Introduction to "practical" NMR spectroscopy

by Prof. Dušan Uhrín

- Fourier transform NMR, Free Induction Decay
- Spin-relaxation
- Parameters to consider when acquiring 1D spectra
- Processing of 1D spectra
- Selective excitation
- Spin-spin interactions relevant to liquid-state NMR, chemical-shift correlations
- Multipulse 1D NMR experiments
- Acquiring 2D spectra, non-linear sampling
- 2D NMR experiments: COSY, TOCSY, NOESY, ROESY, HSQC, HSQC-TOCSY, HMBC, INADEQUATE/ADEQUATE
- Pure shift NMR spectroscopy
- Parameters for acquisition and processing of 2D NMR spectra
- Solving structures by NMR

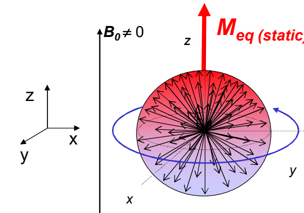
Scottish NMR Users Group 2024 Postgraduate Course in NMR Spectroscopy

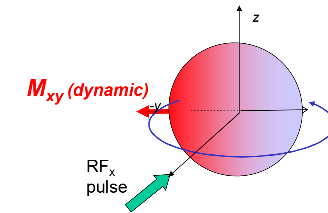
School of Chemistry, University of Edinburgh, December 2-3, 2024

Fourier transform NMR and Free Induction Decay (FID)

Longitudinal (z) macroscopic magnetisation.
More nuclei point parallel with the static magnetic field. The sum of their individual magnetic moments creates **static macroscopic magnetic moment, M_{eq}.**

Transverse (xy) macroscopic magnetization.
Magnetic moments of nuclei interact with the magnetic moment of a radiofrequency pulse and are flipped into the xy plane.





At the end of the radiofrequency pulse macroscopic magnetisation, M_{eq}, is in the xy plane and spins begin to precess around B_o with their **nuclear Larmor frequencies** all with the same phase.

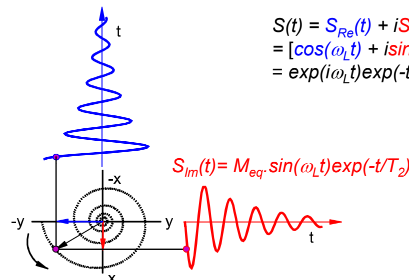
FT NMR – measurement of the **F**ree **I**nduction **D**ecay (**FID**).
Macroscopic magnetic moments rotating in the xy plane at the Larmor frequency induce small oscillating electric currents in the detection coil. This is the primary NMR signal called Free Induction Decay (FID).

Free Induction Decay (FID)

$S_{Re}(t)= M_{eq}.cos(\omega_L t)exp(-t/T_2)$

ω_L the Larmor frequency in radians

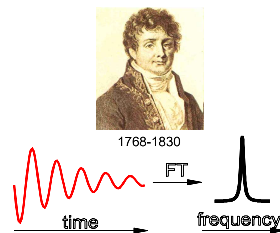
$S(t) = S_{Re}(t) + iS_{Im}(t)$
$= [cos(\omega_L t) + isin(\omega_L t)]exp(-t/T_2)$
$= exp(i\omega_L t)exp(-t/T_2) = exp[-(1/T_2 - i\omega_L)t]$



$S_{Im}(t)= M_{eq}.sin(\omega_L t)exp(-t/T_2)$

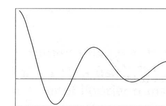
FID is a function of signal intensity *vs* time, *S*(t). Spectrum, a function of signal intensity *vs* frequency, *S*(ω), is obtained by Fourier transformation (FT).

1768-1830



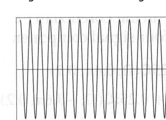
Fourier transformation. An intuitive approach.

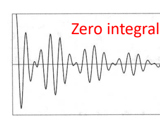
NMR signal *S(t)*

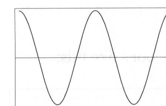


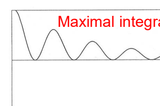
$S_{guess}(t) = cos(\omega_{guess}\, t)$

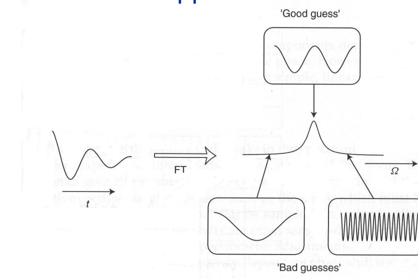
$S(t)^* Sguess(t)$











1. Guess a frequency and construct a "guess FID"
2. Multiply the real FID with the "guess FID"
3. Determine integral
4. Repeat 1-3, changing the frequency of the "guess FID"
5. Integral values constitute an NMR spectrum

Fourier transformation. A mathematical approach.

$S(t) = \exp[-(1/T_2 - i\omega_L)t]$ ← FID

Guess function

$$S(\omega) = \int_0^\infty S(t)\exp(-i\omega t)dt$$

FID * Guess

$$S(\omega) = \int_0^\infty \exp\{-[1/T_2 + i(\omega - \omega_L)t\}dt$$

$$S(\omega) = \frac{1}{(1/T_2) + i(\omega - \omega_L)} =$$

$$S(\omega) = \frac{1}{(1/T_2) + i(\omega - \omega_L)} \frac{(1/T_2) - i(\omega - \omega_L)}{(1/T_2) - i(\omega - \omega_L)}$$

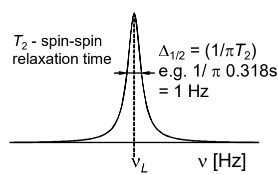
$$S(\omega) = \frac{(1/T_2)}{(1/T_2)^2 + (\omega - \omega_L)^2} + i\frac{(\omega_L - \omega)}{(1/T_2)^2 + (\omega - \omega_L)^2}$$

$= A(\omega) + iD(\omega) =$

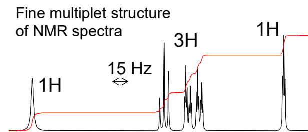
Absorption + Dispersion Lorentzian lines

Absorption Lorentzian line, the desired NMR signal

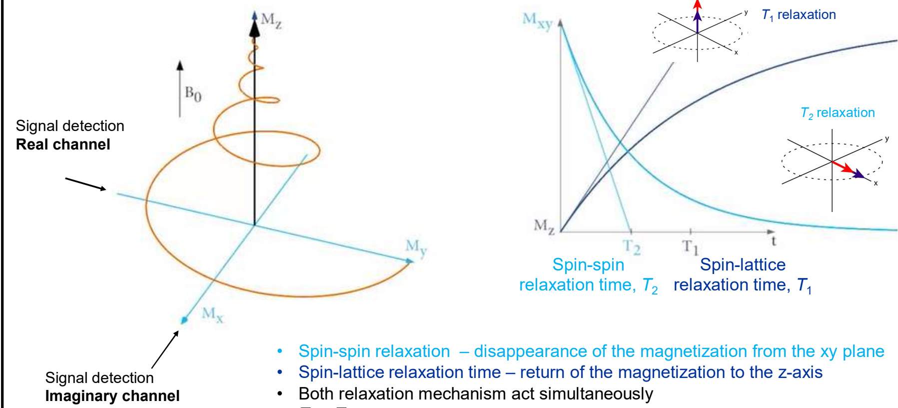
$$S(\nu) = \frac{(1/T_2)}{(1/T_2)^2 + 4\pi^2(\nu - \nu_L)^2}$$



Fine multiplet structure of NMR spectra



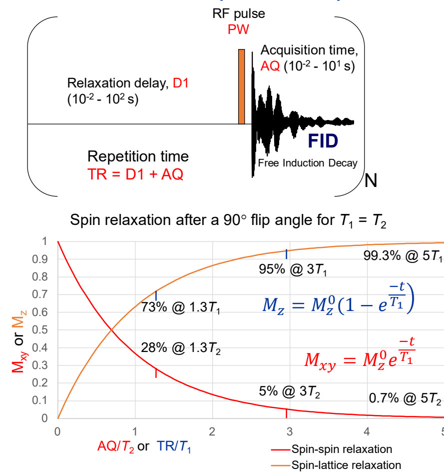
Return of an off-resonance signal to the z-axis after a 90° r.f. pulse



- Spin-spin relaxation – disappearance of the magnetization from the xy plane
- Spin-lattice relaxation time – return of the magnetization to the z-axis
- Both relaxation mechanism act simultaneously
- $T_1 \le T_2$

https://youtu.be/ygwESjbb3rQ

Optimal acquisition of 1D FT NMR spectra



1. How long should the repetition delay (TR) be?
2. How large should the flip angle (PW) be?
3. How long should the acquisition time (AQ) be?

The answer depends on:

i. Spin-lattice relaxation time, T_1

ii. Spin-spin-relaxation time, T_2

Maximizing the signal-to-noise-ratio (SNR):

1. For a 90° pulse it is recommended to set TR = $1.3T_1$ (73% recovery)
2. Reducing the pulse to 30° sets TR=0.14 T_1.
3. Consult Ernst angle (http://nmrwiki.org/wiki/index.php?title=Ernst_angle) and https://nmr.chem.ucsb.edu/protocols/fast1pulse.html

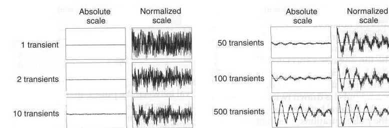
For quantitative spectra:

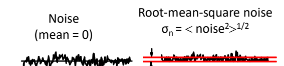
1. ^{1}H: set TR to $5T_1$ ($8T_1$ for >99.9% recovery)
2. ^{13}C: set TR to $10T_1$ and use inverse gated decupling to avoid the heteronucler Nuclear Overhauser Effect (NOE)

Maximizing SNR and resolution:

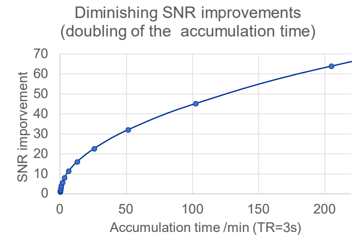
Set AQ = $3T_2$

Improving SNR by signal accumulation





Diminishing SNR improvements (doubling of the accumulation time)



Consider accumulation of two scans with a similar signal and noise levels: [signal(1) ≅ signal(2)], and noise [$\sigma_n(2) \cong \sigma_n(1)$]

Noise:

$\sigma_n(1+2) = \langle [noise(1) + noise(2)]^2 \rangle^{1/2} = \langle noise(1)^2 + 2*noise(1)*noise(2) + noise(2)^2 \rangle^{1/2}$

$= \{\langle (noise(1)^2 \rangle + \langle 2*noise(1)*noise(2) \rangle + \langle (noise(2)^2 \rangle\}^{1/2} =$

$= \{\langle (noise(1)^2 \rangle + \langle (noise(2)^2 \rangle\}^{1/2} = \{2\langle (noise(1)^2 \rangle\}^{1/2} = \sqrt{2}\,\sigma_n(1)$

Noise is random: $\langle 2*noise(1)*noise(2) \rangle = 0$

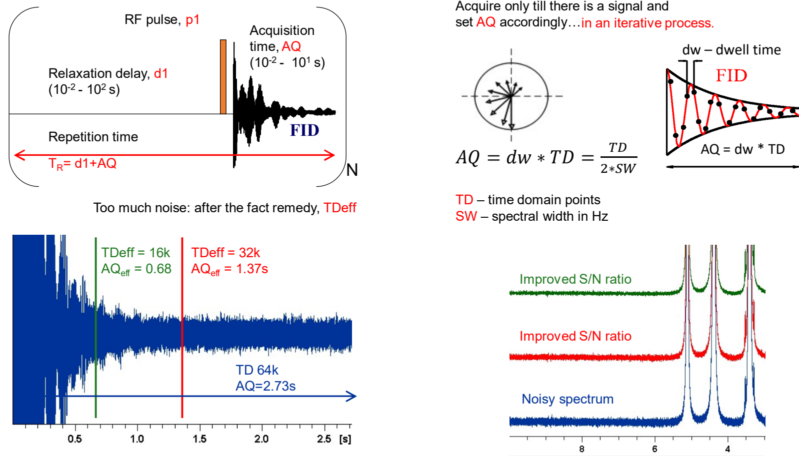
Signal:

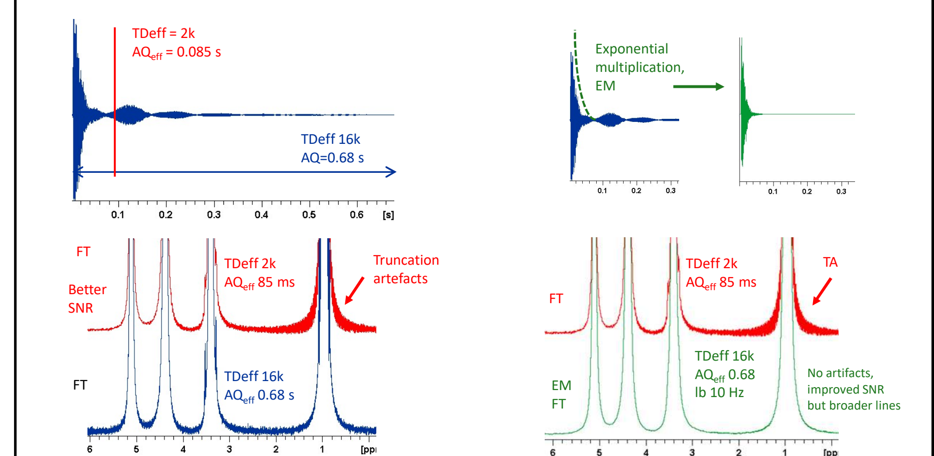
signal(1+2) = signal(1)+signal(2) = 2*signal(1)

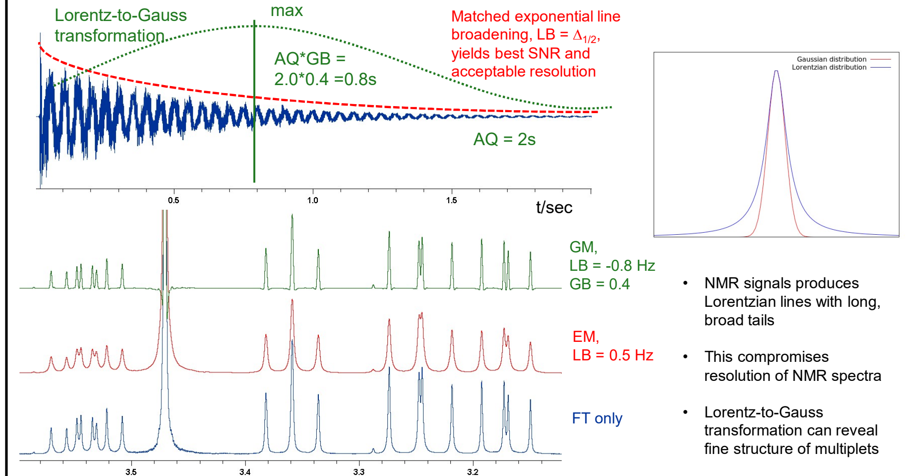
Signal-to-noise ratio, SNR:

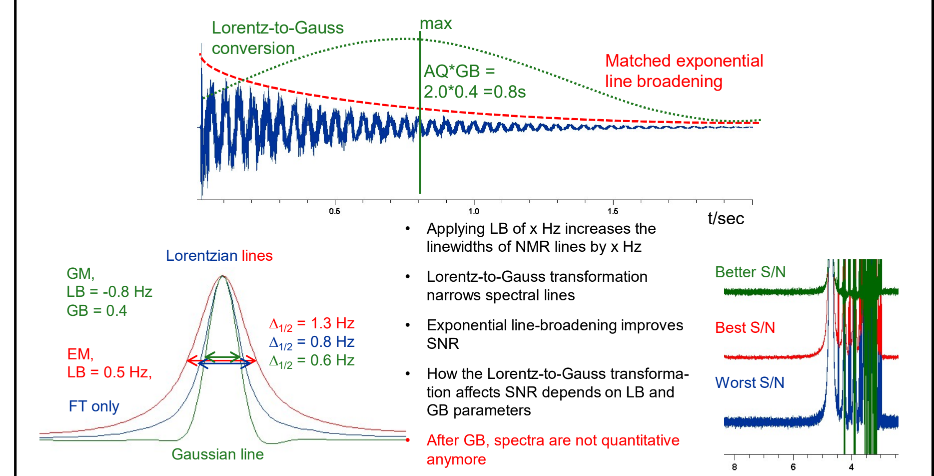
SNR= 2*signal(1)/$\sqrt{2}\,\sigma_n(1)$ = $\sqrt{2}$ signal(1)/$\sigma_n(1)$

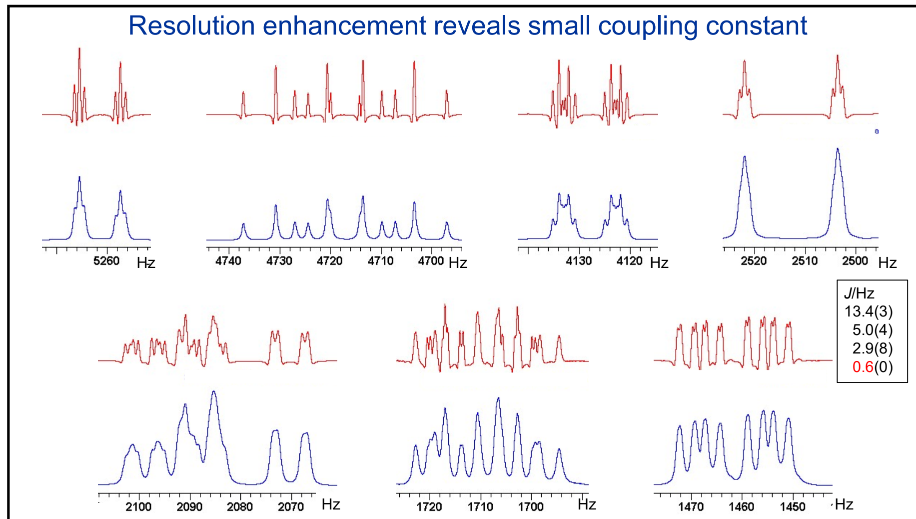
Generalised to N scans: signal(N)/noise(N) = $\sqrt{N}$ signal(1)/$\sigma_n(1)$ or doubling of the SNR requires 4× as many scans.

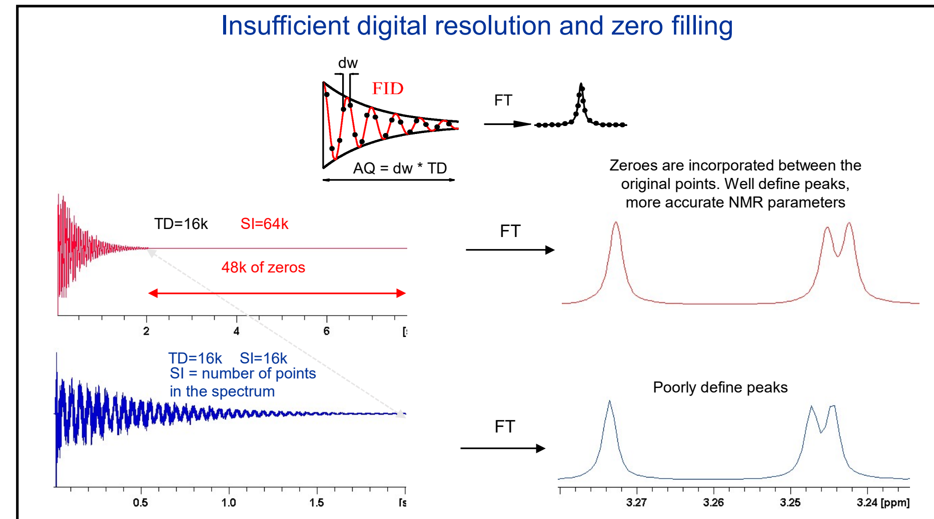

How long should I acquire for? (AQ – acquisition time)
RF pulse, p1
Acquisition time, AQ
(10-2 - 10¹ s)
Relaxation delay, d1
(10-2 - 10² s)
FID
Repetition time
TR= d1+AQ
N
Too much noise: after the fact remedy, TDeff
Acquire only till there is a signal and set AQ accordingly…in an iterative process.
dw – dwell time
FID
AQ = dw * TD
AQ = dw * TD = TD / 2*SW
TD – time domain points
SW – spectral width in Hz
TDeff = 16k
AQeff = 0.68
TDeff = 32k
AQeff = 1.37s
TD 64k
AQ=2.73s
0.5
1.0
1.5
2.0
2.5 [s]
Improved S/N ratio
Improved S/N ratio
Noisy spectrum
8
6
4

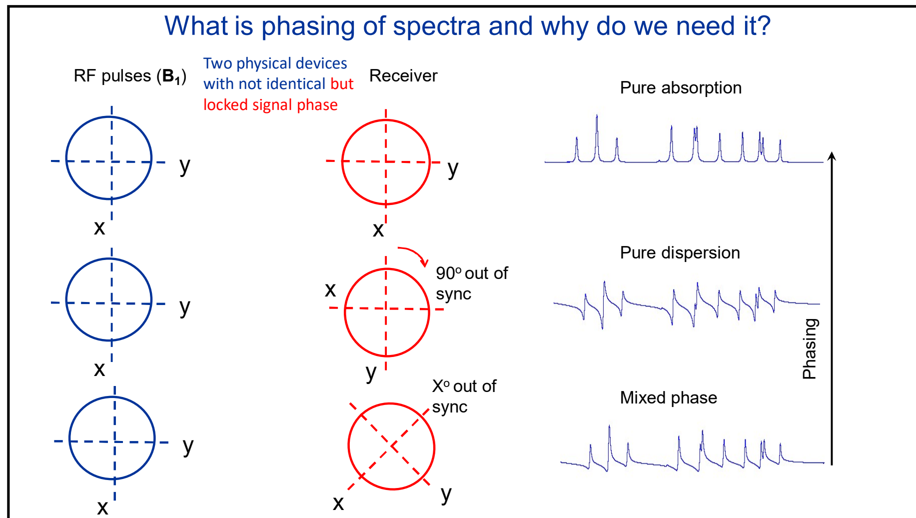

Too short AQ creates truncation artefacts. Apply a weighting function!
TDeff = 2k
AQeff = 0.085 s
TDeff 16k
AQ=0.68 s
0.1
0.2
0.3
0.4
0.5
0.6 [s]
Exponential multiplication, EM
FT
Better SNR
TDeff 2k
AQeff 85 ms
Truncation artefacts
FT
TDeff 16k
AQeff 0.68 s
FT
TDeff 2k
AQeff 85 ms
TA
EM
FT
TDeff 16k
AQeff 0.68
lb 10 Hz
No artifacts, improved SNR but broader lines

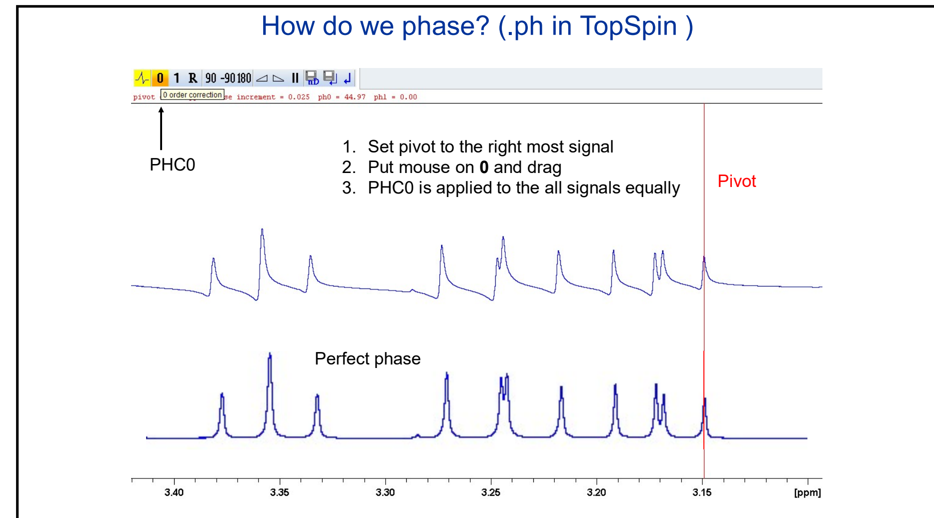

Applying weighting functions to FID before Fourier transformation
Lorentz-to-Gauss transformation
max
AQ*GB = 2.0*0.4 =0.8s
Matched exponential line broadening, LB = Δ1/2, yields best SNR and acceptable resolution
AQ = 2s
0.5
1.0
1.5
t/sec
Gaussian distribution
Lorentzian distribution
GM,
LB = -0.8 Hz
GB = 0.4
EM,
LB = 0.5 Hz
FT only
3.5
3.4
3.3
3.2
NMR signals produces Lorentzian lines with long, broad tails
This compromises resolution of NMR spectra
Lorentz-to-Gauss transformation can reveal fine structure of multiplets


Signal-to-noise or resolution enhancement (or both)?
Lorentz-to-Gauss conversion
max
AQ*GB = 2.0*0.4 =0.8s
Matched exponential line broadening
0.5
1.0
1.5
t/sec
Lorentzian lines
GM,
LB = -0.8 Hz
GB = 0.4
EM,
LB = 0.5 Hz,
FT only
Δ1/2 = 1.3 Hz
Δ1/2 = 0.8 Hz
Δ1/2 = 0.6 Hz
Gaussian line
Applying LB of x Hz increases the linewidths of NMR lines by x Hz
Lorentz-to-Gauss transformation narrows spectral lines
Exponential line-broadening improves SNR
How the Lorentz-to-Gauss transformation affects SNR depends on LB and GB parameters
After GB, spectra are not quantitative anymore
Better S/N
Best S/N
Worst S/N
8
6
4

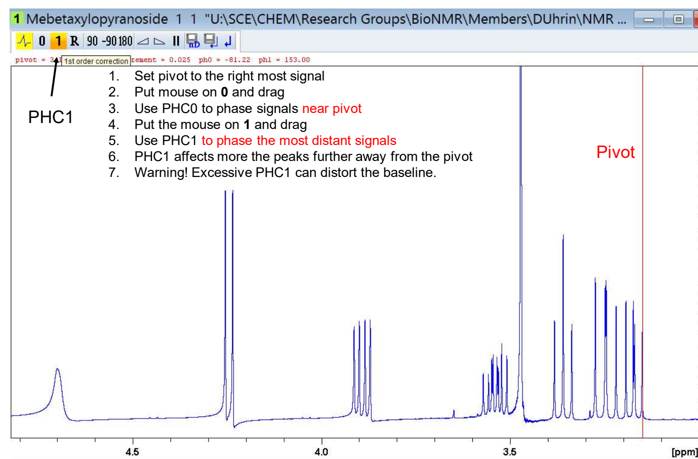

Resolution enhancement reveals small coupling constant
5260 Hz
4740 4730 4720 4710 4700 Hz
4130 4120 Hz
2520 2510 2500 Hz
J/Hz
13.4(3)
5.0(4)
2.9(8)
0.6(0)
2100 2090 2080 2070 Hz
1720 1710 1700 Hz
1470 1460 1450 Hz


Insufficient digital resolution and zero filling
dw
FID
AQ = dw * TD
FT
Zeroes are incorporated between the original points. Well define peaks, more accurate NMR parameters
TD=16k SI=64k
48k of zeros
FT
TD=16k SI=16k
SI = number of points in the spectrum
Poorly define peaks
FT
3.27 3.26 3.25 3.24 [ppm]

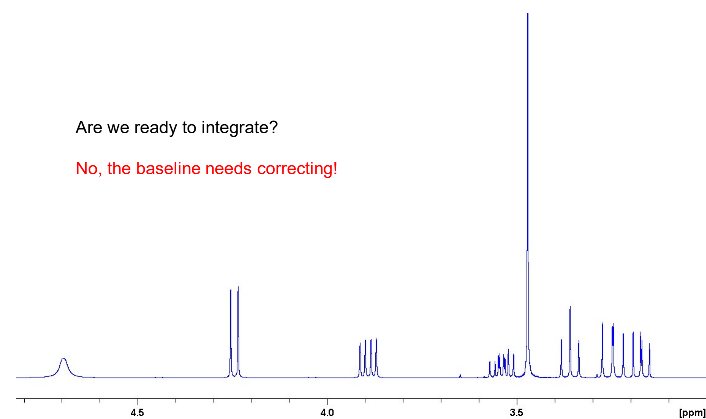

What is phasing of spectra and why do we need it?
RF pulses (B1)
Two physical devices with not identical but locked signal phase
Receiver
Pure absorption
Pure dispersion
Mixed phase
Phasing
90° out of sync
X° out of sync
x
y


How do we phase? (.ph in TopSpin)
PHC0
1. Set pivot to the right most signal
2. Put mouse on 0 and drag
3. PHC0 is applied to the all signals equally
Pivot
Perfect phase
3.40 3.35 3.30 3.25 3.20 3.15 [ppm]

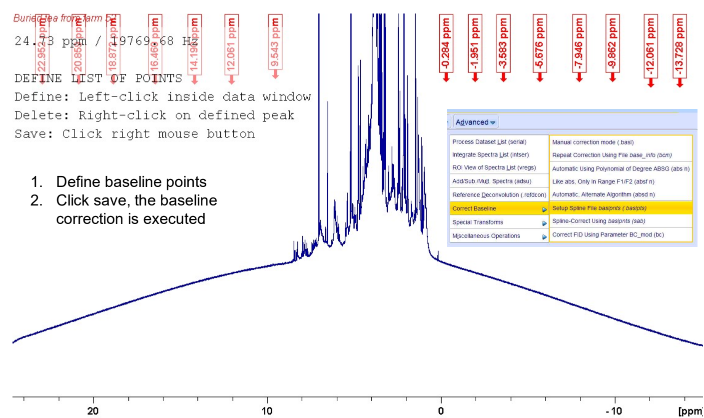
What if PHC0 is not sufficient? The first order correction



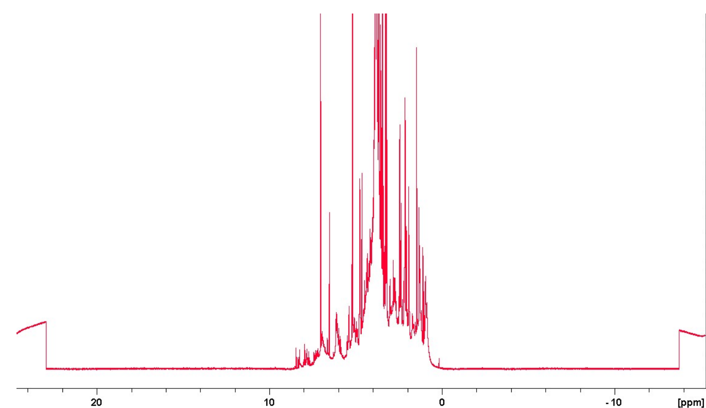
Perfect phase, all signals are in pure absorption mode



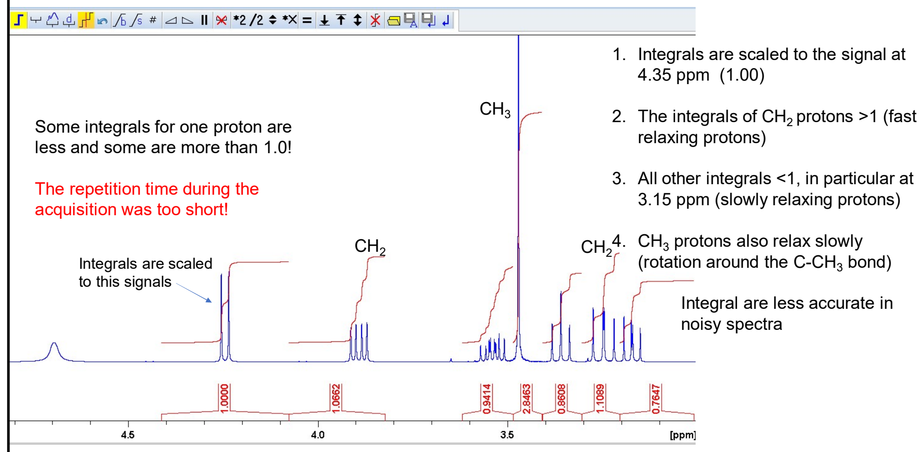
An example of a baseline correction: Set up a spline file



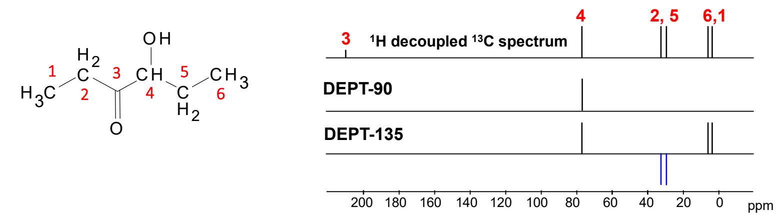
Flat baseline is required for integration



I did everything correctly, but integrals do not make sense!

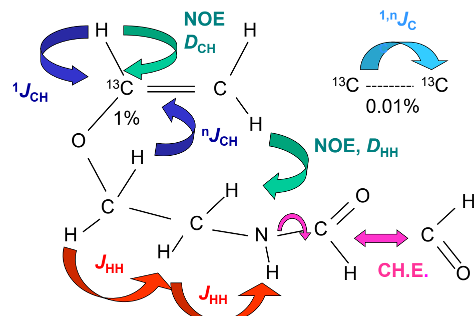


Multiplicity editing of ^{13}C NMR spectra – DEPT pulse sequence



- **DEPT-90**: Spectrum shows only CH signals, positive.
- **DEPT-135**: Spectrum shows CH and CH_3 signals positive, CH_2 negative
- No quaternary carbons in **DEPT** spectra
- Quaternary carbons appear as negative signals in **DEPTQ** spectra

Summary of spin-spin interactions



Type of spin-spin interactions relevant to liquid–state NMR:

- through bond (scalar or *J* couplings)
- through space (dipolar *D*, Nuclear Overhauser Effect, NOE)
- chemical exchange (covered in a separate lecture)

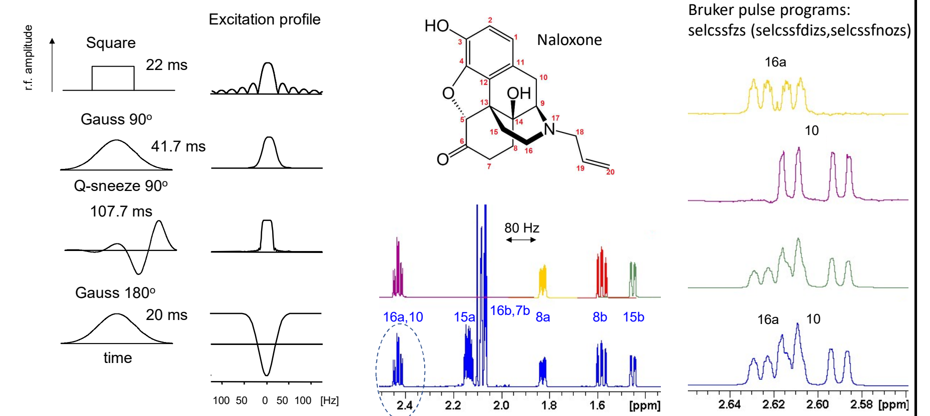
Every interaction (coupling) between spins can be used to transfer signal between spins – or by other words, correlate them.

Modern NMR is about chemical-shift correlation – walking along the skeleton of molecules

Classification of spin-spin interactions and corresponding NMR experiments:

- homonuclear (HH, CC, FF)
- heteronuclear (CH, NH, HF, CF)

Selective excitation – rectangular and shaped pulses for isolated multiplets
Chemical-shift-selective filters (CSSF) for overlapping multiplets



COSY(COrrelation SpectroscopY)

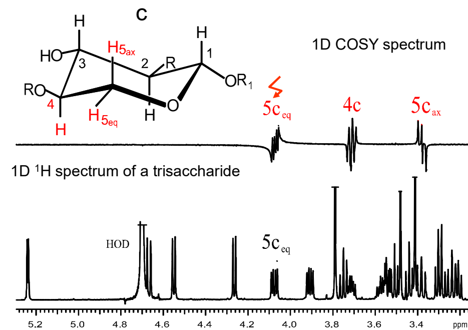
Correlates ^{1}H spins via J_{HH} couplings.
Only a *single* transfer of signals occurs between spins.

1D COSY spectrum of a monosaccharide **c** from a trisaccharide.

After selective excitation of proton H5c_{eq} the signal is transferred by a 90° pulse only to protons that are *J*-coupled to H5c_{eq}.

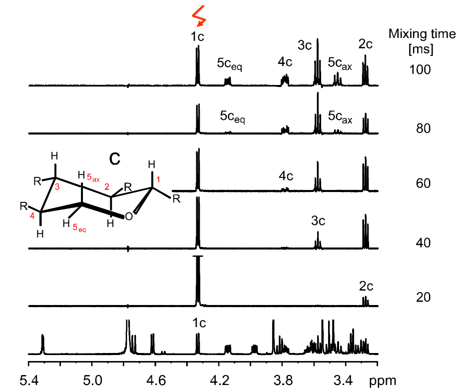
The starting proton appears as a pure in phase dispersive signal.

The correlated peaks appear as a pure antiphase absorption signal with the active coupling constant in antiphase and the passive coupling constants as an in phase splittings.



TOCSY(TOtal Correlation SpectroscopY)

Correlates ^{1}H spins via J_{HH} couplings.
Enables *multiple* signals transfers between spins.



1D TOCSY spectra of a monosaccharaide **c** from a trisaccharide.

After selective excitation of proton H1c, its signal is during the TOCSY mixing time (spin-lock time), transferred along the chain of the coupled spins.

The extent of the transfer of magnetisation is a function of the mixing time. For long mixing times all signals of a spin system can be observed.

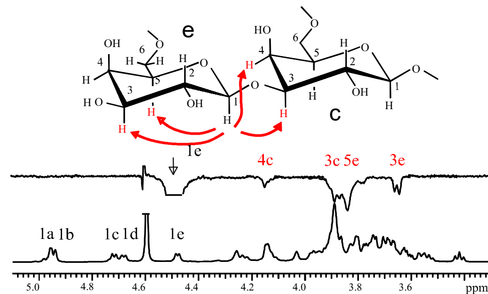
The efficiency of the TOCSY transfer is higher when large coupling constants are involved.

NOESY(Nuclear OvErhauser SpectroscopY)

Correlates spins that are close in space.
Typically, *single* signal transfers between spins is observed.
Multiple signal transfers are possible (spin diffusion).

The Nuclear Overhauser Effect in Structural and Conformational Analysis

1D NOESY spectrum of a disaccharide fragment from a repeating unit of a bacterial polysaccharide



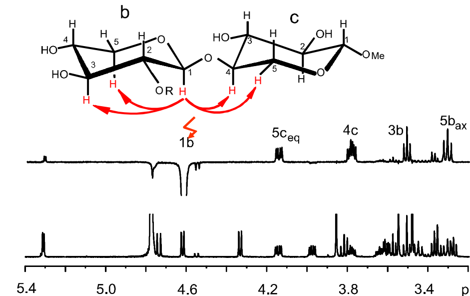
The **nuclear Overhauser effect** is defined as a change of the intensity of a signal of a nucleus when another nearby spin (within ~5 Å) is perturbed from equilibrium and relaxes back. Dipolar couplings make NOE possible.

- Small molecules show small positive NOEs (signal has opposite phase relative to the signal of the perturbed proton)
- Large molecules show large negative NOEs (all signals have equal phase, typically shown as negative signals)
- Medium-sized molecule can show zero NOE, this depends on the magnetic field strength
- Intensity of NOEs depends on the mixing time (10-800ms). For long mixing times:
- Large molecules – possibility of spin-diffusion
- Small molecules show alternating sign of NOEs
- NOE – important tool in conformational analysis

ROESY(Rotating frame nuclear OvErhauser Effect SpectroscopY)

Correlates ^{1}H spins that are close in space.
Typically, *single* signals transfers between spins is observed.
Multiple signal transfers possible – spin diffusion.

1D ROESY spectrum of a disaccharide fragment of a trisaccharide.



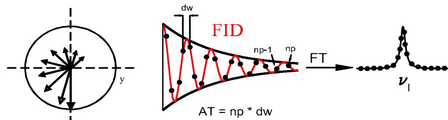
After selective excitation of proton H1b, the signals is transferred during the ROESY spin-lock interval to protons coupled with the selected proton via dipolar couplings (close in space).

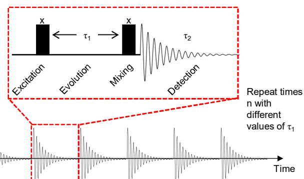
- ROESY peaks are always positive irrespective of the size of the molecule.
- ROESY peaks appear also in cases where NOE goes through zero.
- ROESY is a more demanding experiments; there is a possibility of TOCSY artifacts.
- Typically performed on small to medium size molecules.

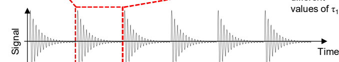
Sampling of NMR signal in1D and 2D experiments

1D
One **directly** detected period
(physical detector)

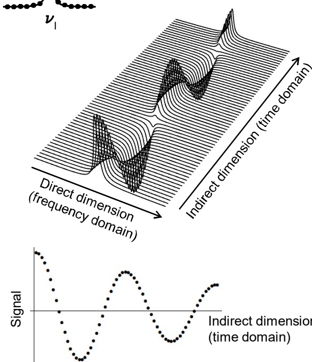
2D
One **indirectly** and one **directly** detected period







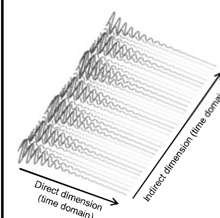
128 – 512 times



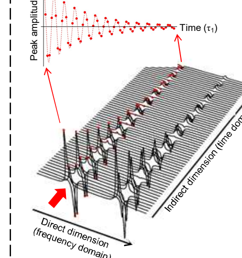
Curtesy of Andrew Hall

2D NMR processing

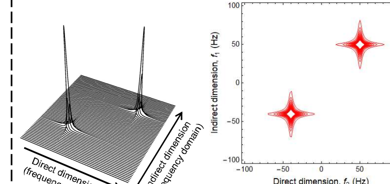
Raw data:



1st Fourier transform:



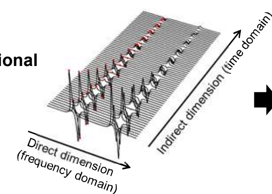
2nd Fourier transform:

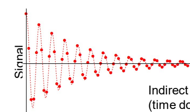


Curtesy of Andrew Hall

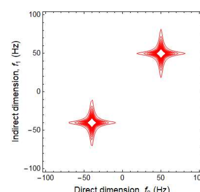
Non-uniform sampling (NUS)

Conventional

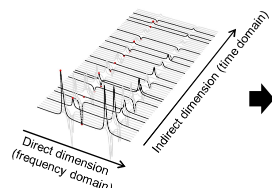


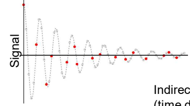


FT

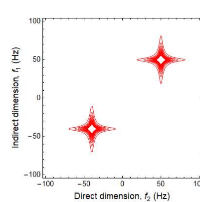


NUS





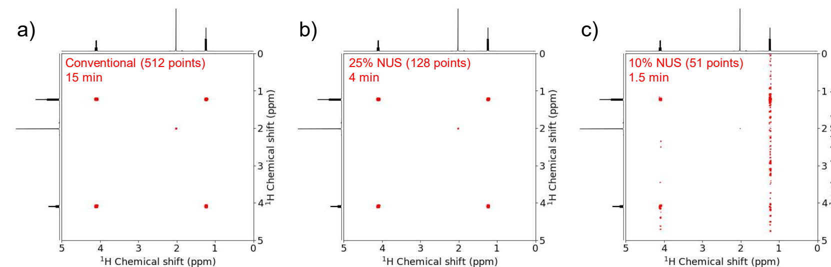
Special processing



Non-uniform sampling (NUS)

- Reduce experimental time
- Minimal loss of resolution
- Needs good SNR

- Aggressive NUS produces artifacts
- For crowded spectra more dense sampling is required
- Very useful for 3D and 4D NMR experiments on biomolecules (2-20% NUS)

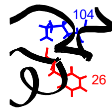


2D homonuclear chemical shift correlation spectra. Symmetry across diagonal

- Start on a diagonal (e.g.H1b) and draw a horizontal line till you hit a cross peak.
- Draw a vertical line till you hit a diagonal.
- Draw a horizontal line from this point, look for a new cross peak. Ignore a symmetry related cross peak. Look both ways
- Repeat the process until you cannot find any more correlations.

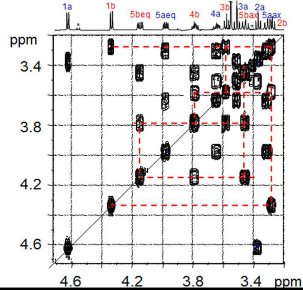
- Connect cross peaks by drawing horizontal lines
- This spectrum contains only two spin systems but is full of cross peaks – redundant information.
- Focus on isolated signals and acquire spectra with different mixing times to assign signals.

- Connect cross peaks by drawing horizontal lines

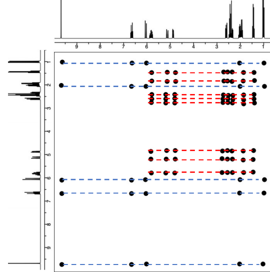


Amino acids that are remote in a sequence can be close in space NOE detects this, enabling structure determination of proteins.

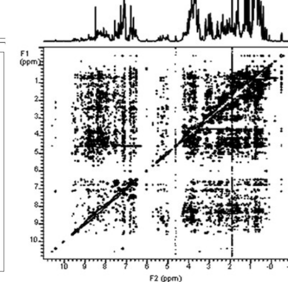
2D COSY



2D TOCSY



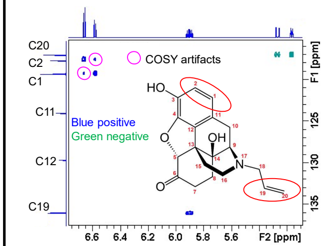
2D NOESY



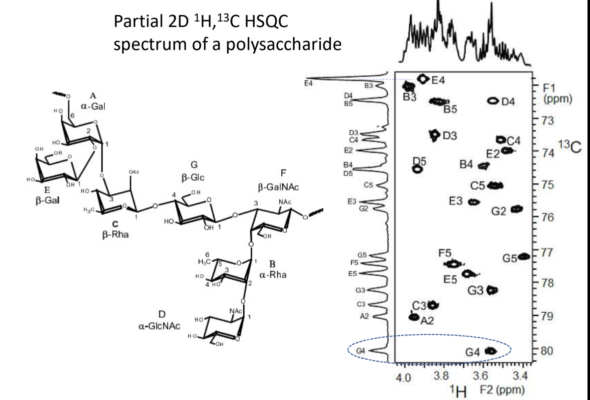
2D Heteronuclear Single-Quantum Correlation, HSQC

Correlates ^{1}H and X (^{13}C, ^{15}N) chemical shifts via $^1J_{XH}$ coupling constants

- 2D HSQC uses the chemical shift of X nuclei to separate overlapping ^{1}H signals.
- Only show signals of protonate carbons.
- Can be acquired to indicate carbon multiplicities (CH, CH_3 positive, CH_2 negative signals).
- Spectra can contain HSQC-COSY cross peaks



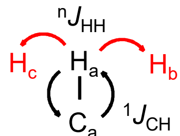
Partial 2D ^{1}H,^{13}C HSQC spectrum of a polysaccharide



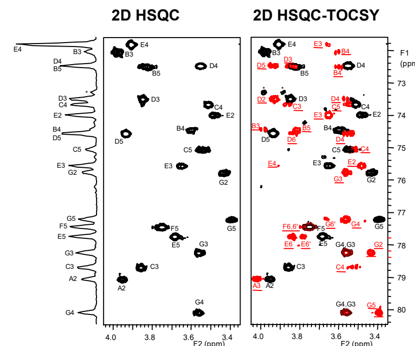
2D HSQC-TOCSY

Correlates ^{1}H and X (^{13}C, ^{15}N) chemical shifts via $^1J_{XH}$ coupling constants as 2D HSQC, but in addition also contains signals of neighbouring *J*-coupled protons at the ^{13}C chemical shift of the starting proton.

- 2D HSQC-TOCSY is typically acquired using a short TOCSY mixing time (~20ms) and therefore mostly show protons 2-3 bonds away.



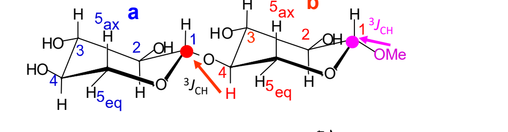
- Very useful for structure elucidation of complicated molecules and mixtures as it uses ^{13}C chemical shifts to separate signals that would overlap in a HH COSY (TOCSY) spectra.



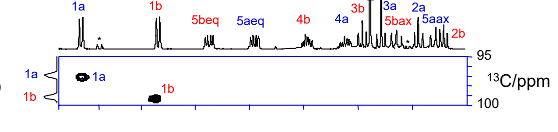
2D Heteronuclear Multiple-Bond Correlation, HMBC

Correlates ^{1}H and X (^{13}C, ^{15}N) chemical shifts via $^nJ_{XH}$ coupling constants, n = 2,3,(4)

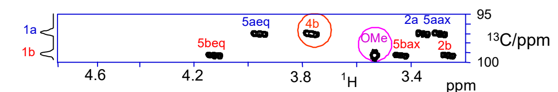
- HMBC contains many more cross peaks than HSQC.
- HMBC connects molecular fragments that cannot be joined by proton-proton couplings.
- One-bond correlations cross peaks are suppressed, but can appear. They can be easily recognised as pairs of cross peaks separated by $^1J_{CH}$.



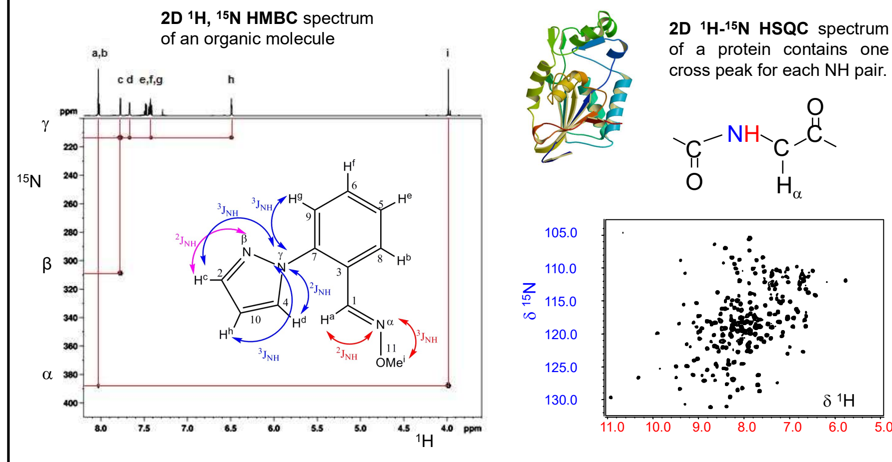
2D ^{1}H,^{13}C HSQC (anomeric carbons only)



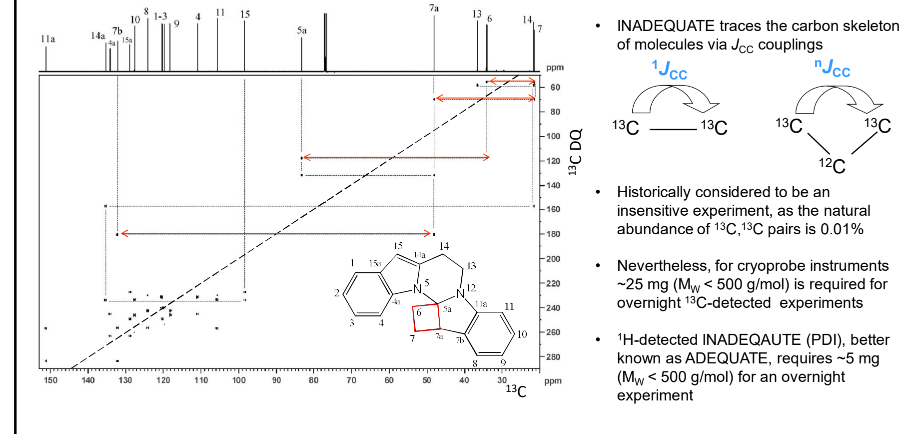
2D ^{1}H,^{13}C HMBC (anomeric carbons only)



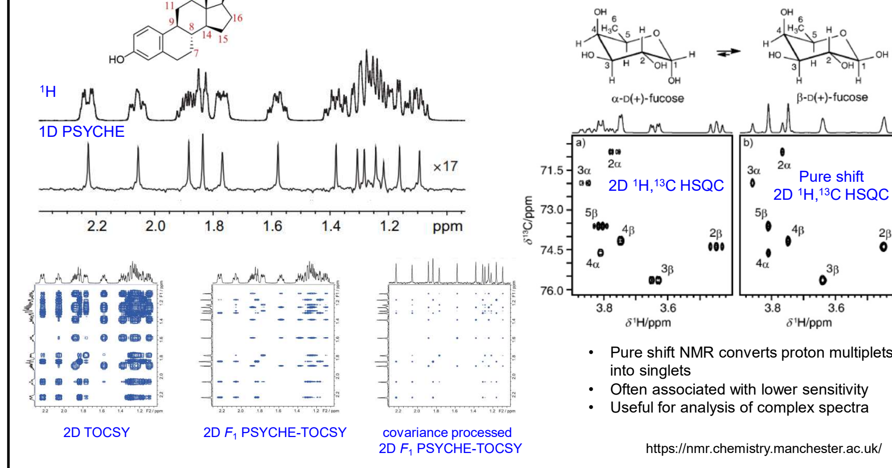
2D ^{1}H-^{15}N correlation experiments



Incredible Natural Abundance Double Quantum Transfer Experiment
2D INADEQUATE



Pure (chemical) shift NMR



Practical aspects of the acquisition and processing of 2D spectra

Typical acquisition parameters for both dimensions

	Directly acquired	Indirectly acquired
Spectral width	SW (chemical shift range +20%)	SW1 (chemical shift range +5%)
Time domain points	TD (512-4k, adjust once SW and AQ set)	TD1 (128-2k, adjust once SW and AQ set, 2^n recommended)
Acquisition time	AQ (typically 10^2 ms)	AQ1 (typically 10^1 ms)

Typical processing parameters for phase-sensitive experiments

Spectral points	SI(1k-8k)	SI1(512-8k, use linear prediction to the nearest 2^n + zero fill once)
Weighting function (phase sensitive)	Cosine-squared	Cosine-squared
Weighting function (magnitude mode)	Sine-squared	Sine-squared

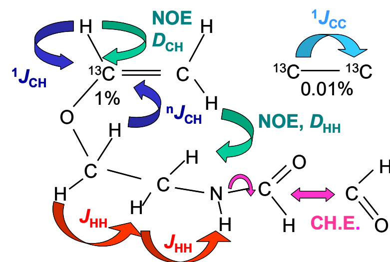
NMR spectroscopy is an indirect method that uses spin-spin interactions to walk along the skeleton of molecules and in the process determines their structures.

Solving structures by NMR is like solving a puzzle.

A typical approach

1. Molecular fragments are identified by proton-proton $^{n}J_{HH}$ couplings (COSY, TOCSY)
2. Proton and carbon chemical shifts are correlated via $^{1}J_{CH}$ couplings (HSQC)
3. Fragments identified by $^{n}J_{HH}$ are joined using $^{n}J_{CH}$ couplings (HMBC)
4. If a molecule contains nitrogen $^{1}J_{NH}$ and $^{n}J_{NH}$ couplings can used (HSQC, HMBC)
5. If any ambiguity remain these can be investigated by $^{1,n}J_{CC}$ couplings (INADEQUATE, ADEQUATE)
6. Stereochemistry is investigated by nuclear Overhauser effect (NOESY)

If you are stuck, you can always ask for more clues.
This is your tool box.



What was no not covered here and you need know to become an accomplished NMR user

1. NMR resonance condition, energy diagrams, Boltzmann equilibrium, frequency and sensitivity
2. Chemical shift, definition and relationship to molecular structure
3. *J* coupling constants, proton multiplets, extraction of coupling constants from spectra
4. Chemical and magnetic equivalence, properties of *J* coupling constants and their relationship to molecular structure
5. Read deeper on (some) topics presented in this lecture and more ☺

Happy NMRing!