the 'inverse' approach observing ^{1}H (along the x axis) and indirectly detecting ^{19}F (along the y axis). Both of these examples are shown below;

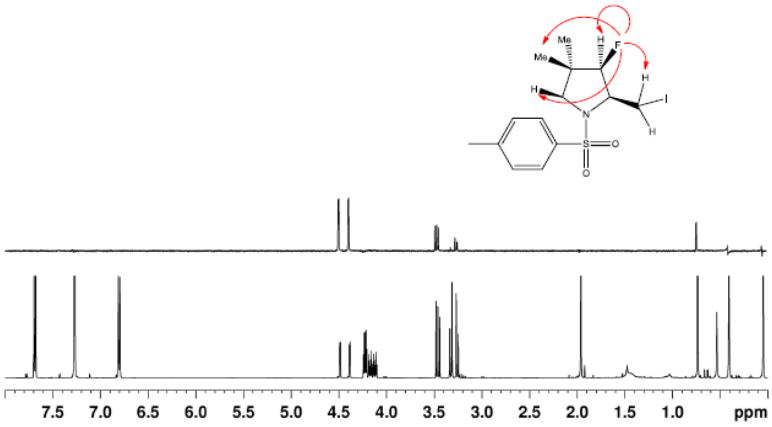


2-D HOESY experiments: left: $^{19}F\{^{1}H\}$ and right: $^{1}H\{^{19}F\}$

The 'inverse' approach $^{1}H\{^{19}F\}$ is preferred due to two advantages over $^{19}F\{^{1}H\}$; the enhanced sensitivity by observing proton and superior resolution in the proton dimension which is usually more crowded than the ^{19}F dimension. These 2-D HOESY experiments are also useful when there is more than one ^{19}F signal.

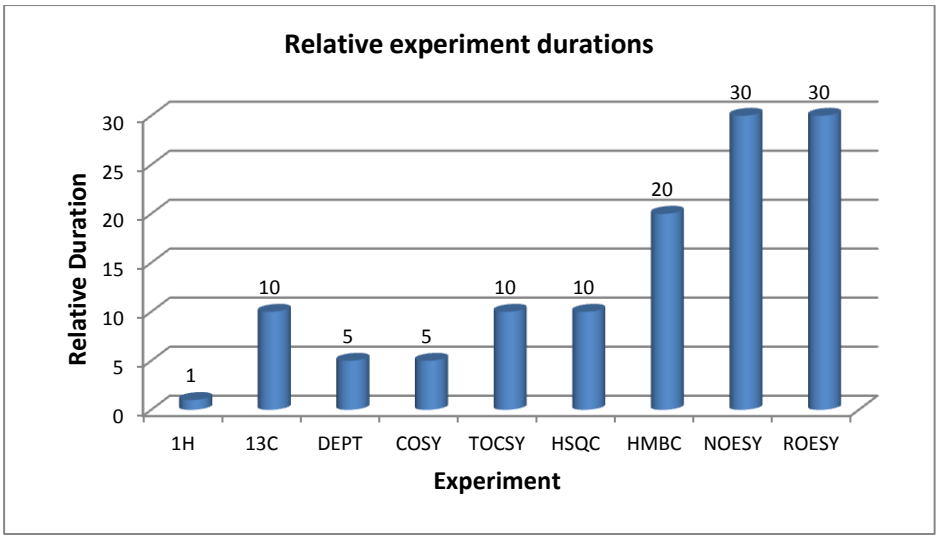
The alternative 1-D transient gradient HOESY experiment employing ^{1}H observation, however, is now usually the method of choice due to the fact it is the most time efficient compared to the 2-D HOESY analogues and it also provides as high a resolution proton spectrum. It is also possible to generate HOE build-up curves from which quantitative distances can be estimated. The example below shows how well resolved and clear the HOEs are from irradiating the ^{19}F signal in the fluoropyrrolidine.

1-D transient gradient HOESY experiment irradiating ^{19}F in the fluoropyrrolidine, recorded with mixing time of 800msec



8. Experiment durations

When requesting experiments through the NMR service or when setting up experiments yourself, it is useful to have some feel for the relative sensitivity of different experiments and therefore the relative number of scans required and how long each is likely to take to collect. The graph below provides an approximate indication of the relative duration of routine experiments for samples that are not severely mass limited versus that of a 1D proton spectrum. Typical numbers of scans for reasonable small molecule samples (> 5 mg) on our ^{13}C-optimised cryoprobe would be ^{1}H (8), ^{13}C (256), DEPT (128), COSY (2), TOCSY (4), HSQC (4), HMBC (8), NOESY (8), ROESY (8).



Typical NMR experiment relative running times for routine experiments. Variable parameters that influence these would include TOCSY (typical τ_m= 80 ms), NOESY (typical τ_m = 800 ms) and ROESY (typical τ_m = 400 ms).