KU LEUVEN

qNMR – The do’s and don’ts

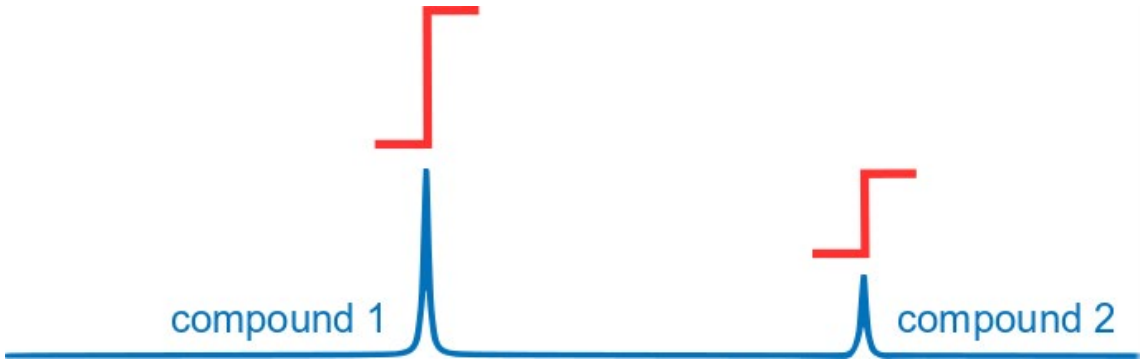
Gert Steurs

February 10th 2022

Quantitative NMR (qNMR)

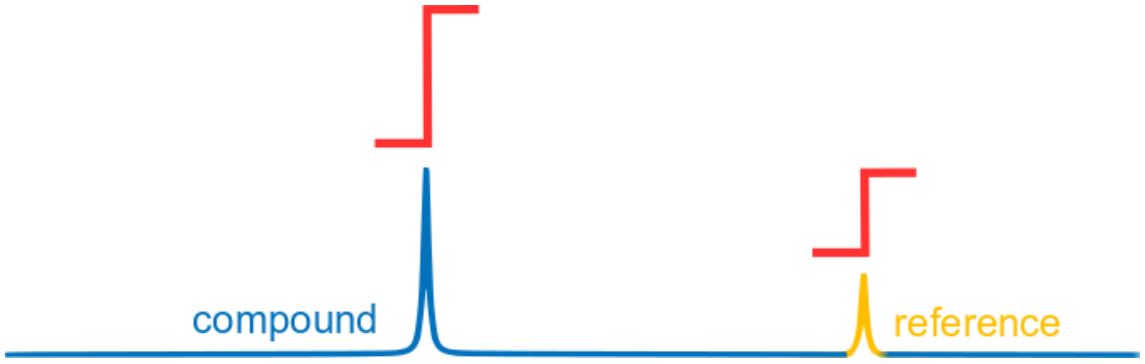
Relative concentration determination

- Concentration ratio of different compounds
  - Purity determination
  - Determination of isomer ratio



Absolute concentration determination

- Absolute concentration of one or more compounds
- With internal standard (or using Eretic2)



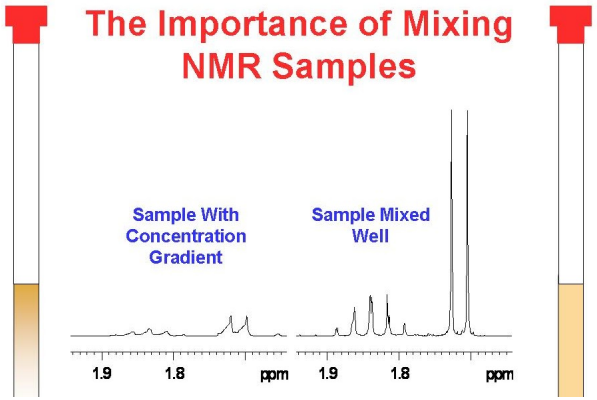
Internal standards

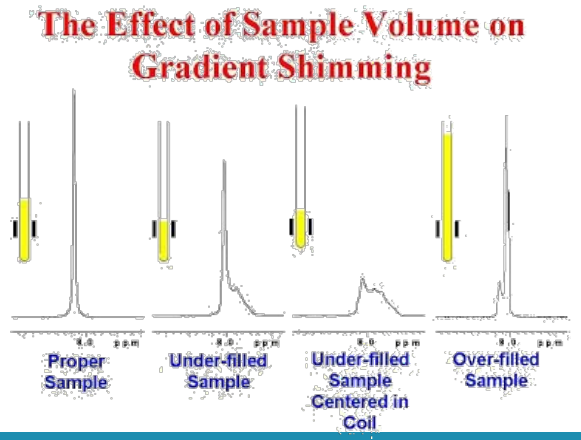
- Calibrant properties
  - No structural relation to compound of interest necessary
  - Must contain nucleus of interest
  - Relatively simple spectrum (preferably only singlets)
  - Must have peak(s) that don’t overlap with compound of interest
  - Short T_1
  - Known purity
  - Chemically inert
  - Non-hygroscopic
  - Highly soluble in deuterated solvent
  - Low volatility
- List of calibrants: https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/marketing/global/documents/101/854/qnmr-brochure-rjo.pdf

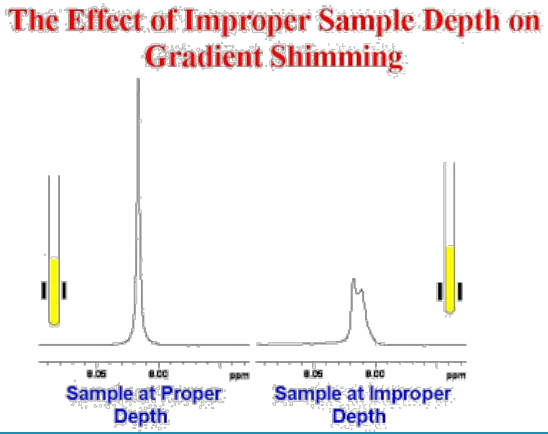
qNMR – Considerations

❶ Sample preparation

- Homogeneous sample
- When using a standard: known amounts of internal standard
- Close sample tube properly to avoid evaporation
- Clean the tube very well
- Sample must be proper volume (4.2 cm = 500 µL!) and **exactly** in middle of TX coil



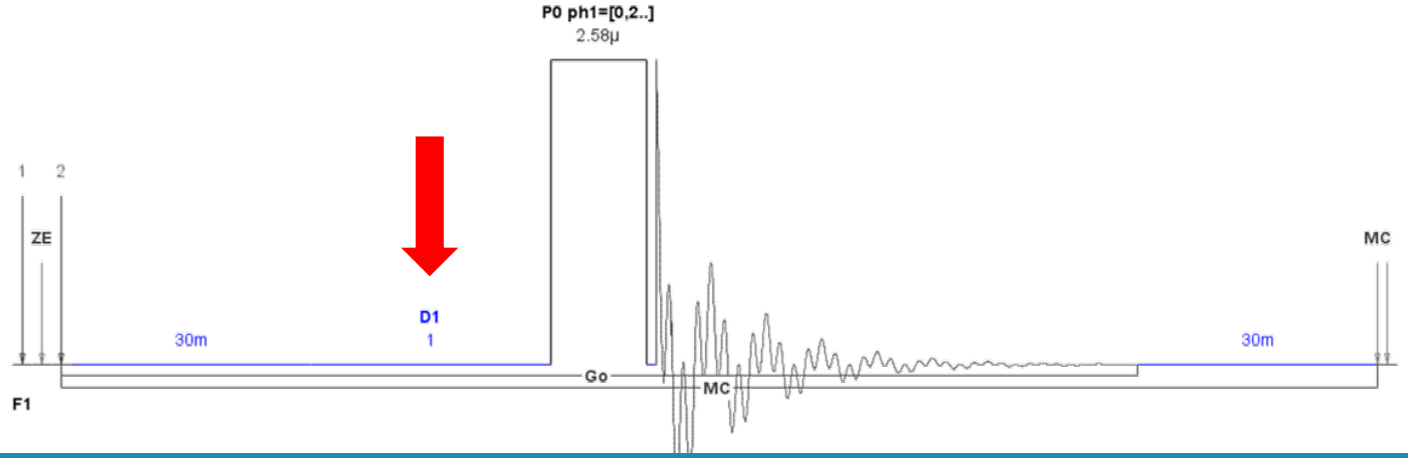




Images from: http://u-of-o-nmr-facility.blogspot.com

❷ Data acquisition – the infamous ‘d1’

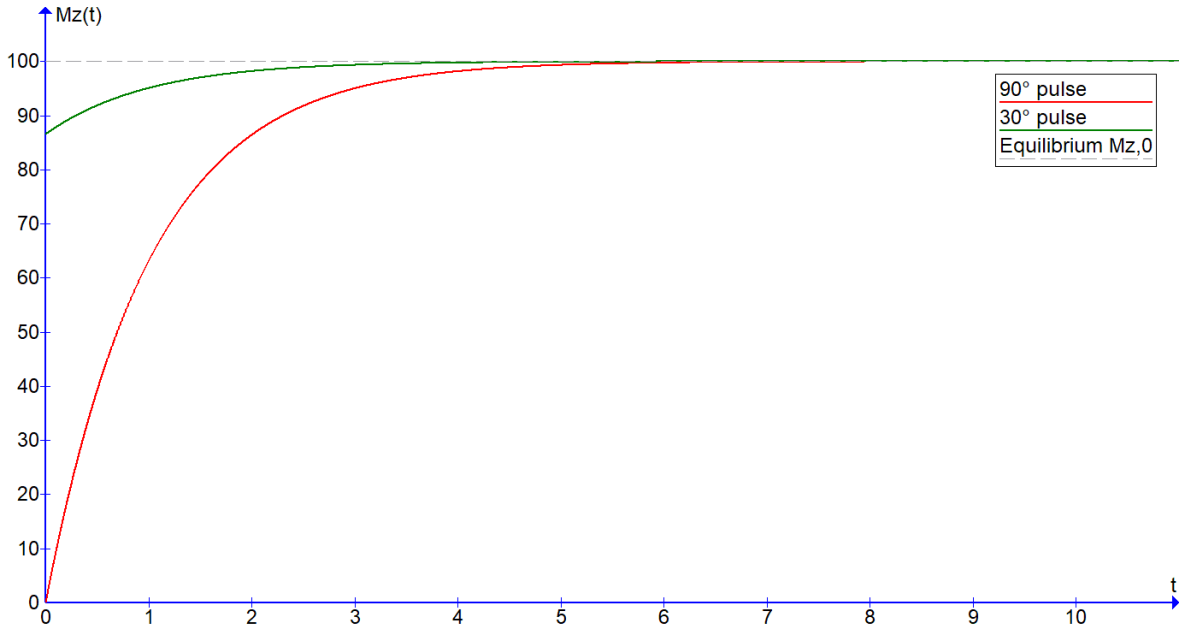
- Relaxation delay in sec
- At the very beginning of every scan
- Allows for all magnetization to relax back to z-equilibrium
- For qNMR d1 ≥ 5*T_1 for 90° pulses or d1 ≥ 3*T_1 for 30° pulses
  - T_1 is the longitudinal relaxation constant



❷ Data acquisition – the infamous ‘d1’

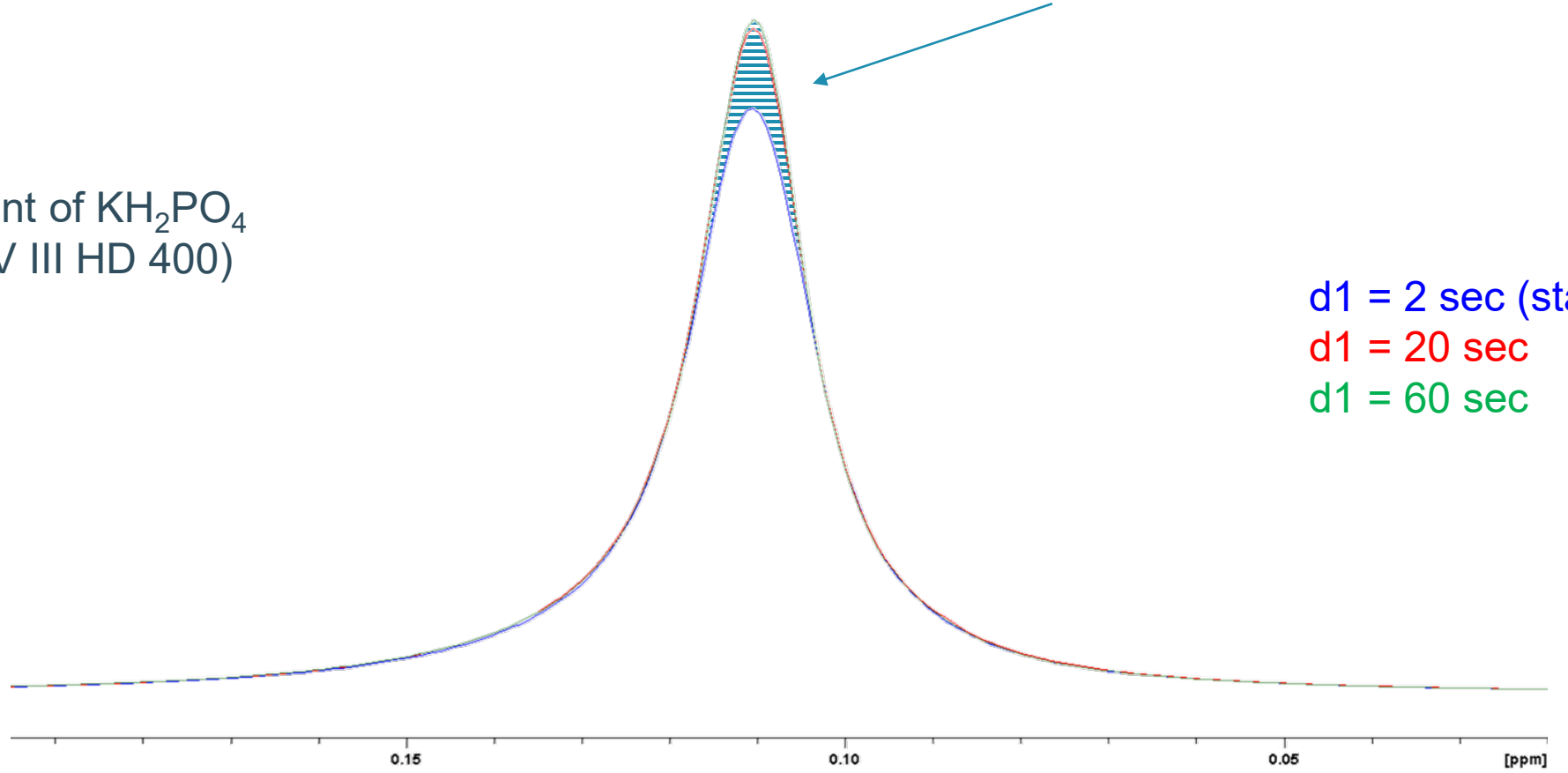
$$M_z(t) = M_z^0\left[1 - \exp\left(-\frac{t}{T_1}\right)\right] \qquad M_z(t) = M_z^0 \exp\left(-\frac{t}{T_1}\right)\left[\frac{\sqrt{3}}{2} - 1\right] + M_z^0$$

d1 (*T_1)	Recovery of z-magnetization for 90° pulse (%)	Recovery of z-magnetization for 30° pulse (%)
1	63.212055882856	95.071350041305
2	86.466471676339	98.186851007466
3	95.021293163214	99.332979761866
4	98.168436111127	99.754616967545
5	99.326205300092	99.909728627148
10	99.995460007024	99.999391756274
20	99.999999793885	99.999999972386



❷ Data acquisition – the infamous ‘d1’

^{31}P experiment of KH_2PO_4 in D_2O (on AV III HD 400)



d1 = 2 sec (standard)
d1 = 20 sec
d1 = 60 sec

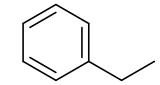
Pulse sequence = zg30. NS = 1.

❷ Data acquisition – the infamous ‘d1’

Nucleus	Typical T_1 (sec)	Standard pulseprogram on AV III HD 400	Standard d1 on AV III HD 400 (sec)	Recommended d1 if T_1 if unknown (sec)
^{1}H	0.5-10	zg30 (30°)	1	30
$^{13}C\{^{1}H\}$	0.1-30	zgig30 (30°)	2	90
^{19}F	0.2-15	zgflqn (90°)	1	75
^{31}P	0.1-60	zg30* (30°)	2	180

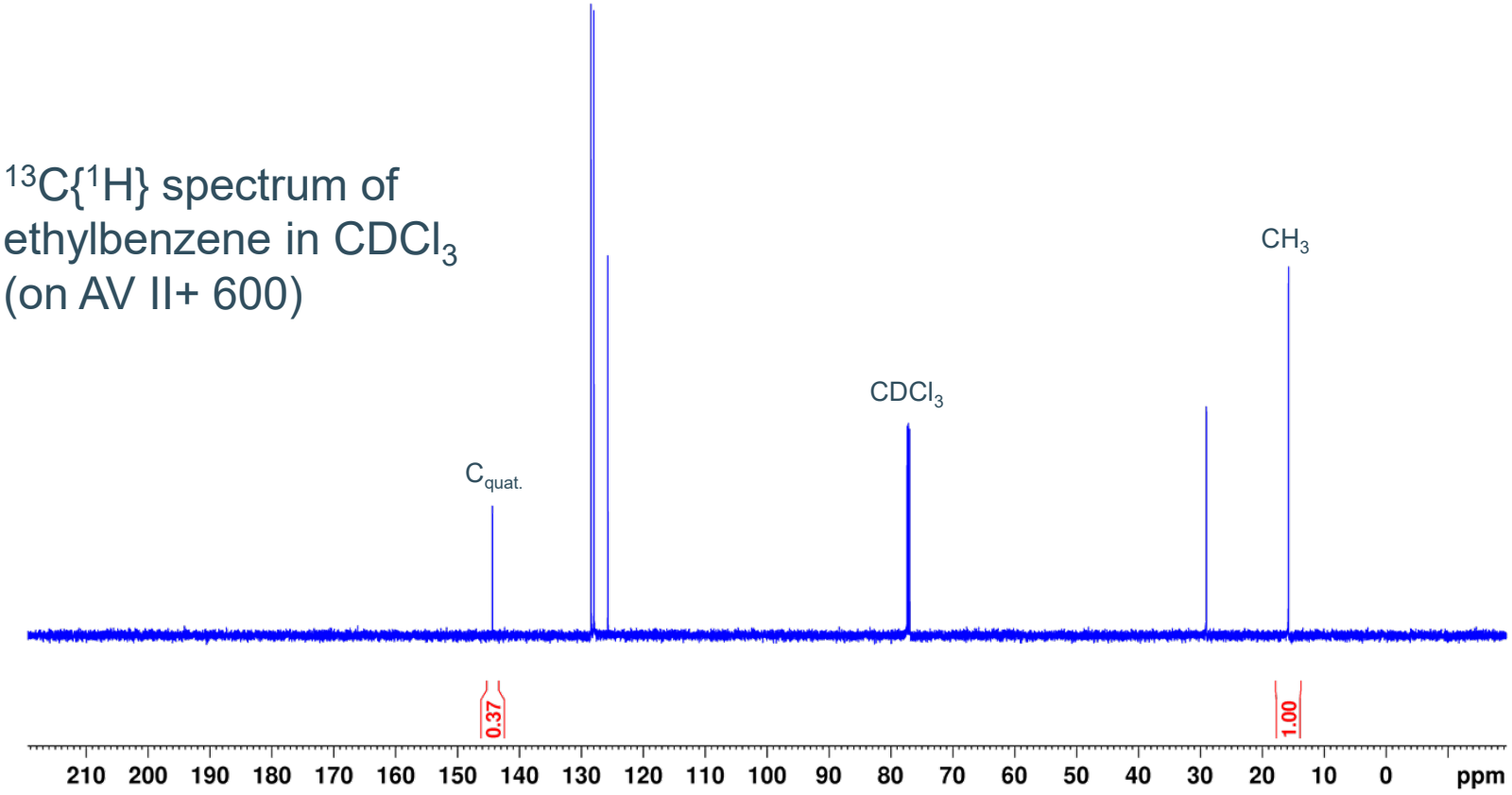
*No pulseprogram for ^{1}H-decoupled ^{31}P spectra ($^{31}P\{^{1}H\}$) is available for qNMR at the moment!

❷ Data acquisition – the infamous ‘d1’



ethylbenzene

$^{13}C\{^{1}H\}$ spectrum of ethylbenzene in $CDCl_3$ (on AV II+ 600)



Integration ratio of $C_{quat.}/CH_3$ is 0.37.

Theoretically, this should be 1.

Clearly, d1 was too low for the full relaxation of the $C_{quat.}$

Pulse sequence = zgig. NS = 8. D1 = 2 sec.

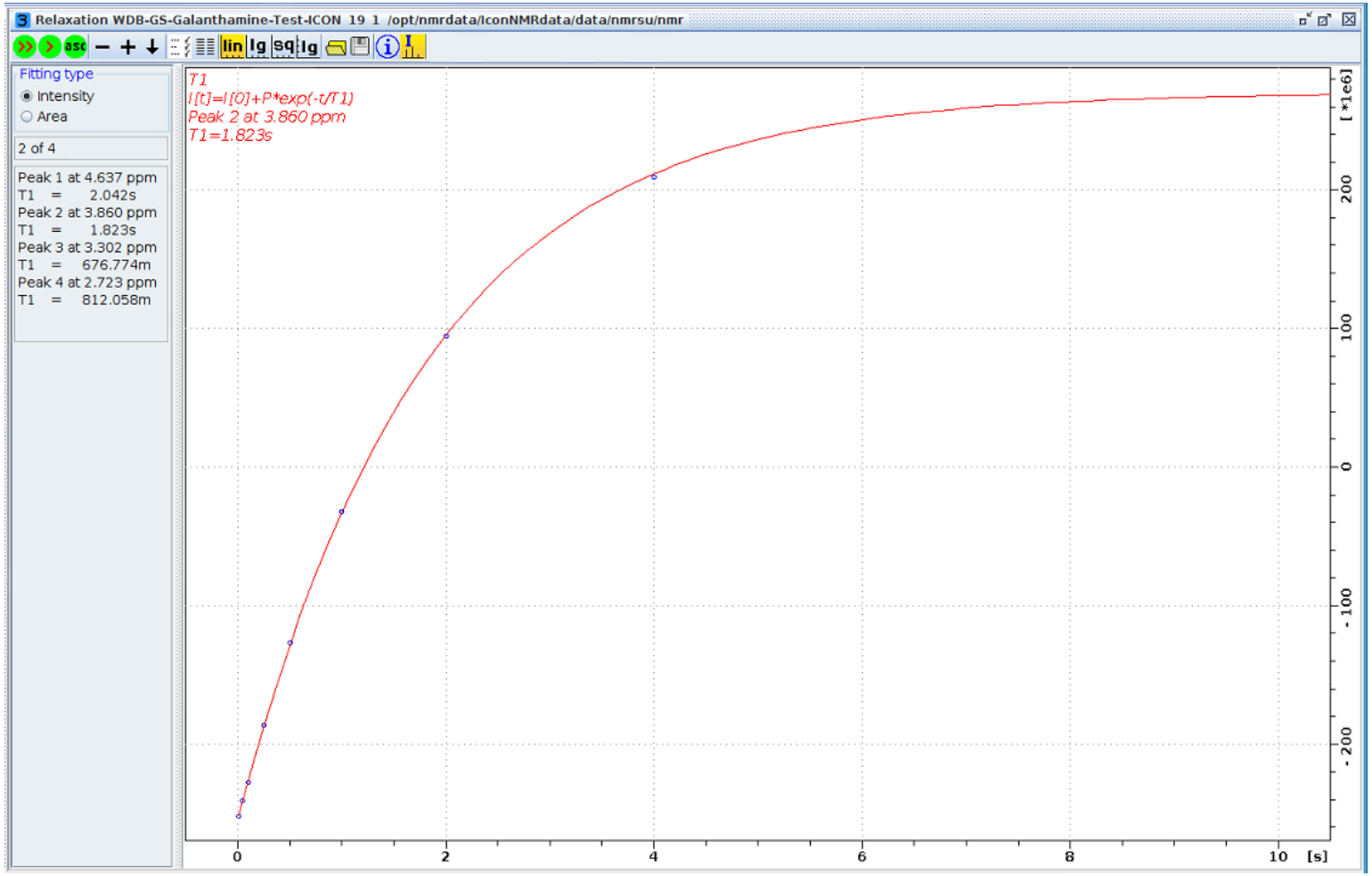
^{1}H T_1 experiment *via* inversion recovery

- Experiment **2D 1H T1 (IR)** (Bruker AV III HD 400)
- Pseudo-2D experiment
- Via inversion-recovery experiment
- Tuning and matching (*atma*) with 'exact' parameter (may take long!)
- Performs fast pulse calibration before acquisition
- Only for
  - ^{1}H nuclei
  - Non-dilute samples
  - Samples with T_1 < 5-10 sec
- Long experiment (minimal parameters = 26 min + *atma exact*!)

^{1}H T_1 experiment *via* inversion recovery

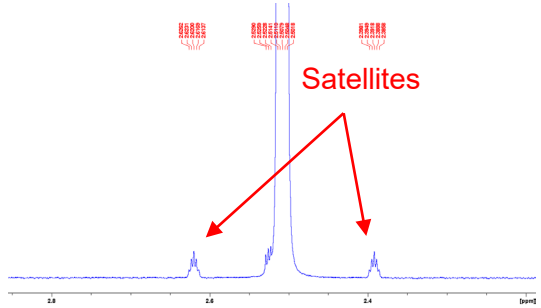
- Acquisition
  - Choose experiment '2D 1H T1 (IR)'
  - Do NOT touch TD, AQ, DS (4*n), NS (8*n)
  - Set O1P to middle of peak range or middle of peak of interest
  - Set D1 to (estimated) $5*T_1$ (90 ° flip angle)
- Processing
  - Execute **xaup**, *or*
  - Follow TopSpin's 'Advanced NMR Experiments Manual', section 'T1 Experiment' if you want to know what you are doing
  - Do NOT perform regular 2D or 1D processing, or your data will be lost!

^{1}H T_1 experiment *via* inversion recovery

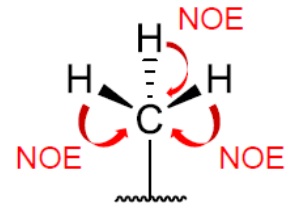



```
Dataset :
 /opt/nmrdata/IconNMRdata/data/nmrsu/nmr/WDB-GS-Galar
INTENSITY fit :
 I[t]=I[0]+P*exp(-t/T1)

10 points for Peak 1,  Peak Point at 4.637 ppm
Results     Comp. 1

I[0]  =    9.866e-01
P     =   -1.872e+00
T1    =      2.042s
SD    =    2.177e-02

    tau    ppm     integral    intensity

   10.000m     4.636  -1.3218e+08  -2.5693e+07
   50.000m     4.636  -1.2549e+08  -2.4427e+07
  100.000m     4.636   -1.177e+08  -2.2955e+07
  250.000m     4.636  -9.6332e+07  -1.8821e+07
  500.000m     4.636  -6.5856e+07  -1.2899e+07
    1.000s     4.636  -1.8829e+07  -3.7634e+06
    2.000s     4.636   4.1821e+07   7.9352e+06
    4.000s     4.636   1.0153e+08   1.9417e+07
   15.000s     4.636   1.4857e+08   2.8504e+07
   60.000s     4.636   1.4923e+08   2.8616e+07


10 points for Peak 2,  Peak Point at 3.860 ppm
Results     Comp. 1

I[0]  =    9.956e-01
P     =   -1.933e+00
T1    =      1.823s
SD    =    4.402e-03

    tau    ppm     integral    intensity

   10.000m     3.857  -4.0787e+08  -2.5237e+08
   50.000m     3.857  -3.8805e+08  -2.4074e+08
  100.000m     3.857  -3.6529e+08  -2.2752e+08
  250.000m     3.857  -2.9885e+08  -1.8636e+08
  500.000m     3.857   -2.011e+08  -1.2696e+08
    1.000s     3.857  -4.3528e+07  -3.1943e+07
    2.000s     3.857   1.6645e+08   9.4609e+07
    4.000s     3.857   3.5644e+08   2.0929e+08
   15.000s     3.857   4.5467e+08   2.7137e+08
   60.000s     3.857   4.5582e+08    2.703e+08
```

❷ Data acquisition – pulse sequence: decoupling or not?

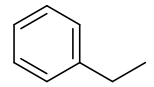


- No decoupling (hence, 'with coupling')
  - Spectra are more complex
  - Experimentally easier
  - Take satellite peaks into account during analysis! (*e.g.* ^{13}C-decoupled ^{1}H spectra)
- Decoupling (during acquisition of FID)
  - Spectra are easier to analyze
  - Appropriate decoupling power necessary
  - **No NOE enhancement**! Hence, decoupling may only happen during acquisition time.
    - No power-gated (zg**pg** or zg**pg**30) or gated decoupling (zg**gd** or zg**gd**30)
    - Only inverse-gated decoupling (zg**ig** or zg**ig**30)



❷ Data acquisition – pulse sequence: decoupling or not?

Example: $^{13}C\{^{1}H\}$ spectrum

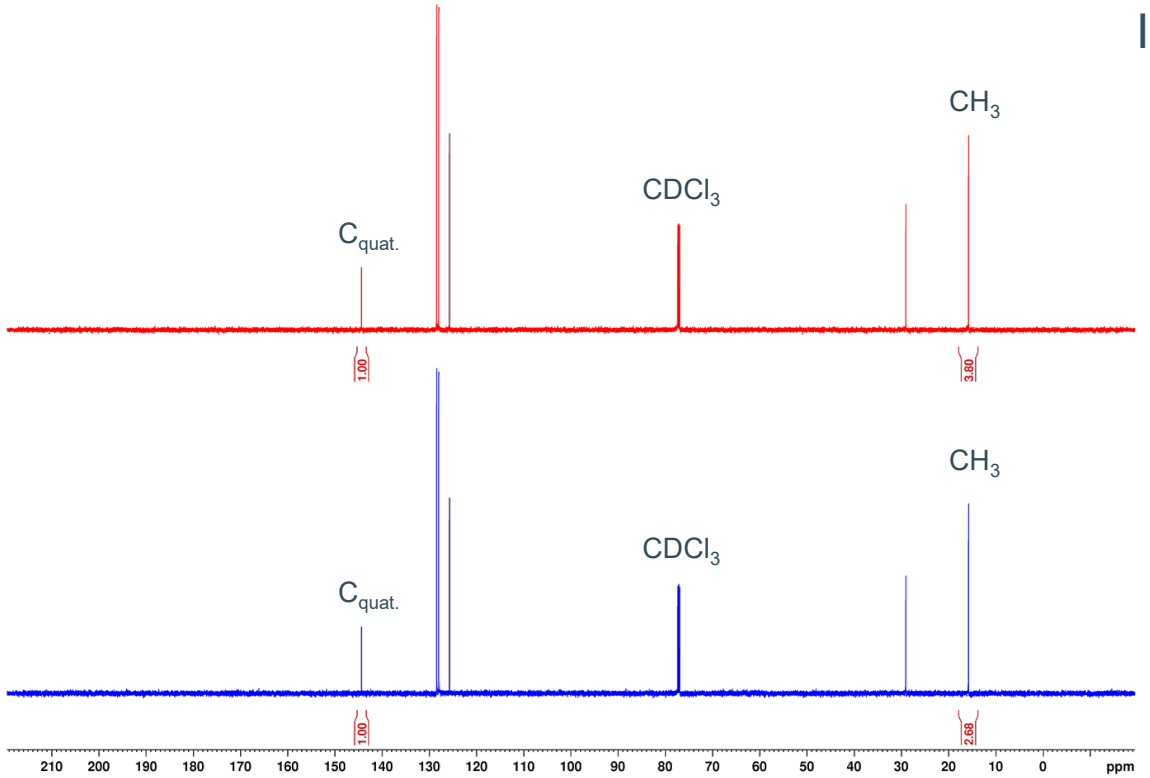


ethylbenzene

Integration ratio of $\mathbf{C}H_3/\mathbf{C}_{quat.}$

zgpg (with NOE enchancement)

zgig (without NOE enchancement)



3.80

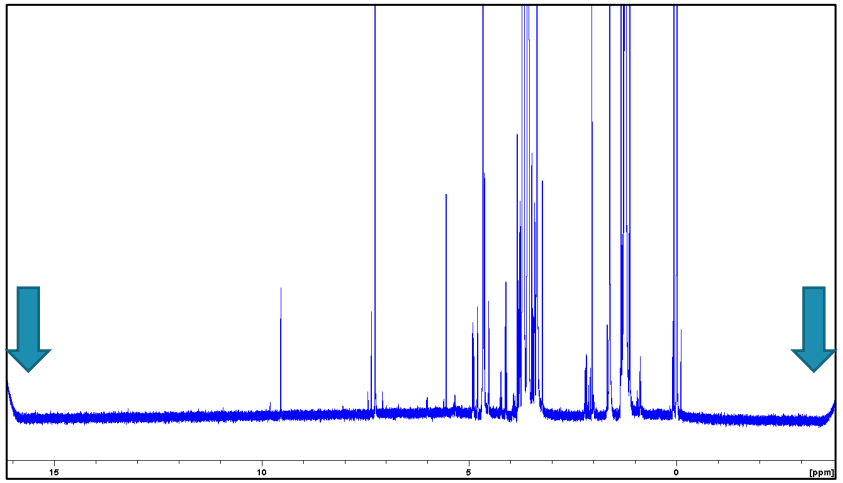
2.68

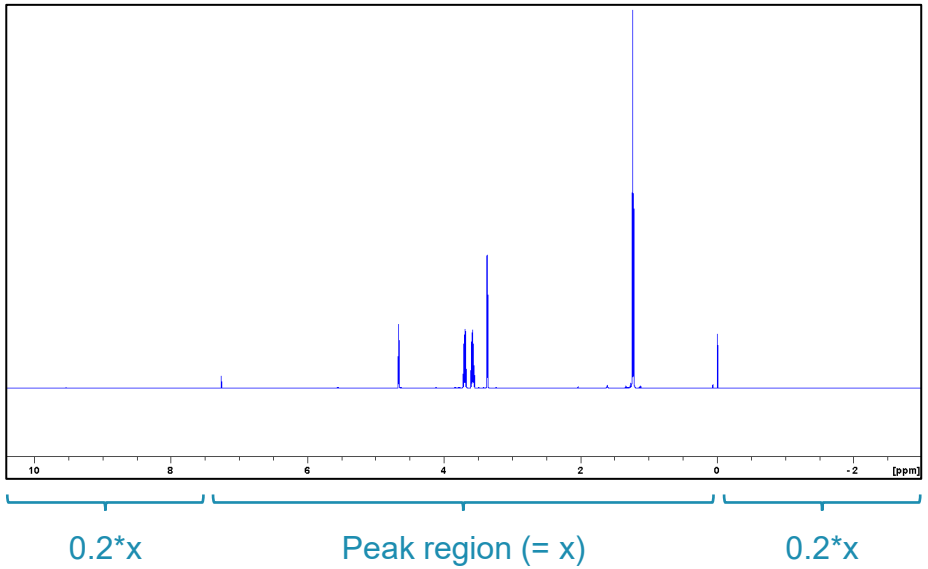
Difference of 40% only due to NOE enhancement

For the zgig experiment, the theoretical expectation is a ratio of 1. The fact that this is not the case now, means D1 was also set incorrectly.

❷ Data acquisition – spectral width (SW)

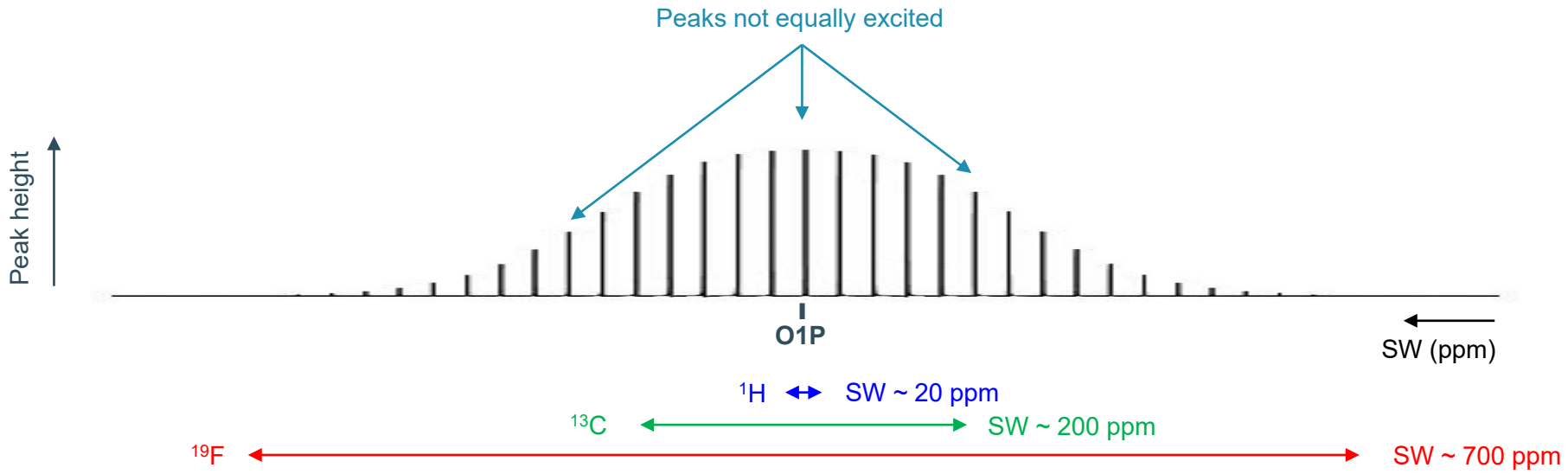
- SW should be large enough to avoid attenuation of peaks at the edges of the spectrum due to receiver filters
- Large SW aides with baseline corrections
- SW should contain 20% of the peak region of baseline on both sides





❷ Data acquisition – excitation bandwidth

- Uniform excitation of entire SW necessary
- Problem: only (small) part of bandwidth is equally excited during pulse



❷ Data acquisition – excitation bandwidth

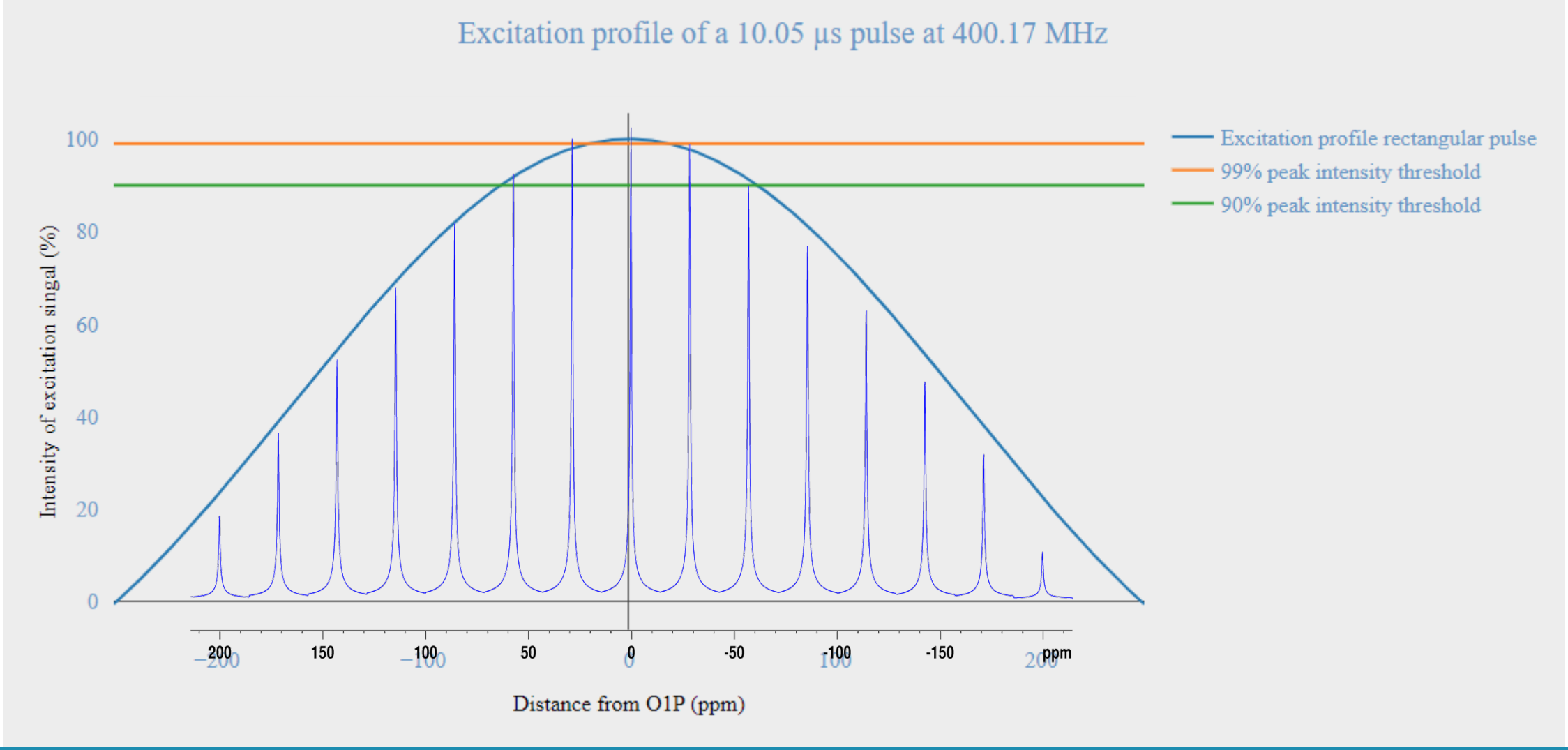
- Excitation profile (centered around O1P) follows $f(\delta) = sinc\left(\frac{\pi \times \delta \times SF \times p}{10^6}\right)$

  with SF = spectrometer frequency (MHz), p = pulse length (µsec)

- For uniform excitation (error < 1%), $SW \lessapprox 156\,000 \times \frac{1}{SF \times p}$
- Choose calibrant with similar δ to analyte
- Set middle of the spectrum (O1P) at **exact** center between peaks of calibrant and analyte, so that excitation error is equal for both signals (diminishes relative error)
- Uniform excitation problematic for high-frequency nuclei with large SW (*e.g.* ^{19}F)
  e.g. For ^{19}F on AV III HD 400: SW < 26 ppm for uniform bandwidth (error < 1%)
- https://nmrfacilities.chem.kuleuven.be/applet/excitation_profile

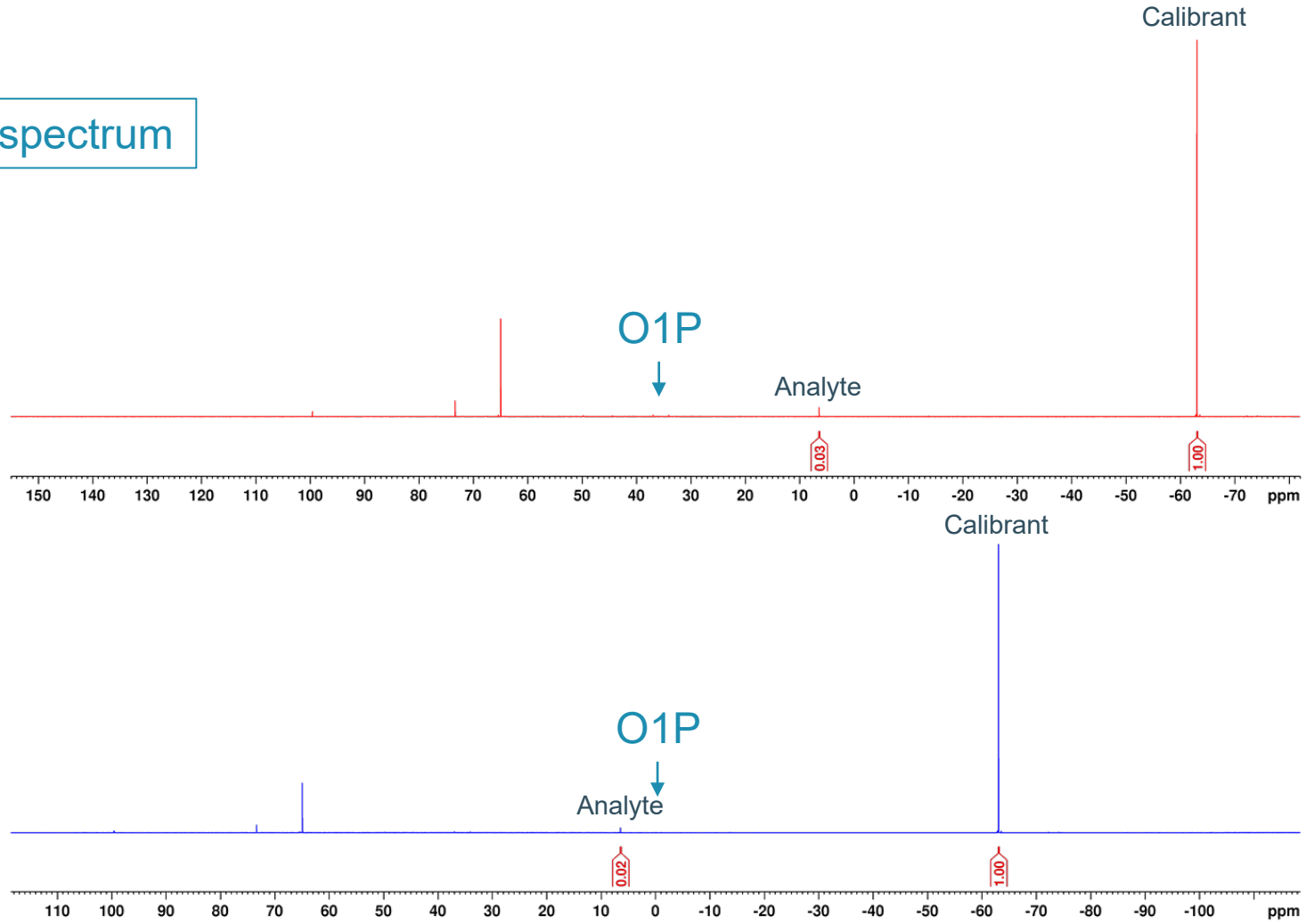
For more information about quantitative ^{19}F NMR: https://onlinelibrary.wiley.com/doi/abs/10.1002/cmr.a.21422

❷ Data acquisition – excitation bandwidth



❷ Data acquisition – excitation bandwidth

Example: $^{19}F\{^{1}H\}$ spectrum



Integration ratio of analyte/calibrant

O1P = 36.5 ppm

0.03

Difference of 50% only due to shift of O1P!

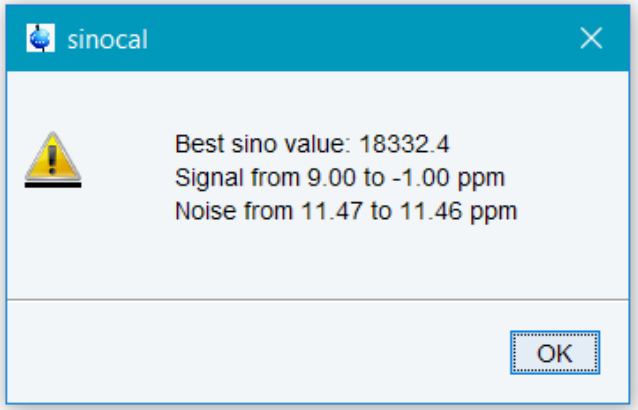
O1P = 0 ppm

0.02

Two consecutive measurements on same sample, without ejection from the magnet.

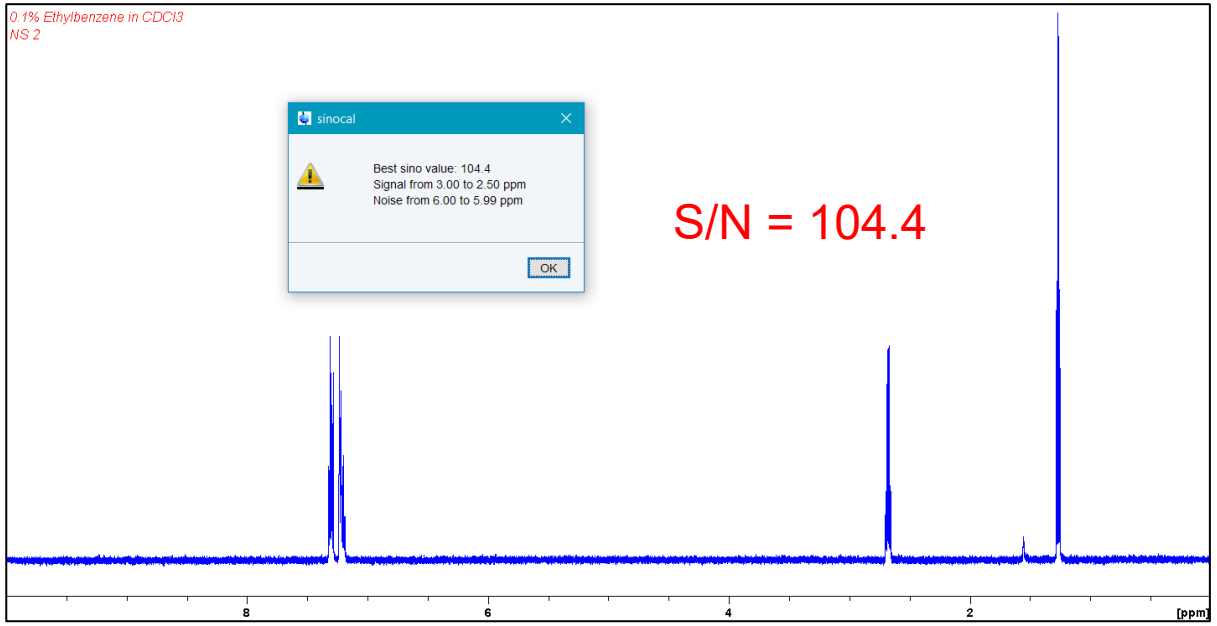
❷ Data acquisition – signal-to-noise ratio (S/N)

- Good S/N essential for accurate integration
- Remember that acquiring N times more scans, increases the S/N only $\sqrt{N}$ times
- For integration errors < 1%, S/N must be ≥ 150/1 (S/N instrument dependent!)
- Calculate S/N of all the peaks you want to integrate using sinocal



Saito, T.; *et al*.., Practical guide for accurate quantitative solution state NMR analysis. *Metrologia* **2004,** *41* (3), 213-218.

❷ Data acquisition – signal-to-noise ratio (S/N)



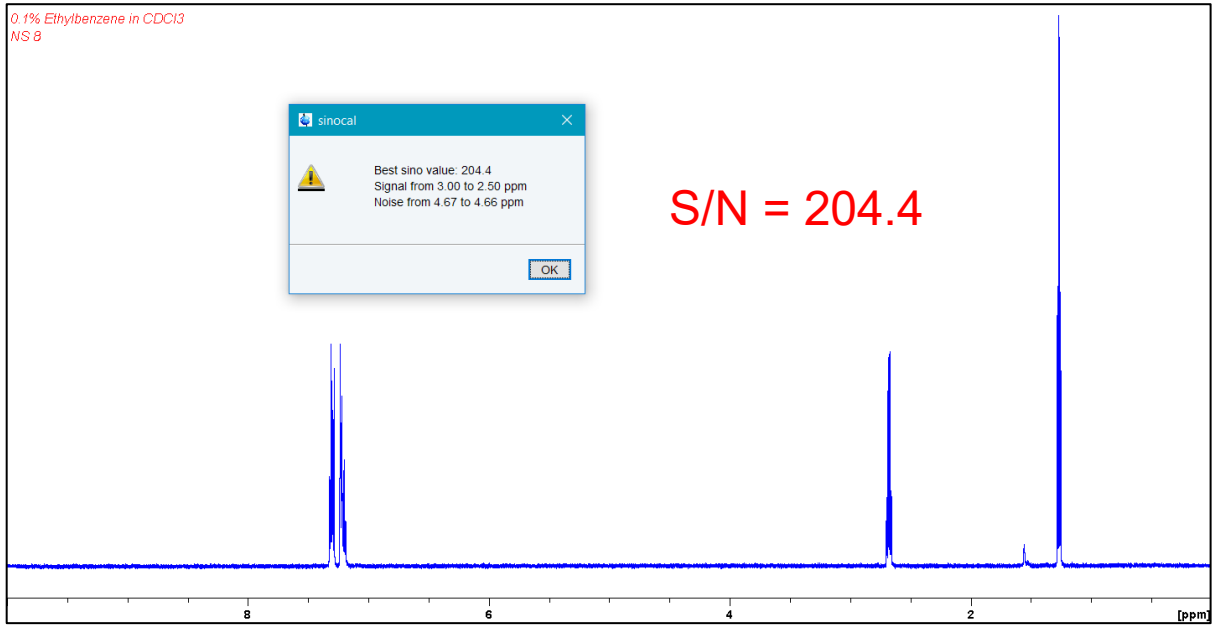
S/N = 104.4

NS = 2

NS x 4

NS = 8

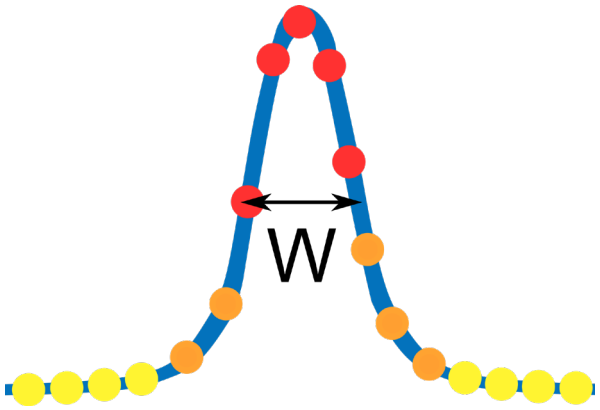
S/N x $\sqrt{4}$ = S/N x 2



S/N = 204.4

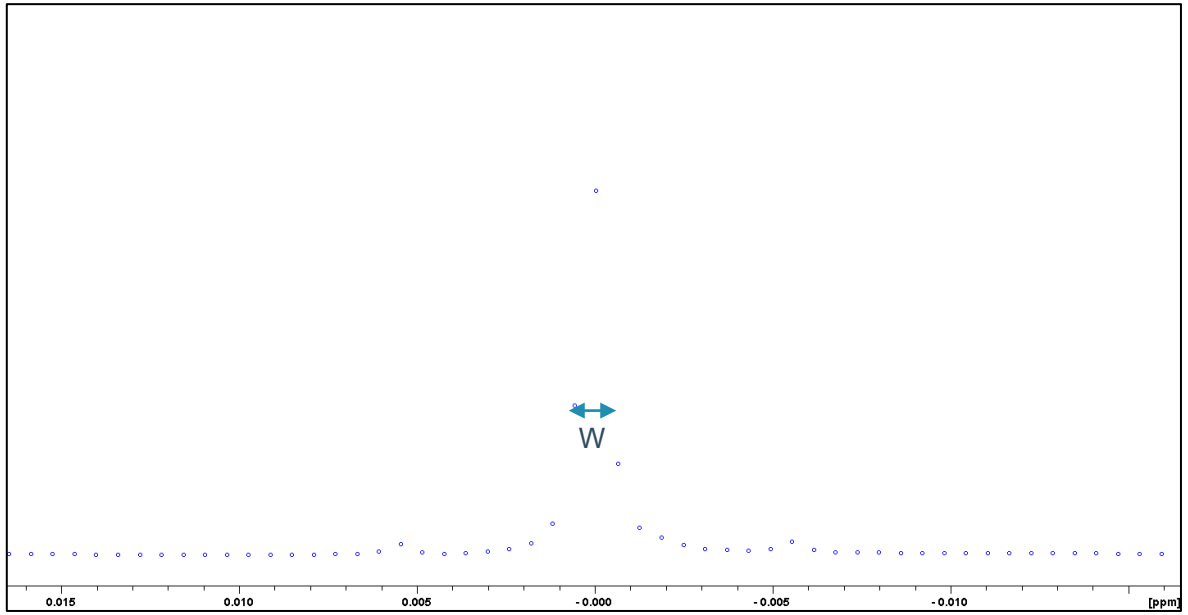
❷ Data acquisition – digital resolution (FIDRES)

- $FIDRES = \frac{spectral\ width\ in\ Hz}{amount\ of\ real\ data\ points} = \frac{SWH}{TD/_2} = \frac{1}{acquisition\ time\ in\ sec} = \frac{1}{AQ}$
- Every peak should consist of ≥ 5 data points above half-height (red points)
  ⇒ every peak consists of ≥ 10 data points in total (red points + orange points)
- Example
  - Peak with W = 1 Hz
  - At least 5 points above half-height
    ⇒ separation between points = 0.2 Hz
  - FIDRES should be ≤ 0.2 Hz

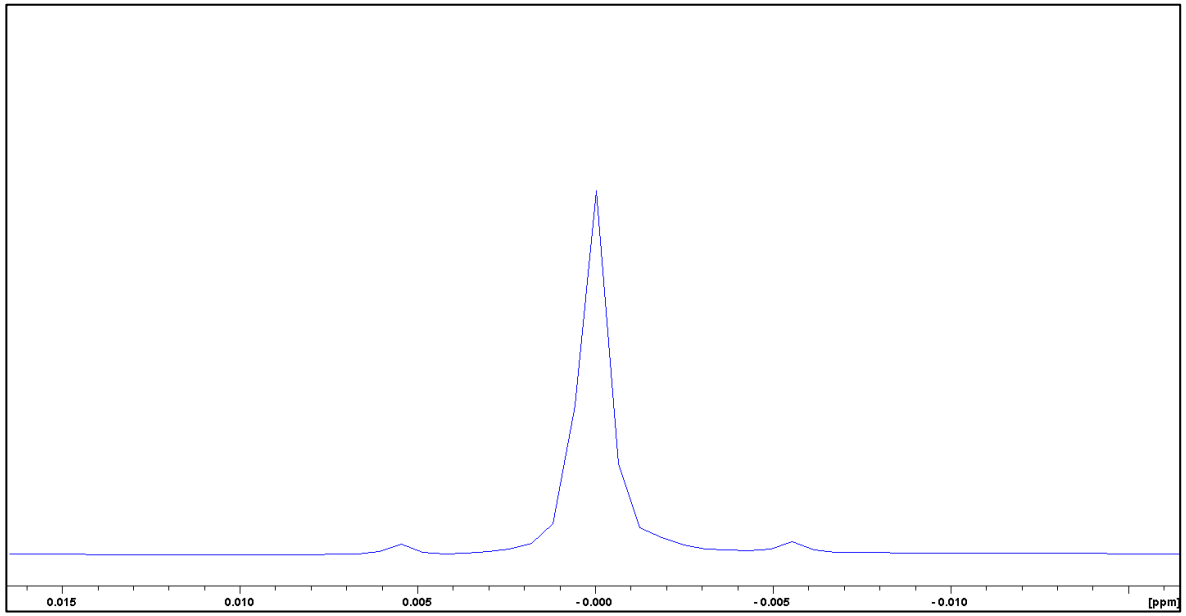


W = linewidth at half-height in Hz.

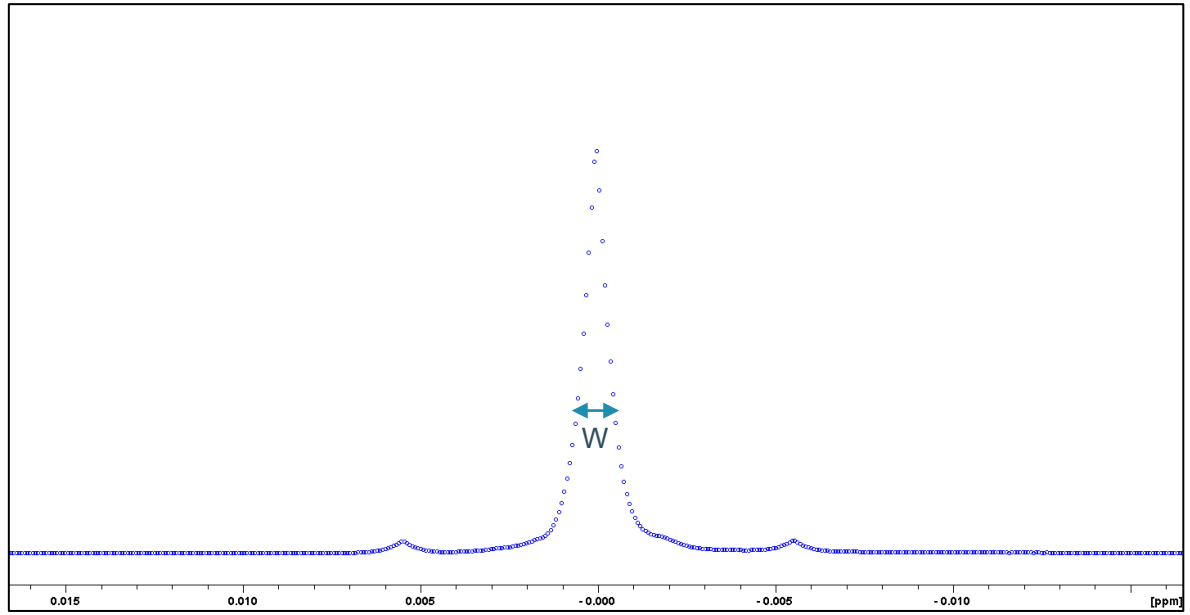
❷ Data acquisition – digital resolution (FIDRES)



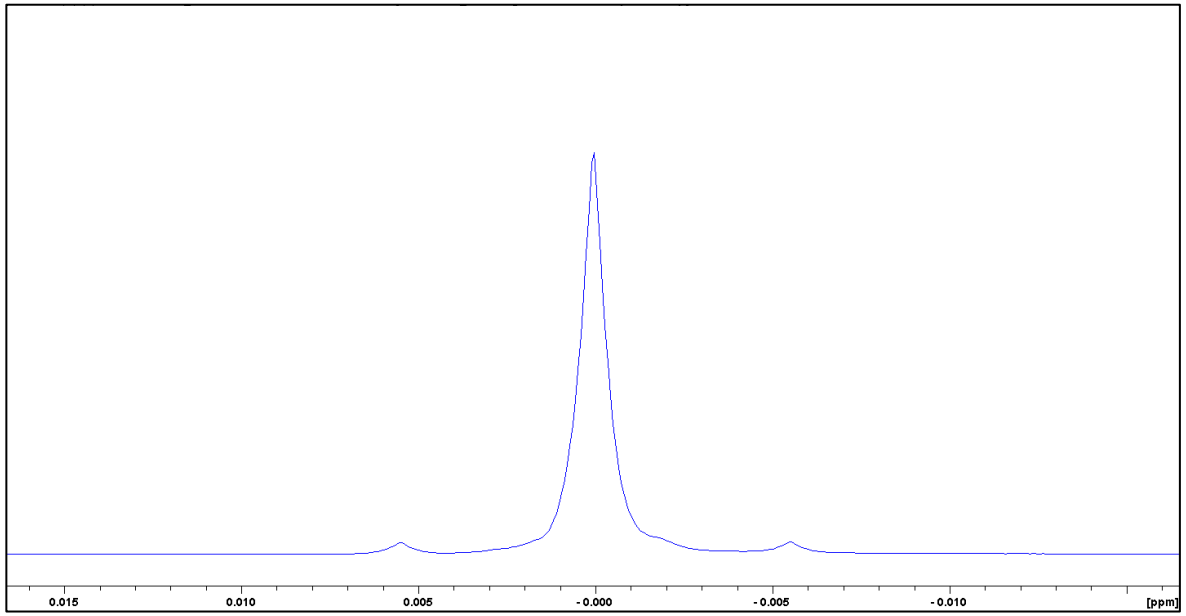
Peak described by 2 data points
covering the linewidth



❷ Data acquisition – digital resolution (FIDRES)



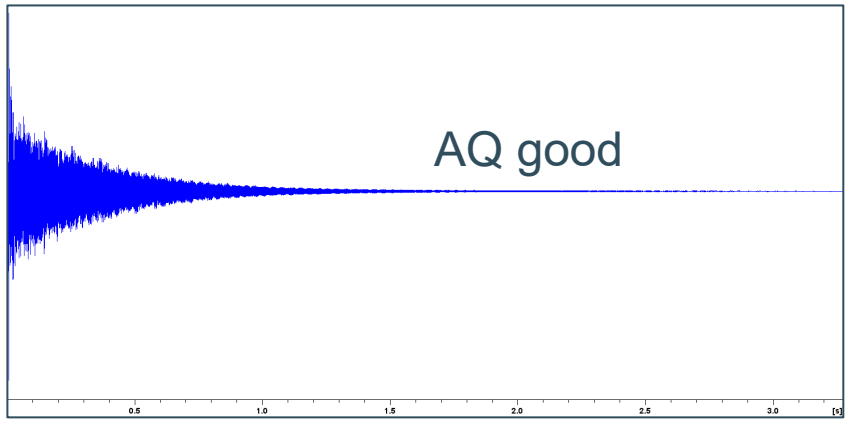
Peak described by 14 data points
covering the linewidth



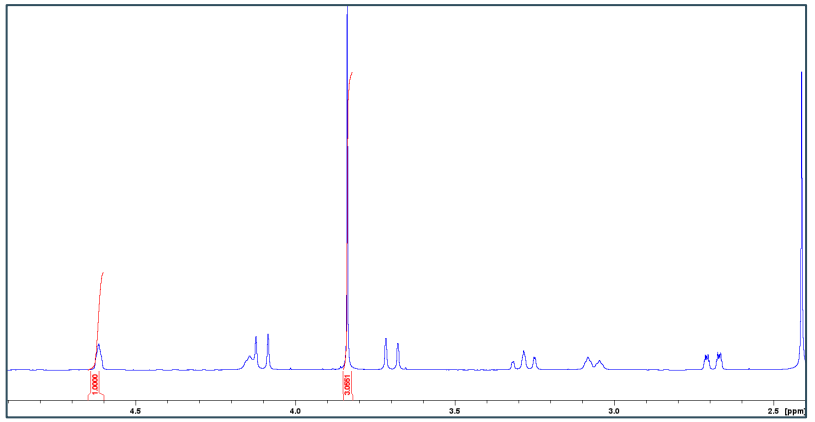
❷ Data acquisition – acquisition time (AQ)

- Acquisition time (AQ) is time during which FID is recorded
- Should be large enough, so that FID is not truncated
- Should not be too large, to prevent additional noise in spectrum
- Truncation of FID will lead to sinc wiggles and improper integration

❷ Data acquisition – acquisition time (AQ)

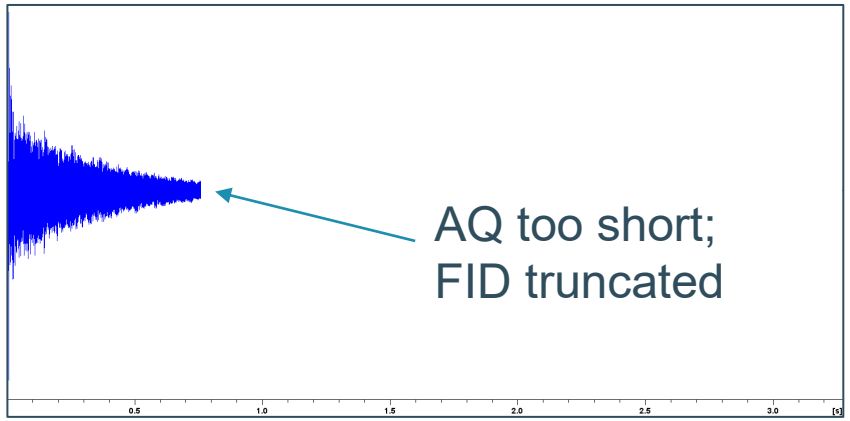


Fourier transform

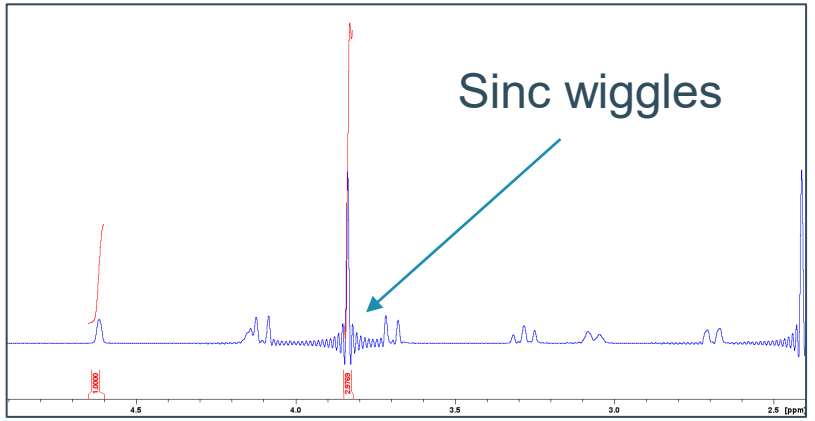


Integration ratio

3.0551



Fourier transform



2.9769

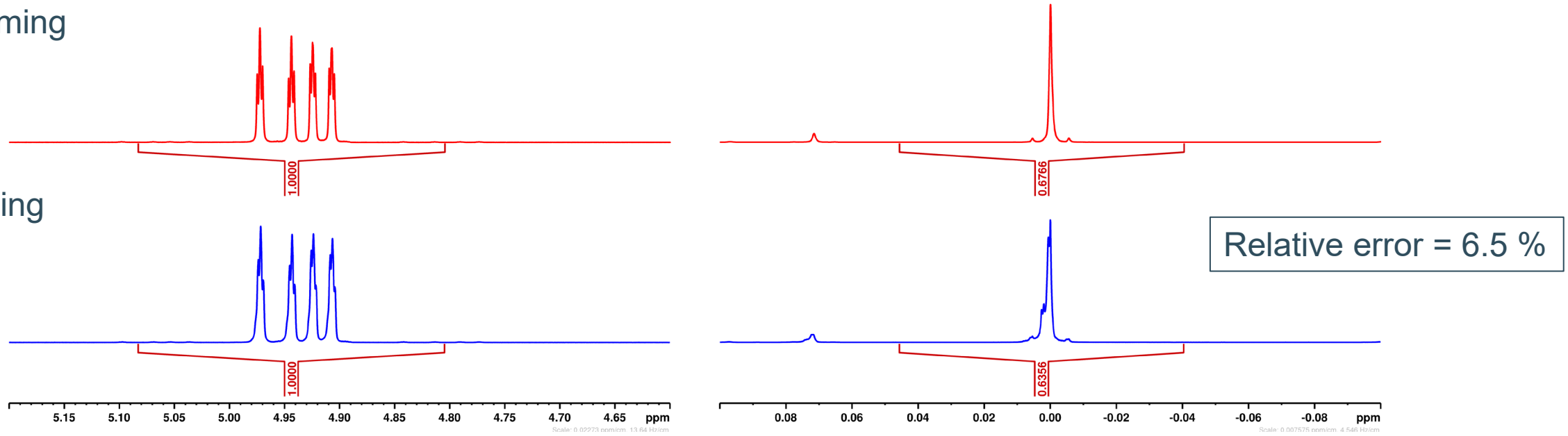
Difference of 3% only due to too short AQ

❷ Data acquisition – shimming the sample

- Accurate shimming is essential for qNMR
- Spinning the sample is discouraged (prevents spinning sidebands)

Good shimming

Bad shimming



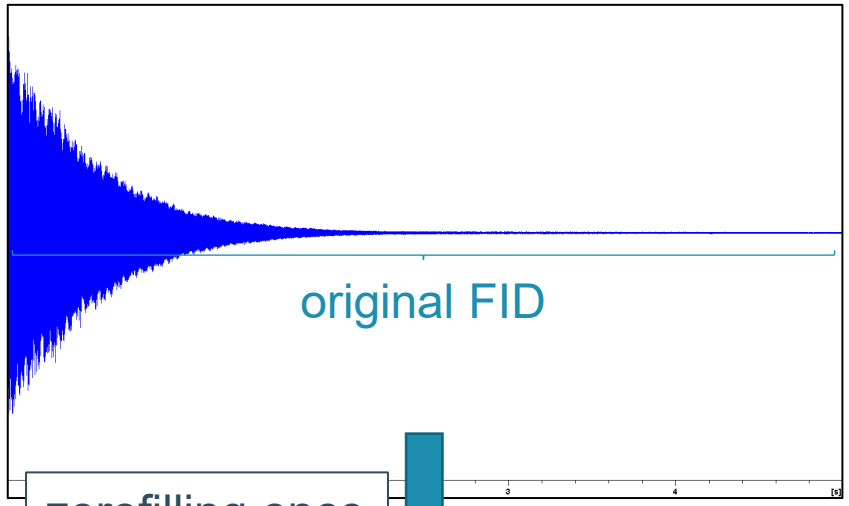
Relative error = 6.5 %

Sample: quinine in $CDCl_3$ at 600 MHz. Both spectra were acquired subsequently without ejecting the sample from the magnet.

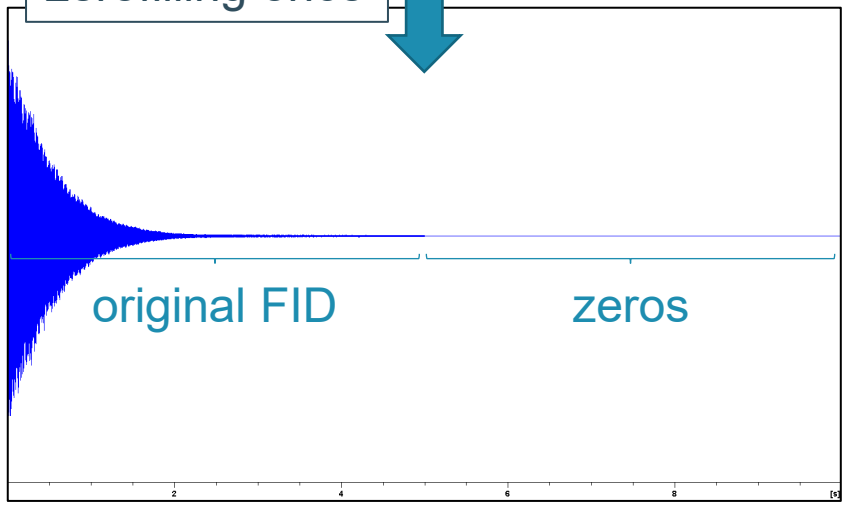
❸ Data processing – zerofilling

- Zerofilling = adding ‘zeros’ after the FID signal
- Increases spectral resolution significantly
- Aides with integral precision
- Can be performed by setting SI to a value larger than TD/2 (you will zerofill once for every increase of SI by TD/2). Perform Fourier transform (ft) and phase correction (apk) afterwards.

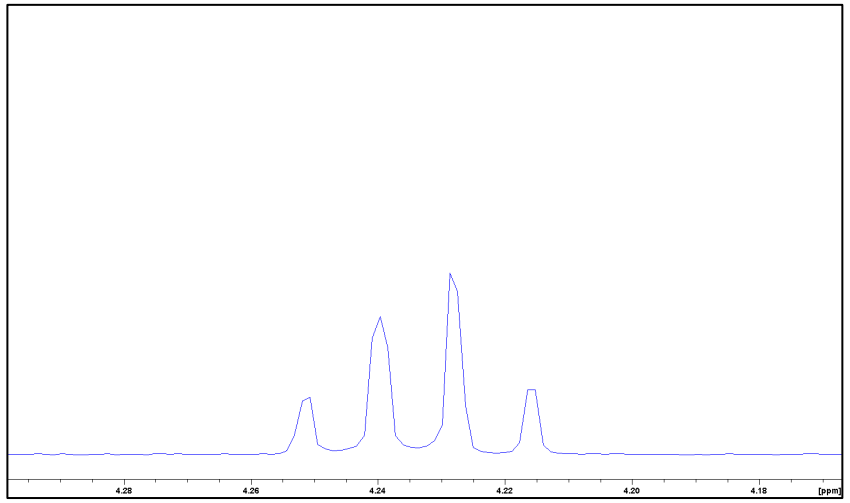
❸ Data processing – zerofilling



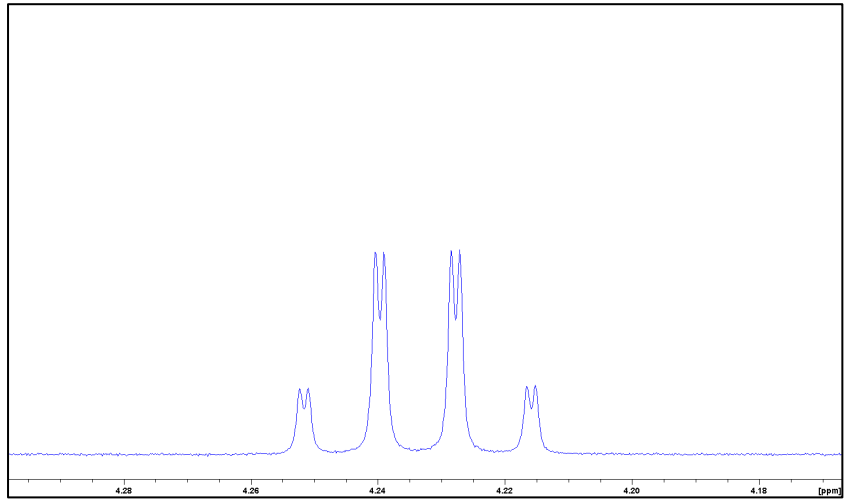
zerofilling once



Fourier transform

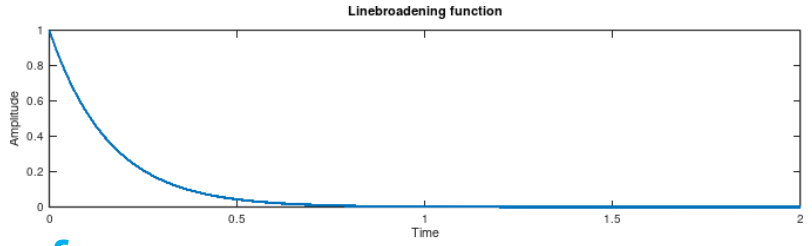


Fourier transform

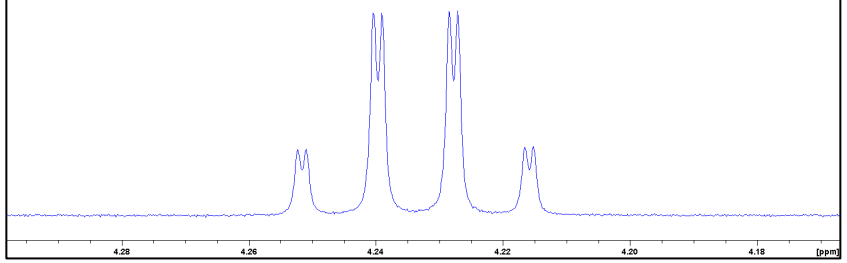


❸ Data processing – exponential weighting function

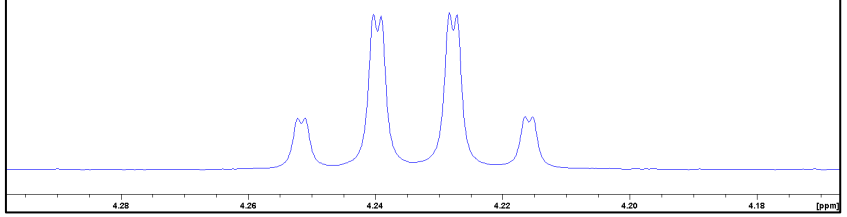
- Forces FID signal quicker to zero by multiplying it with $W_{LB} = \exp(-\pi \times LB \times t)$
- Increases S/N
- Decreases resolution (peak broadening)
- Specify LB (line broadening factor) in Hz and perform efp



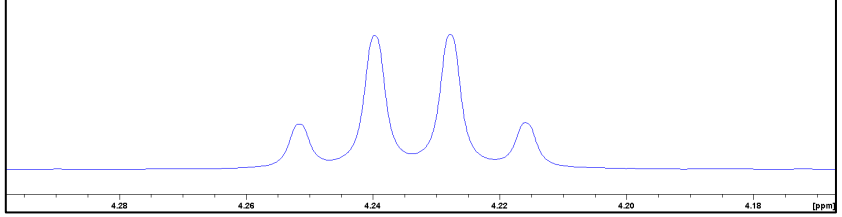
No weighting function (LB = 0 Hz)
S/N = 190.8



Weighting function (LB = 0.3 Hz)
S/N = 711.5
Standard in HT zone

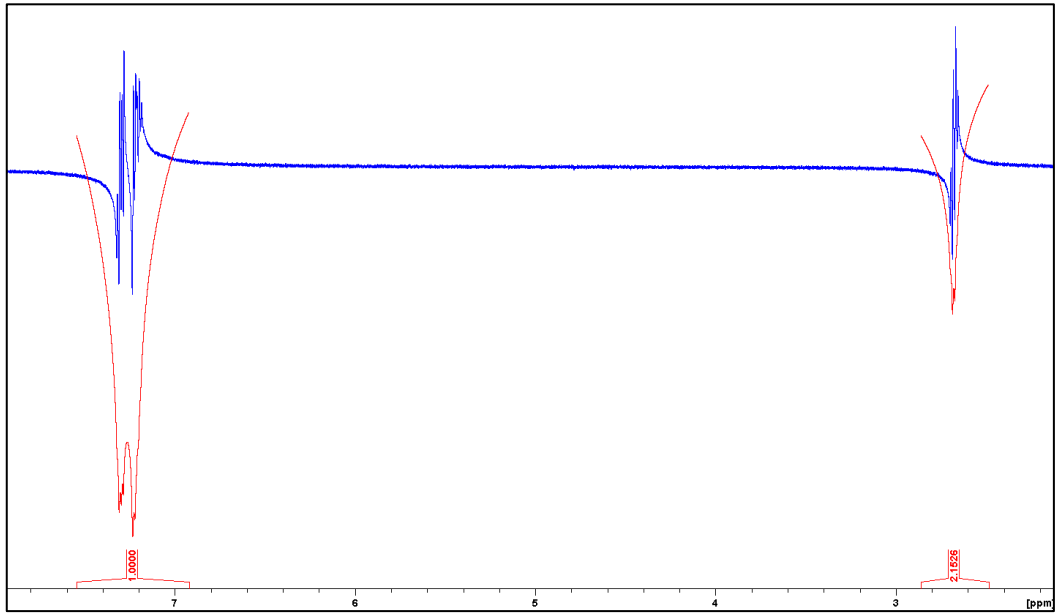


Weighting function (LB = 1 Hz)
S/N = 1347.4

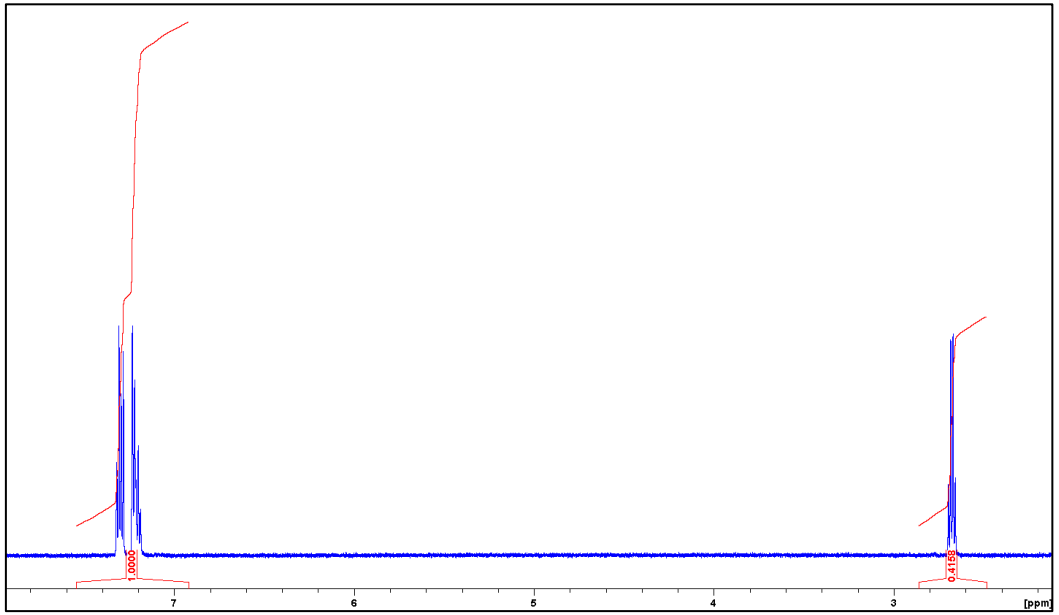


❸ Data processing – phase correction

- Drastic impact on integration
- Correct the phase automatically (apk, apks, apkm) or manually
- For specific heteronuclei ^{13}C, ^{19}F and ^{31}P: apbk (phase + baseline correction)

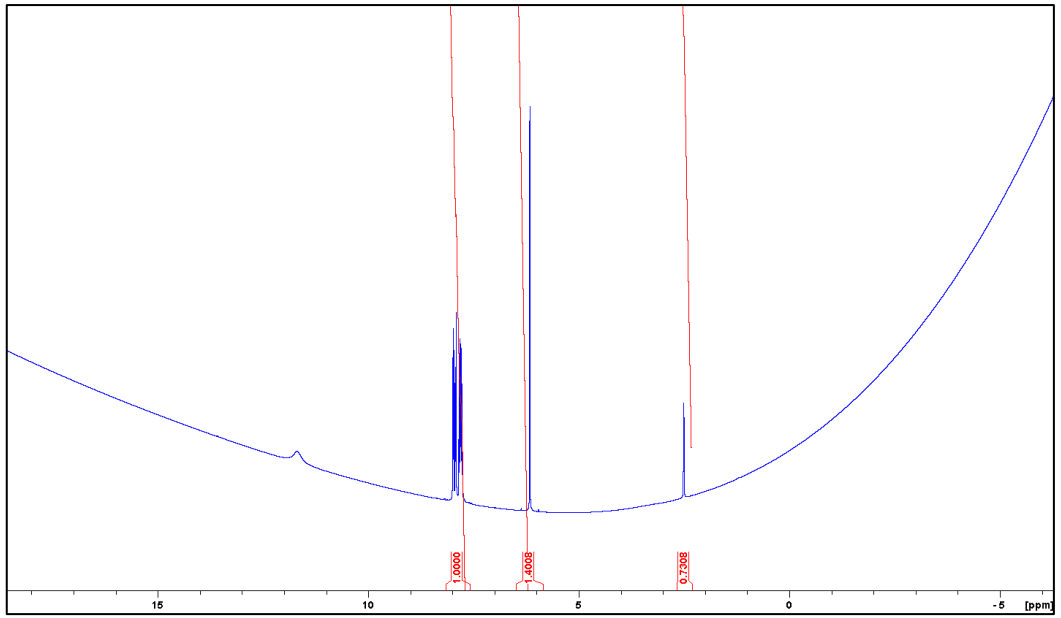


Phase correction

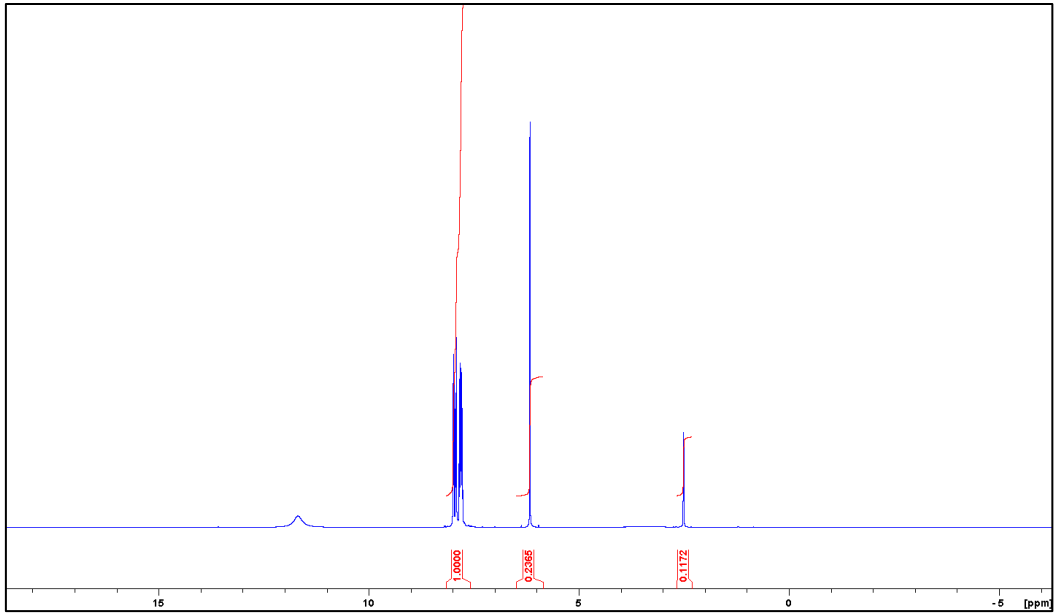


❸ Data processing – baseline correction

- Drastic impact on integration
- Correct the spectral baseline automatically (abs, abs n) or manually
- For specific heteronuclei ^{13}C, ^{19}F and ^{31}P: apbk (phase + baseline correction)



Baseline correction



❹ Data analysis – choosing the right peak

- Quantification signals should
  - be unambiguously assigned
  - be as simple as possible (*i.e* singlet > doublet > … > complex multiplet)
  - show as little overlap as possible
  - NOT be from exchangeable nuclei (*e.g.* R-C(O)-N**H**-R, R-O**H**, R-N$\mathbf{H}_2$, …)

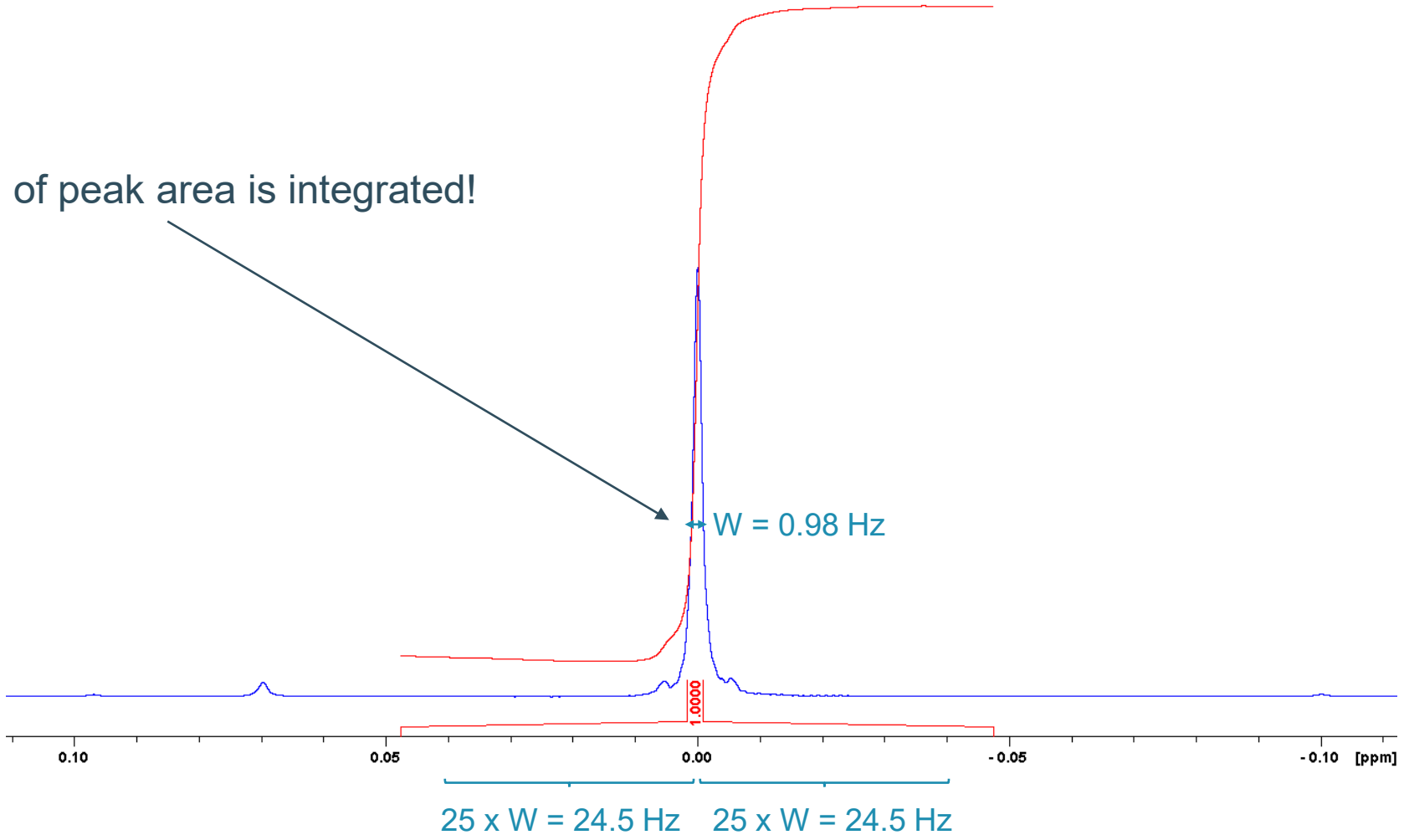
❹ Data analysis – integration

- Peaks are Lorentzian according to $L(x) = \frac{h}{1+\left(\frac{x}{W}\right)^2}$ (h = height of the peak; W = linewidth at half-height)
- In theory, peaks should be integrated from $-\infty$ to $+\infty$
- In practice, integral regions should cover ≥ 25 x W in both directions in order to cover 99% of the area or ≥ 75 x W in both directions in order to cover 99.9% of the area

Griffiths, L., Assay by nuclear magnetic resonance spectroscopy: quantification limits. *Analyst* **1998,** *123* (5), 1061-1068.

❹ Data analysis – integration

Only 99% of peak area is integrated!



Griffiths, L., Assay by nuclear magnetic resonance spectroscopy: quantification limits. *Analyst* **1998,** *123* (5), 1061-1068.

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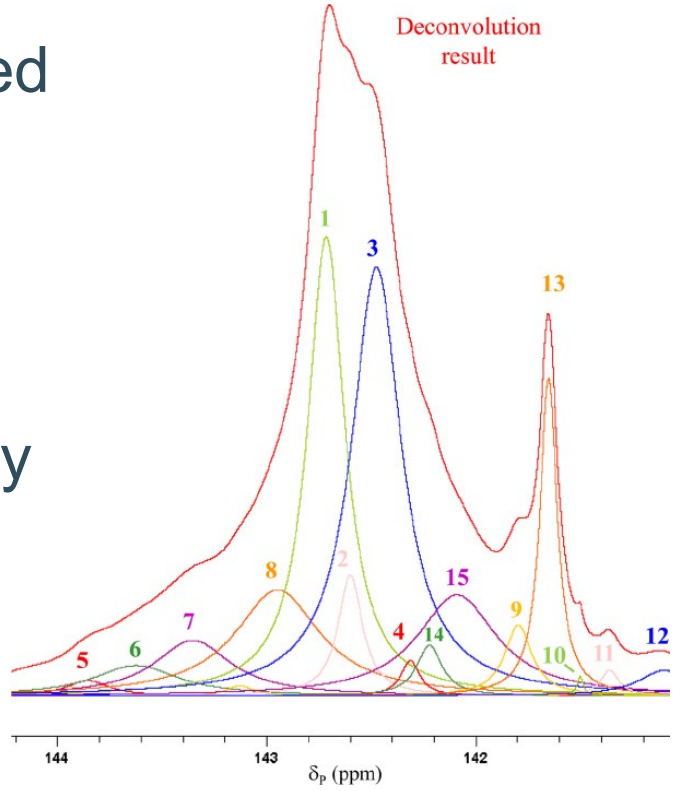
❹ Data analysis – deconvolution

- For < 1% integration error, two (singlet) peaks should be separated by at least 50 times the linewidth W
- Not-well-separated signals or multiplets can be deconvolved
- Deconvolution comprises
  - fitting all the lines of the peaks in the multiplet
  - reproducing each of the peaks individually
- After deconvolution, the peaks can be integrated separately

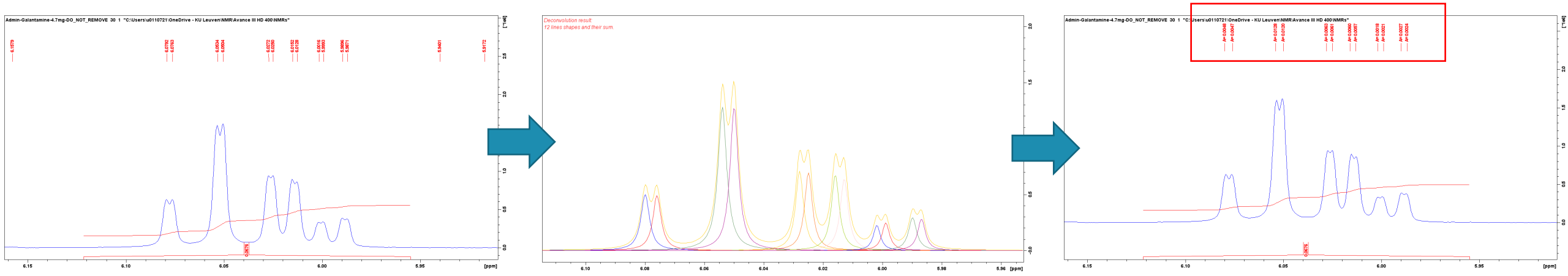


Picture from: Prinsen, P.; *et al.*, Modification of the Lignin Structure during Alkaline Delignification of Eucalyptus Wood by Kraft, Soda-AQ, and Soda-O2 Cooking. *Industrial & Engineering Chemistry Research* **2013,** *52* (45), 15702-15712.

❹ Data analysis – deconvolution

- TopSpin can easily deconvolve peaks and display corresponding integral areas
- In Integration mode, right click on integral → ‘Deconvolusion’ → ‘Deconvolve and Display Integrals’
- For advanced deconvolution, go to ‘Analyse’ → ‘Line Shapes’ → ‘Fit Lorenz/Gauss function (dcon)’

Relative areas of deconvolved peaks



Omitting the internal standard – Eretic2

Eretic2 – What is Eretic2?

- Electronic Reference To *In vivo* Concentration 2[1]
- Quantification method allowing to (absolutely) quantify an NMR sample, without adding a standard to the sample
- Determine concentration of unknown sample by correlating absolute integral values to those of a reference spectrum with known concentration

Eretic2 allows the user to 'calibrate the spectrometer' and use this calibration for absolute quantification of a(nother) sample.

[1]Based on PULCON; Wider G. & Dreier L., *J. Am. Chem. Soc.*, **2006**, 128 (2571-2576).

Eretic2 – How does it work?

- Eretic2

$$c_{unk} = \mathrm{k} c_{ref} \frac{A_{unk} T_{unk} \vartheta_{unk} NS_{ref}}{A_{ref} T_{ref} \vartheta_{ref} NS_{unk}}$$

with

- indices unk and ref representing unknown and reference sample, resp.
- c concentrations
- A integration values
- k correction factor (different RG, incomplete relaxation, …)
- T measurement temperature (K)
- ϑ pulse length (µsec)
- NS number of scans

Eretic2 – What do you need?

1. For calibration

- ANY reference sample with known
  - Concentration (in mM),
  - Molar mass (g/mol)
  - (total) sample volume (mL)*
  - Assignment for at least one peak
- Quantitative measurement conditions!

2. For quantification

- Your solubilized compound with known
  - Molar mass (g/mol)
  - (total) sample volume (mL)*
  - Assignment for at least one peak
- Quantitative measurement conditions!

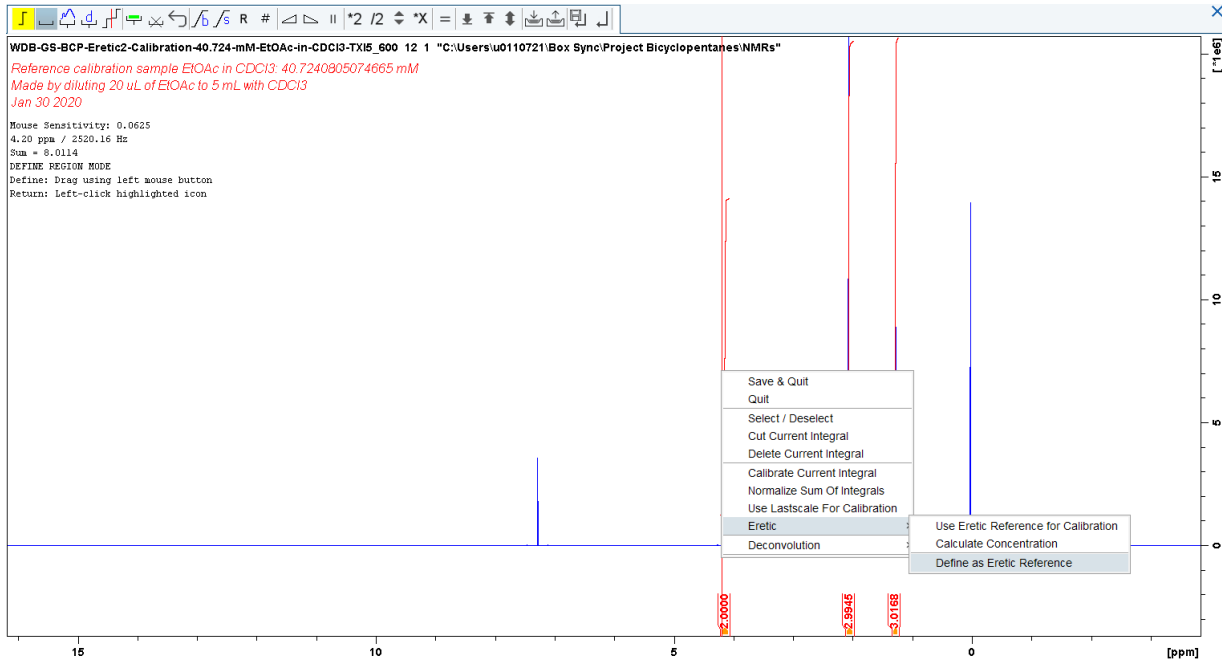
* Only necessary if you want to calculate the mass of compound in your sample.

Eretic2 – What do you need?

- Experiment **1D 1H QNMR (30)** (Bruker AV III HD 400)
- 1D sequence using regular pulse program zg30
- Tuning and matching (*atma*) with ‘exact’ parameter (may take long!)
- Performs fast pulse calibration before acquisition
- Only for
  - ^{1}H nuclei
  - Non-dilute samples
  - Samples with T_1 < 5-10 sec
- All other requirements for qNMR (D1, SINO, SW, O1P, …) must still be set by the user!
- Intended for use with Eretic2 method

Eretic2 – How to use it?

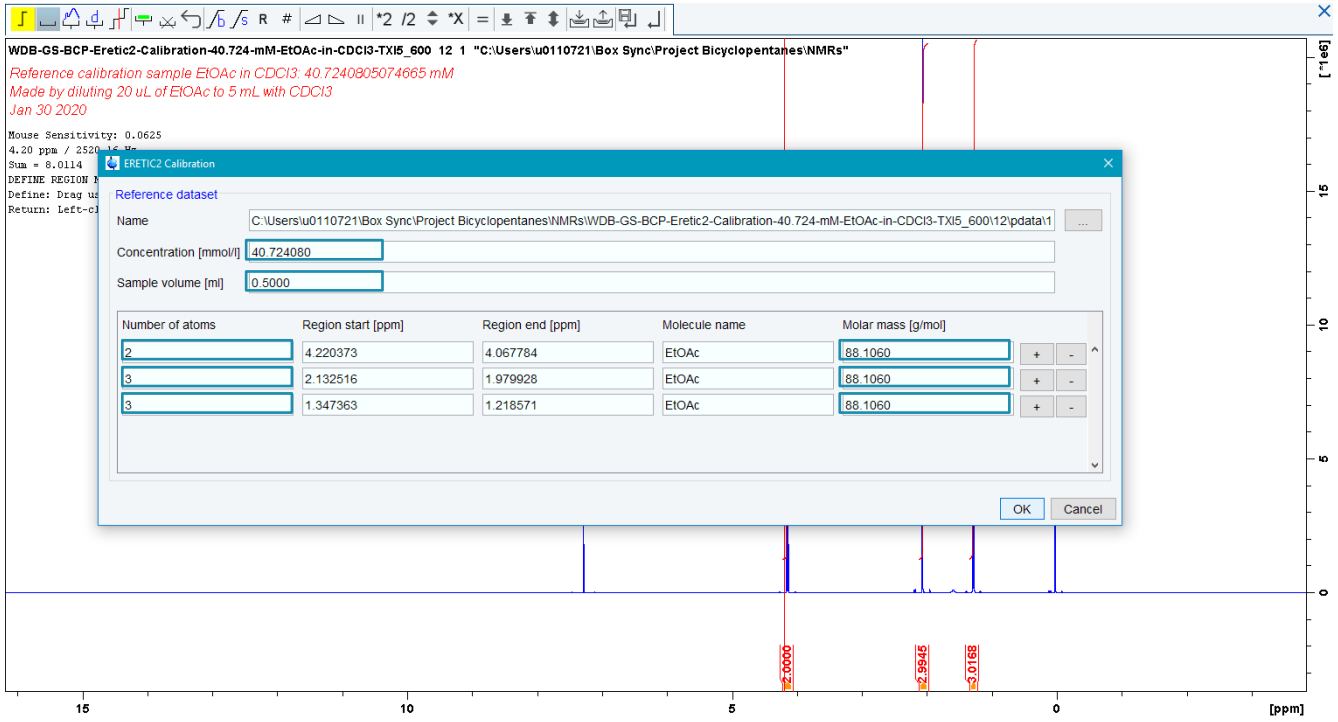
- For measuring reference sample with known concentration
  - Run experiment with **1D 1H QNMR (30)** under quantitative conditions
  - Process the spectrum
  - Integrate as many known peaks as possible (to lower standard deviation)
  - Right-click on the selected peaks and select Eretic → Define as Eretic Reference



@ Leuven Chem&Tech Liquid NMR Core Facilities

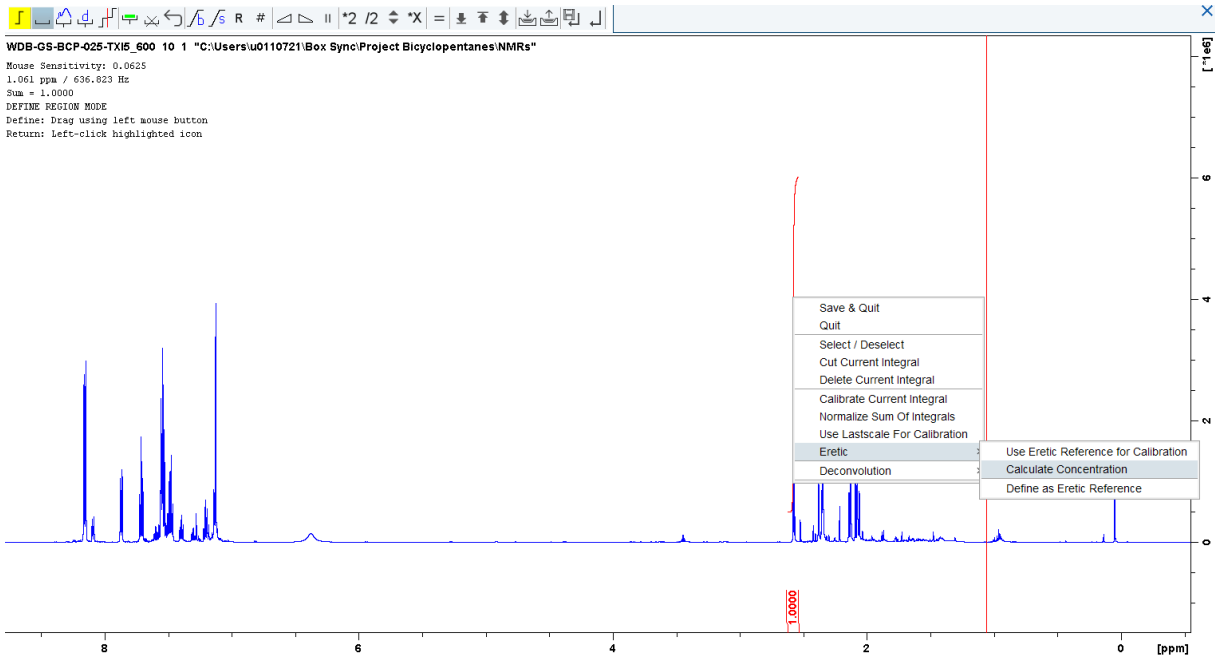
Eretic2 – How to use it?

- For measuring reference sample with known concentration
  - Fill in required sample information (concentration, Mw, sample volume)



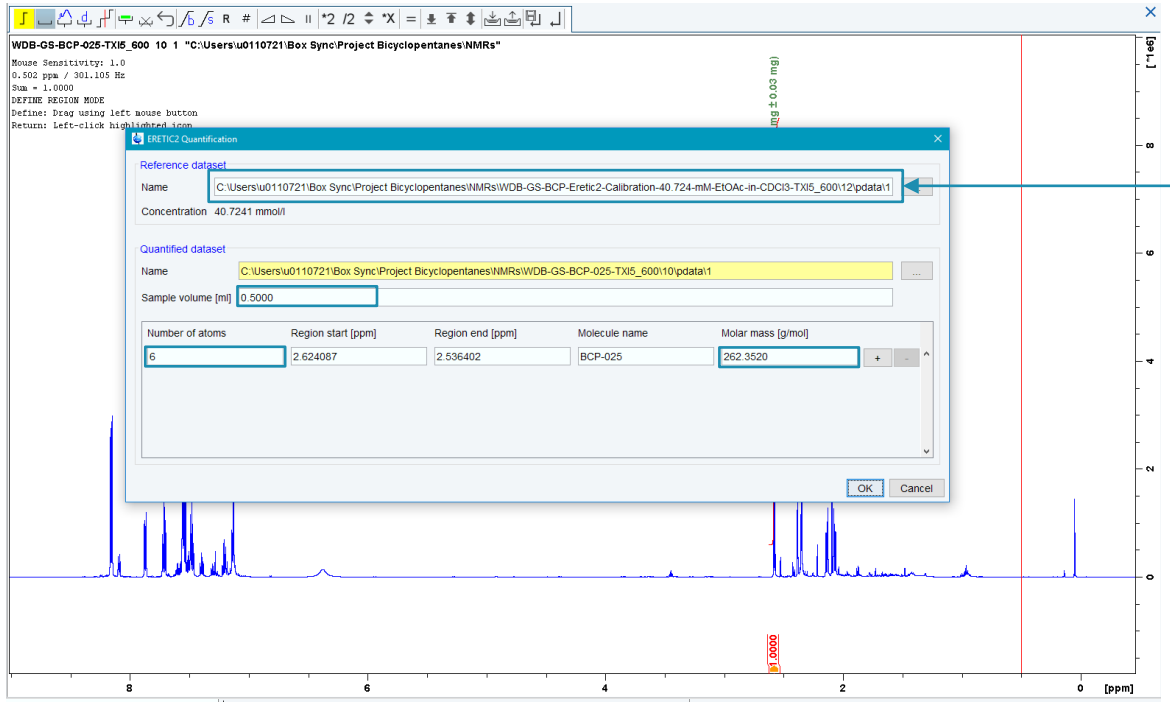
Eretic2 – How to use it?

- For measuring unknown sample
  - Use same(!) acquisition parameters as used for reference spectrum
  - Use same(!) processing parameters as used for reference spectrum
  - Integrate peaks from analyte
  - Right-click on the selected peaks and select Eretic → Calculate Concentration



Eretic2 – How to use it?

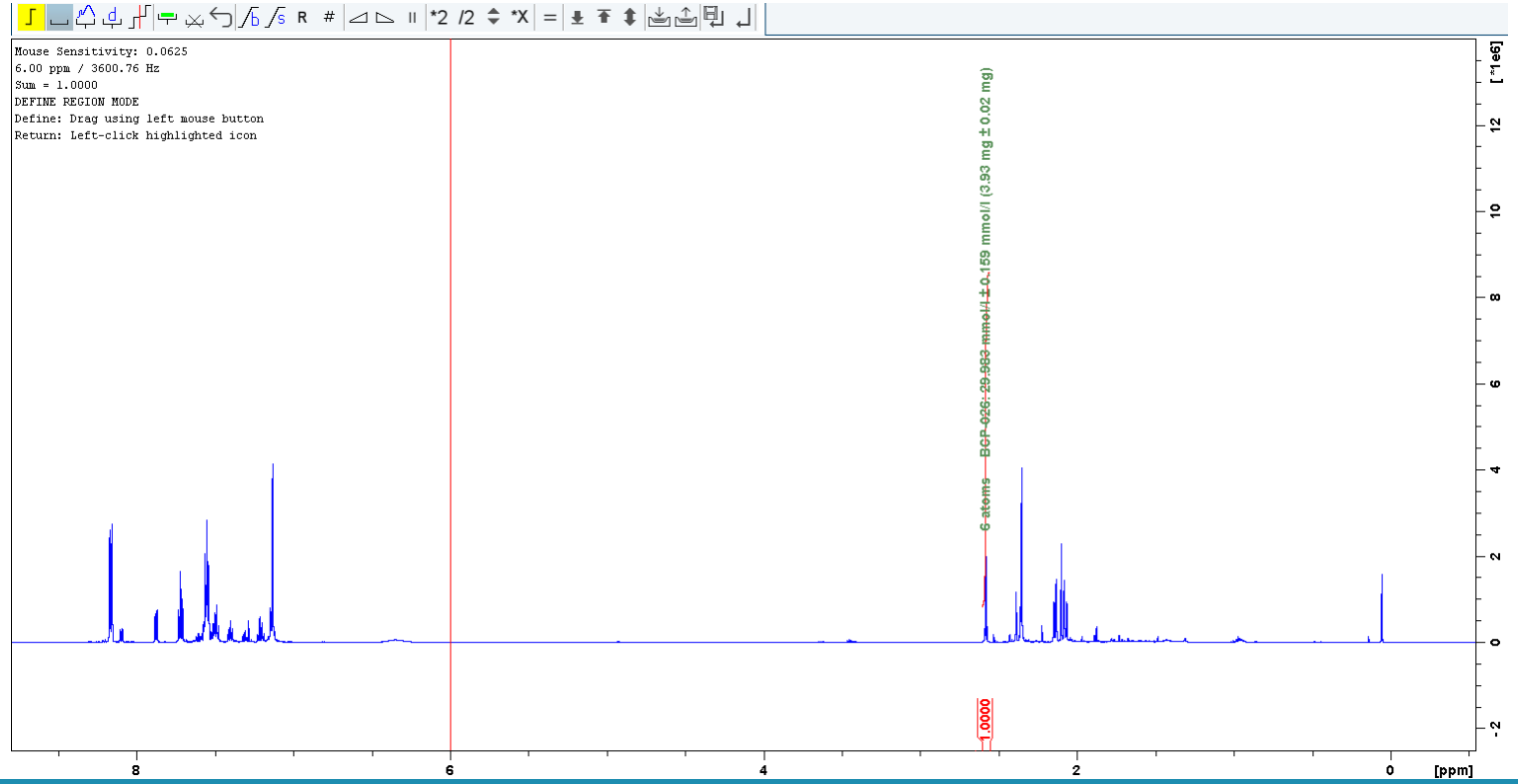
- For measuring unknown sample
  - Fill in required sample information (concentration, Mw, sample volume)



Eretic2 reference dataset
(created in previous slide)

Eretic2 – How to use it?

- For measuring unknown sample
  - Concentrations are displayed above the peaks



Eretic2 – General remarks

- Optimal results are obtained for reference and unknown samples in same solvent and similar concentrations
- Use calibration file from same probe and spectrometer
- Eretic2 reference should be reliable up to 60 °C difference between reference sample and unknown sample
- Eretic2 can also be used with internal reference
- Under normal and correct operational conditions, error should be within 5% (empirically on Bruker AV III HD 400)

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qNMR – The do’s and don’ts

Gert Steurs

February 10th 2022