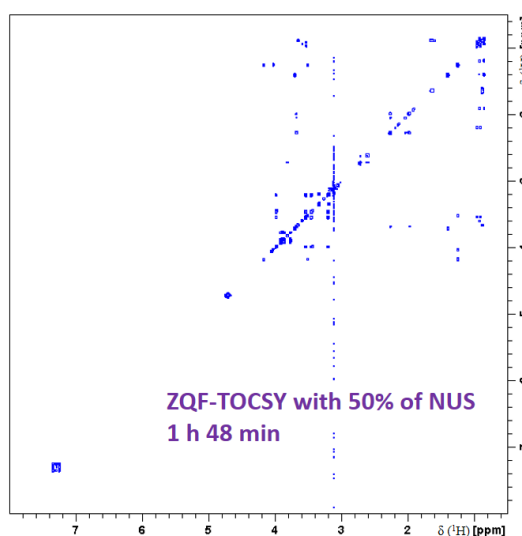
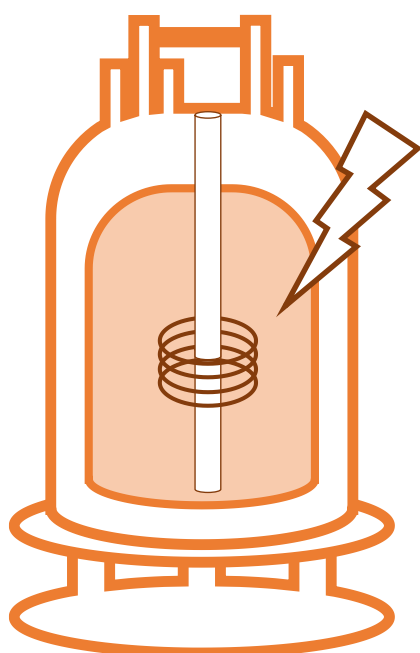


CEISAM, Nantes Université, CNRS

Implementation protocol for NUS ZQF-TOCSY



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1 Sequence implementation

This protocol presents the implementation of the Zero-Quantum Filtered TOtal Correlation Spectroscopy ZQF-TOCSY sequence on Bruker spectrometers (Avance III ulterior versions). This method is based on the following paper: Thrippleton and Keeler, *Angew. Chem. Int. Ed.* 2003, 42, 3938–3941 ([Elimination_of_ZQ_interference_in_2DNMR.pdf](#) dans le Nextcloud). Compared to the classical TOCSY pulse sequence, the ZQ filter makes possible the spectral phase adjustment, with cleaner baselines. The use of Non-Uniform Sampling (or NUS) is optional and allows splitting experiment time by two when 50% NUS is applied. Associated with calibration curves, this experiment allows quantitative results: for more information on quantitative 2D, please read P. Giraudeau, *Magn. Reson. Chem.*, 52, 259-272, (2014). This pulse sequence can also be applied as such for profiling approaches (J. Marchand et al. *Metabolomics*, 14, 60, (2018)). Please note that a version with ^{13}C decoupling exists in order to determine site-specific isotopic enrichment in ^{13}C enriched metabolites mixtures (S. Massou et al. *Metab. Eng.*, 9, 252-257, (2007)).

Acquisition and processing parameters were optimized and adapted in the frame of a previous study at CEISAM.

Before starting implementation, copy the attached files in the following folders:

- Pulse sequences:

[zTOCSY-em-3](#) and [zs_setup.2](#) in `\exp\stan\nmr\lists\pp\user`

On some configurations, use the [zTOCSY-JM-1](#) sequence (since it includes a line with the `acqt0` parameter, in order to prevent an error message)

If it is necessary to apply solvent pre-saturation without using a second channel, the [zTOCSY-JM-5](#) (includes `acqt0`) or [zTOCSY-JM5-2](#) (does not include `acqt0`)

- Adiabatic pulses:

[Crp20,50,10.1-2](#) and [Crp20,30,10.1-2](#) in `\exp\stan\nmr\lists\wave\user`

As a help for the following protocol, pre-filed tables are included with optimized parameters, associated with their name as used in the ZQF-TOCSY sequence.

1.1 Preliminary settings

Insert the NMR tube in the spectrometer (298 K), then wait for temperature stabilization (15 min).

Tune the probe for ^1H (F1). Lock, adjust shimming parameters.

The 2D pulse sequence includes an option for solvent pre-saturation, if necessary. Record a regular ^1H spectrum and determine the exact solvent frequency (SR must be set to 0 for this acquisition).

Then, create a 1D ^1H experiment with pre-saturation of the solvent signal: [zgpr](#), or [zgf2pr](#) which allows pre-saturation on the second channel (In the case where pre-saturation is not required, please ignore this part of the protocol).

For this:

- copy the ^1H standard acquisition created before;
- replace the pulse sequence with [zgpr](#) (ou [zgf2pr](#));
- set DS = 2, NS = 2 (minimum, otherwise water signal pre-saturation is not optimal with a single scan);
- adjust O1 value to the water peak frequency determined earlier (if using [zgpr](#), or O2 if using [zgf2pr](#));

- adjust pre-saturation power (“p19”, or “plw9” or “pldb9” depending on the version of topspin).

Optimized parameters for zTOCSY-em-3	
O2 =	... Hz
PI9 =	... [W] / ... [-dbW]

For all these experiments, set the gain (*rga*, then divide the gain by) before recording a spectrum. After acquisition of the 1D spectrum with solvent pre-saturation, check the quality of the shims on peak shape. If needed, adjust shims.

¹H Hard pulse calibration. Once the quality of the shims is validated, check the 90° hard pulse calibration on F1 (either using *popt* or through manual determination of the 360° angle). Note p1 et pl1 values.

P1 =	... μs
Pl1 =	... [W] / ... [-dbW]

Calibration of the ¹H low power pulses. The 2D sequence also includes low power pulses for the spin lock. These pulses must be carefully calibrated, aiming for a 90° angle with a pulse duration around 35 μs. Generally, this corresponds to a 10 dB attenuation compared to the hard pulse power. The attenuation value to be used must be calculated with a dedicated au program, accessible with the *calcpowlev* commands and the following parameters:

- “new pulse length” = 35 μs

- “reference pulse length” = value corresponding to the 90° hard pulse (ex 13,4 μs)

→ The au program then provides the attenuation to add to hard pulse power (ex : “change power level by 9.50 dB”, means that 9,5 dB must be added to hard pulses power).

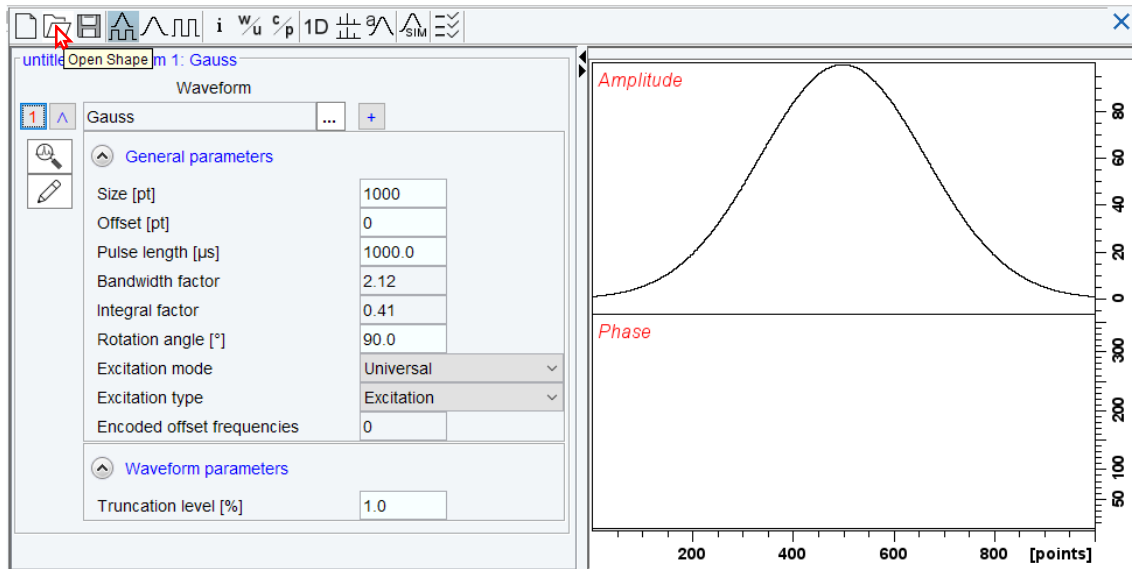
Type the calculated reduced power as pl1 value, then calibrate p1 in the same way as a hard pulse (aiming the 360° flip angle, either manually or through the *popt* command). The 360° flip angle should be around 140 μs. Caution, the low power pulse calibration should be carried out on a signal close to the middle of the desired spectral window, otherwise the calibration is hampered by off-resonance effects. Note the 90° flip angle value (around 35 μs) the corresponding power (that will be used for the ZQF-TOCSY spectrum acquisition).


P6 =	... μs
Pl10 =	... [W] / ... [-dbW]

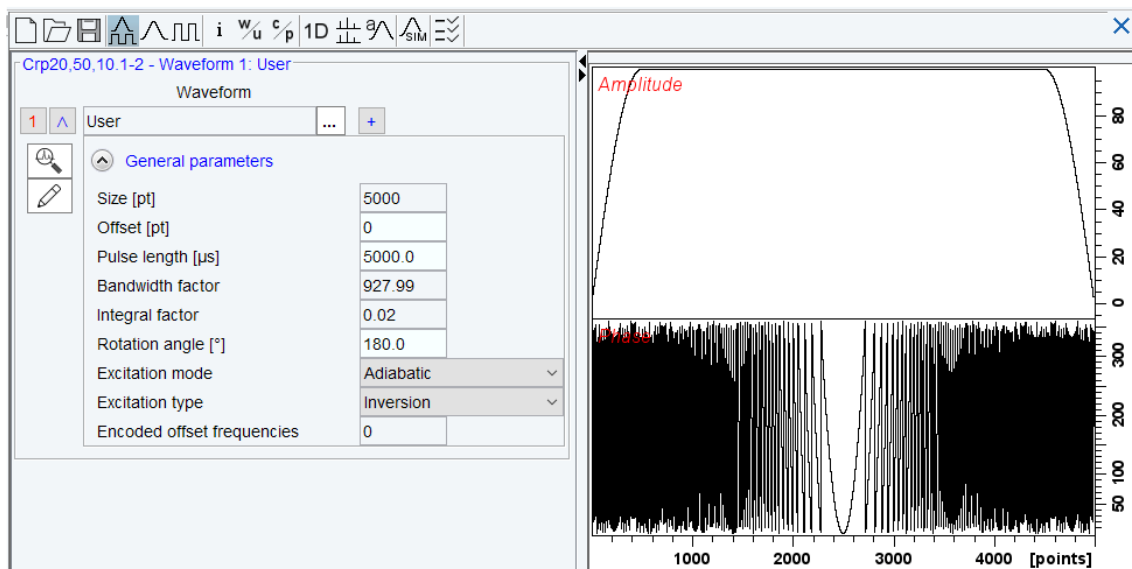
1.2 Calibration of the adiabatic pulses

The ZQF-TOCSY pulse sequence includes two adiabatic pulses (chirp pulse) which require preliminary calibration. These two chip pulses can be optimized thanks to the shapetool. The following steps must be followed for each of the pulses :

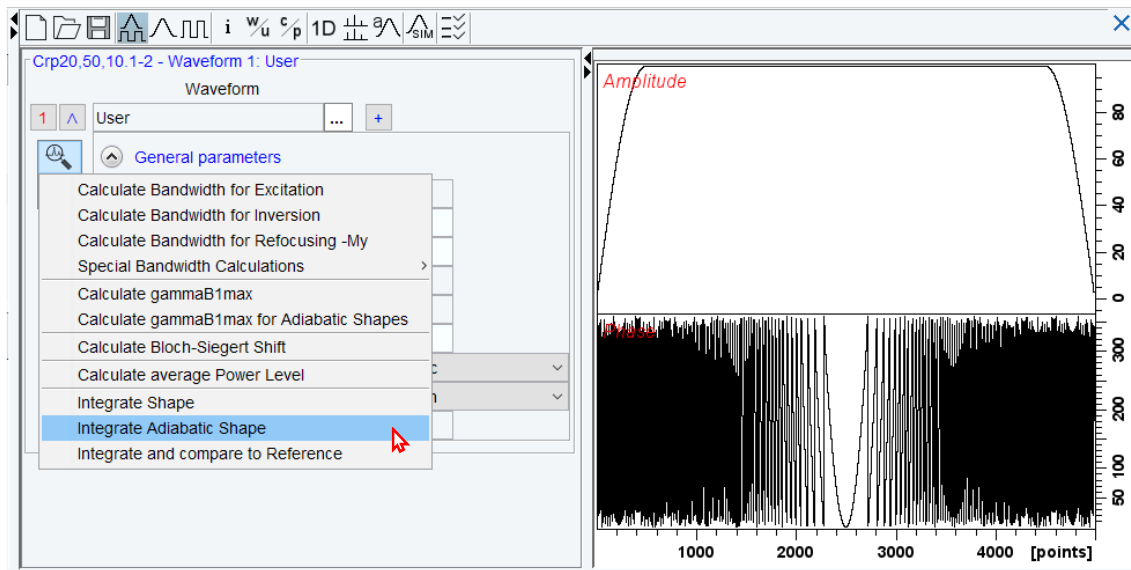
- type “*stdisp*” in the command line to open the shapetool: the following window appears



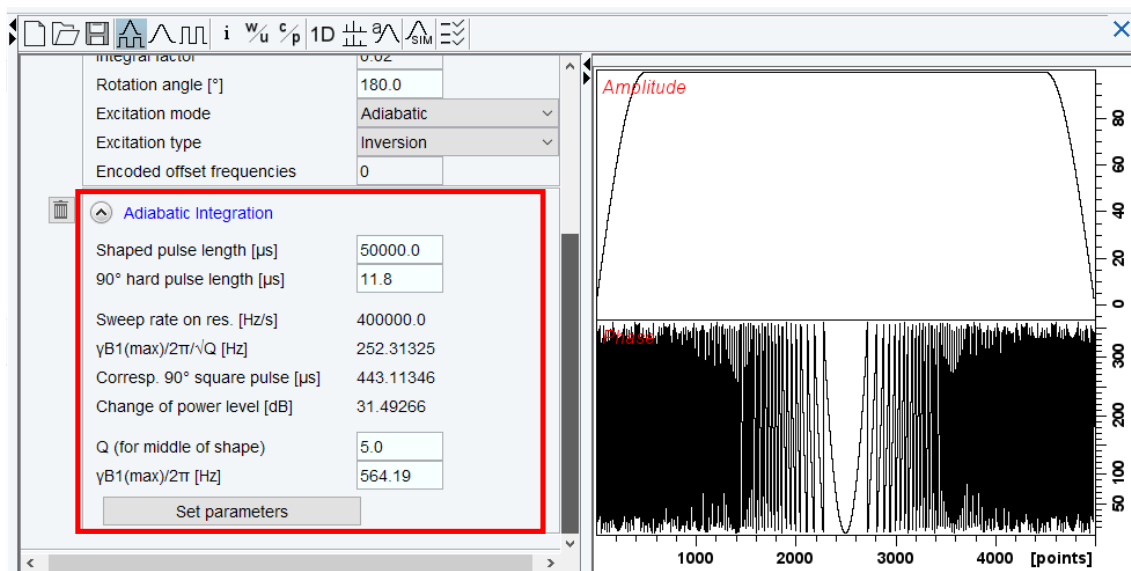
- then, click on "Open shape"  and the desired shape (for instance *Crp20,50,10.1-2*), which will appear on the following window:



- click on *“Analyze Waveform”*, then select *“Integrate Adiabatic Shape”*



The *“Adiabatic Integration”* then opens



- in this interface, replace the *“90° hard pulse length”* by the previously optimized value (see 1.1 Preliminary settings). Pulse duration must also be defined (the proposed attenuations depend on it):

“50 000 µs” for the 50 ms chirp pulse(*Crp20,50,10.1-2*)

and *“30 000 µs”* for the 30 ms chirp pulse (*Crp20,30,10.1-2*)

After clicking on *“Set parameters”*, the change of power is calculated (*“change of power level”*): it corresponds to the power level attenuation that must be applied to the hard pulse power, in order to obtain the power of the chirp pulse.

SPNAM1 =	<i>Crp20,50,10.1-2</i>
sp1 =	... [W] / ... [-dbW]

For example: if $p_1 = 11,7 \mu\text{s}$ for pl_1 (or $pldb_1$) = -4 dB, the software calculates a 31,57 dB power change for **Crp20,50,10.1-2**, hence the power of this pulse is sp_1 (ou $spdb_1$) = -4 + 31,57 = 27,57 dB.

- the same procedure must be applied for the second chirp pulse.

SPNAM2 =	Crp20,30,10.1-2
sp2 =	... [W] / ... [-dbW]

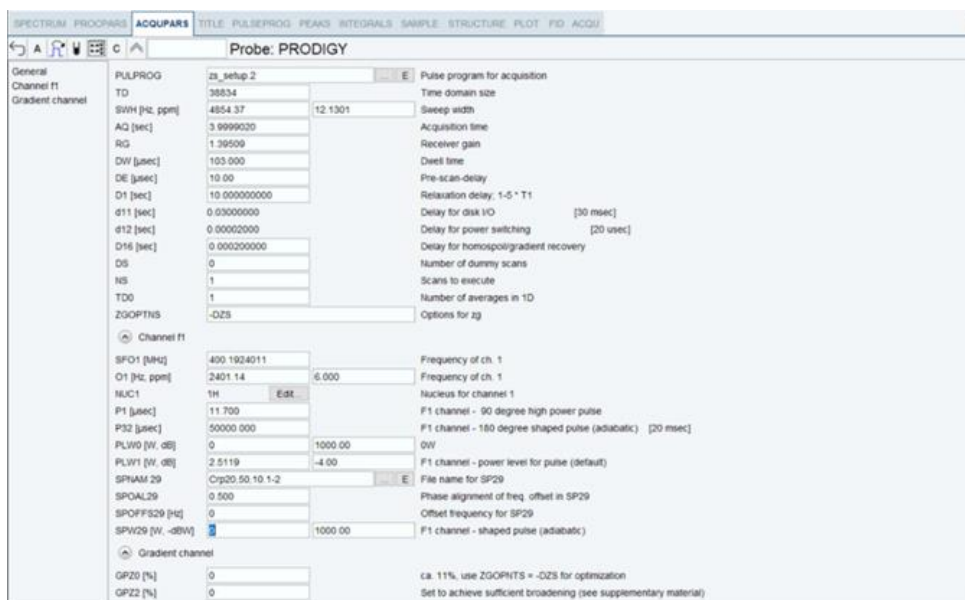
Note: Depending of the Topspin version used, the length of the chirp power is not necessarily indicated in the "pulse length" box \Rightarrow check this parameter before calculating the power change in dB.

1.3 Calibration of the gradient for ZQ suppression

The gradient amplitude used for ZQ suppression must be optimized using the **zs_setup.2** pulse program and the protocol described in the supplementary information of the Thrippleton and Keeler publication (**Elimination_of_ZQ_interference_in_2DNMR-SI**). This calibration must be performed on one of the two chirp pulses that were previously optimized: the same value of gradient strength will be obtained since they have identical bandwidths. In addition, if the optimization is achieved on different experiment files (using **edc** command), it is necessary to uncheck the "automatic processing" option in Topspin (in "Preferences/Processing Preferences"), since the same phase values must be kept for comparing the results from one experiment to the other.

In short:

- copy a standard ^1H 1D experiment;
- replace the pulse program with **zs_setup.2** and modify the following parameters:

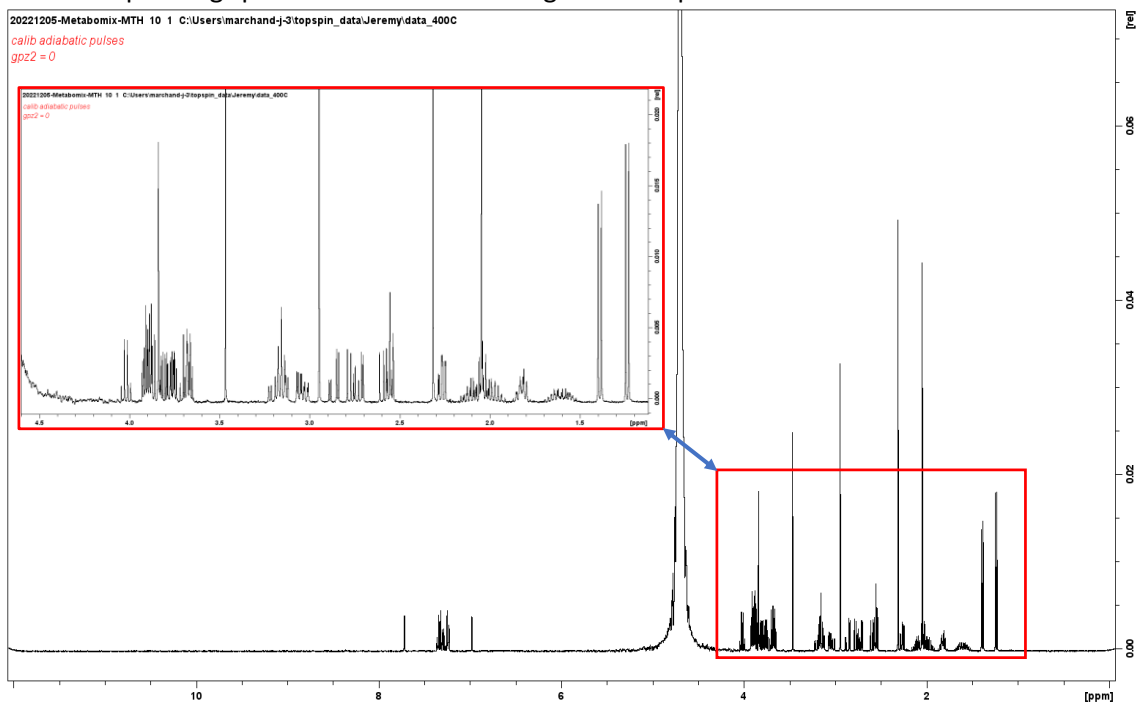


- ZGOPTNS = -DZS
- P32 = 50 000 μs (if la Crp20,50,10.1-2 is used)
- Spnam29: Crp20,50,10.1-2

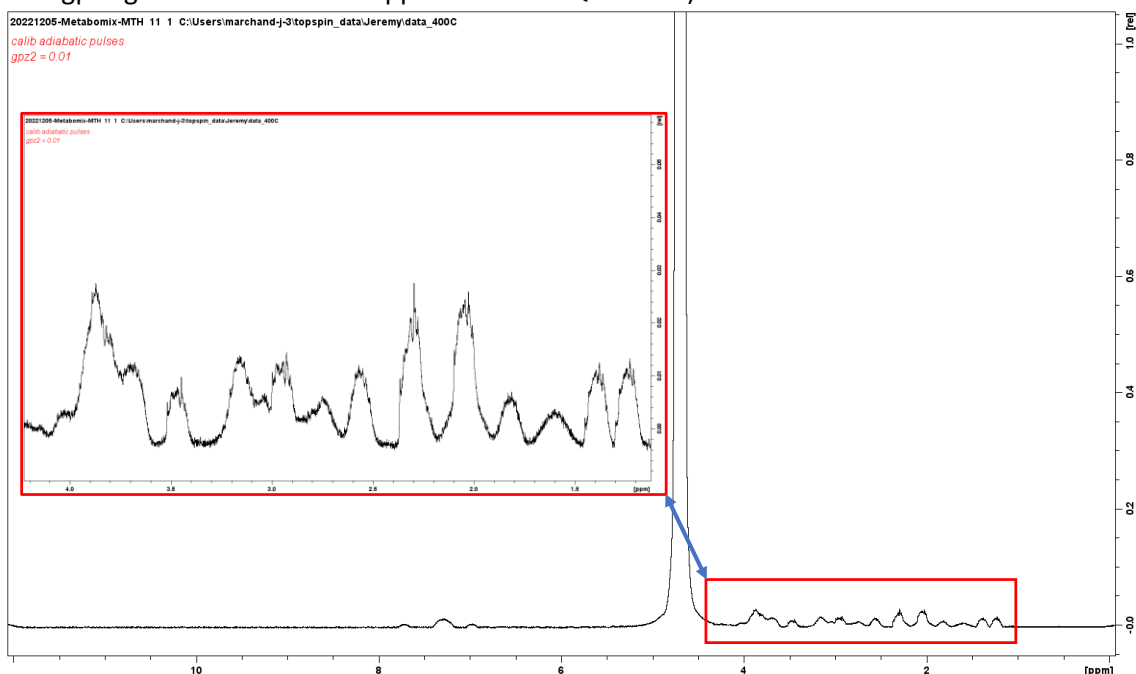
Start with sp_{29} (spw_{29}) = 0 W, gpz_0 = gpz_2 = 0%.

Note: The following spectra were obtained from a metabolite mixture at 10 mM, named mix-MTH » in the rest of the protocol.

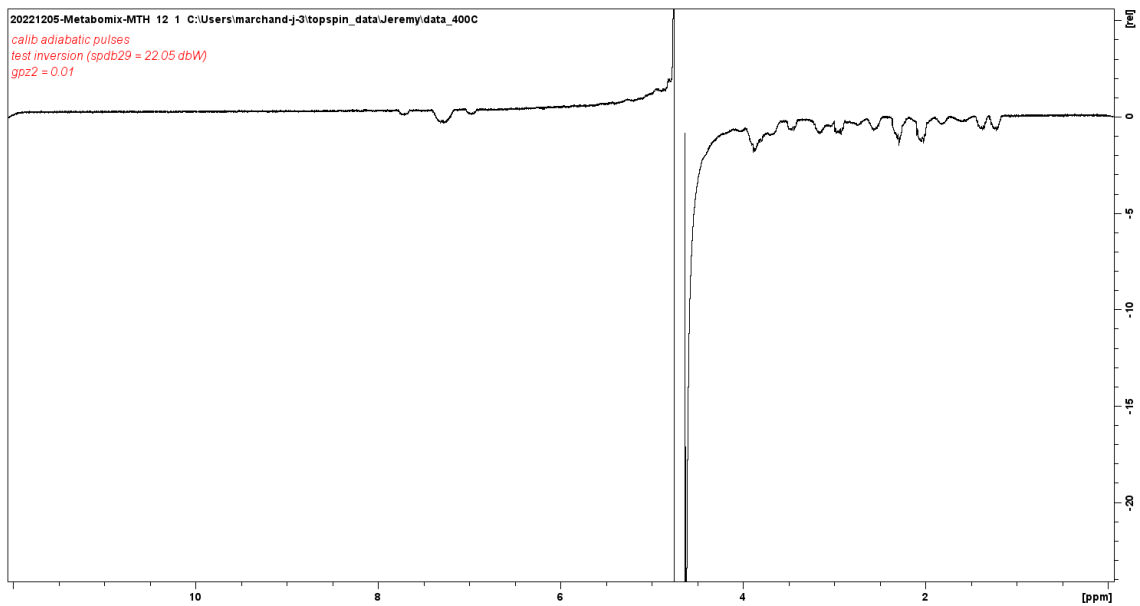
- record the corresponding spectrum. It looks like a regular 1D experiment:



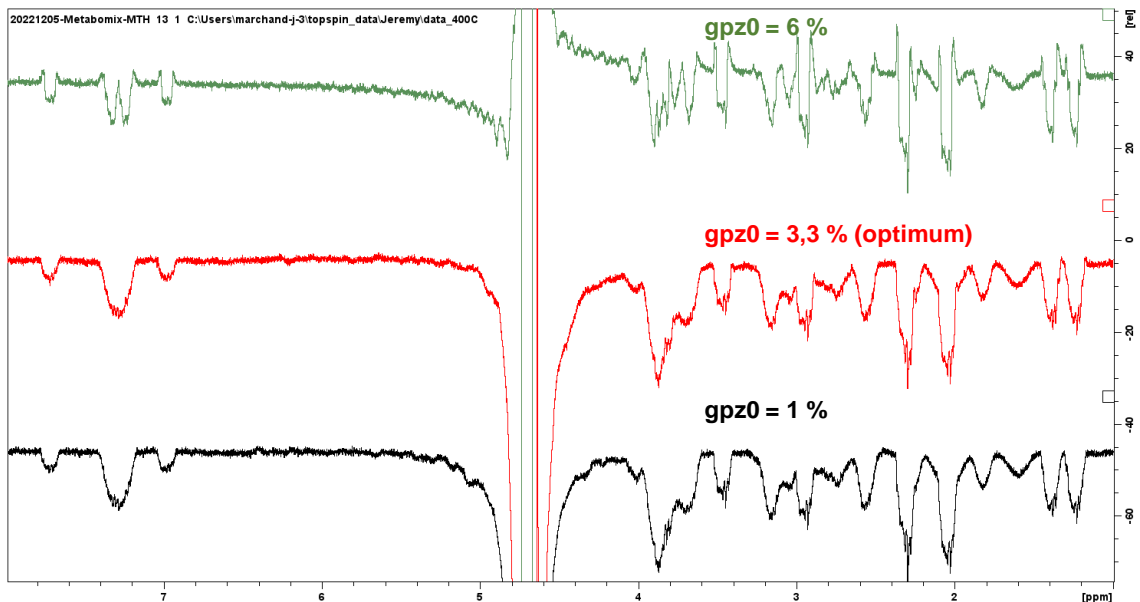
- then, adjust the gpz2 value until the peaks are enlarged in a significant way on the corresponding spectrum (i.e. the multiplets are not clearly visible anymore). Typically, the gpz2 is found between 0.01% and 0.05%. After optimization, keep this value constant for the rest of the study (note: the gpz2 value is only used in order to calibrate the gpz0 gradient that will be applied for the ZQF-TOCSY):

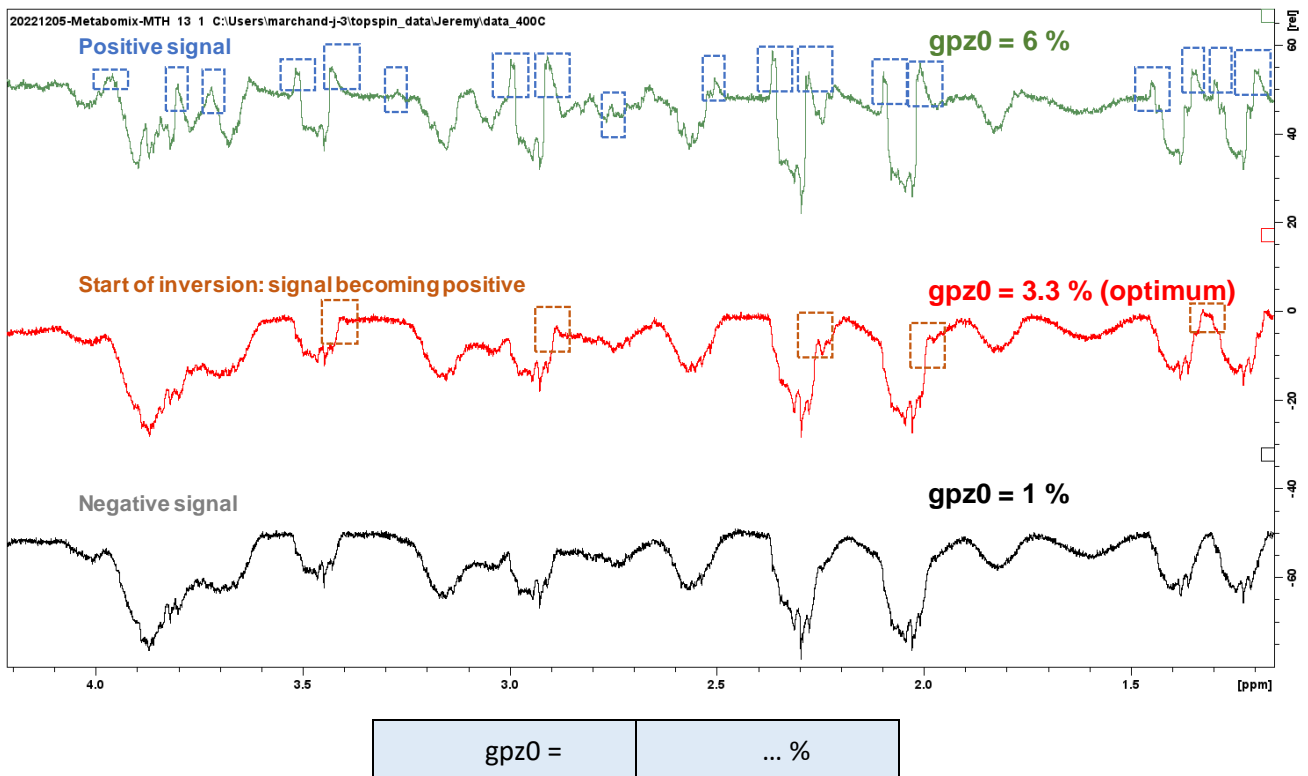


- set sp29 to the previously calibrated value ("sp1" calculated with the *shapetool* for the related chirp pulse) and launch an experiment: the peaks are inverted on the corresponding spectrum. **For this optimization and the following ones, it is important to keep the same phase values;** thus, uncheck the "Automatic Processing" parameter in Topspin.



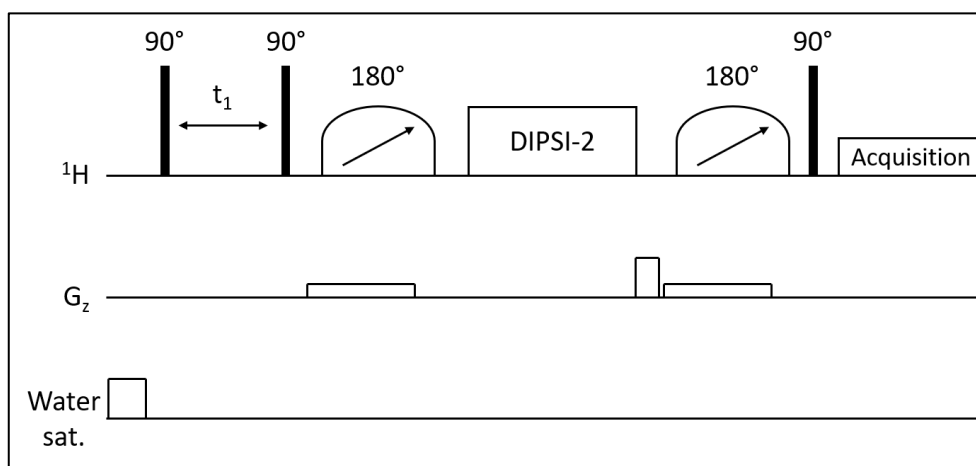
- then, adjust the gpz0 gradient value. Increase the value step by step and record a spectrum each time. The goal here is to increase gpz0 as much as possible while keeping completely inverted– for instance, carry out a first optimization from 0% to 6% by 1% steps in order to determine the moment of inversion, then perform a finer optimization, changing gpz0 by 0.1% steps. If the gradient becomes too strong, only the median part of the baseline is inverted (when the peaks start to become positive again, then it means that the value is too high). In practice, the gradient strength is increased until the moment where the edges of the baseline are affected (the typical values are usually found between **3 and 5%**). The figures below depict the start of the signal inversion (3.3% in this example), corresponding to the optimal value. Once the settings are optimized on a probe in particular, they can be used again without a new calibration.





1.4 Acquisition of the ZQF-TOCSY spectrum

Figure 1 shows the pulse sequence corresponding to the 2D ZQF-TOCSY experiment. The pulse program *zTOCSY-em-3* includes a pre-saturation module on the F2 channel. So as to allow the observation windows to be centered in the relevant part of the spectrum, this module was added on the second channel of the first one. Indeed, depending on the studied matrix, the central frequency of interest can be placed far away from the one to be pre-saturated. Hence, a reduction of the spectral window is possible, allowing a potentially reduced TD(F1), *i.e.* adjusted to the region of interest (thus avoiding wide empty peak regions) and a shorter experiment time, which can be an asset when analyzing a large number of samples.



ZQF-TOCSY pulse sequence

Copy the previously recorded 1D ^1H experiment (with or without pre-saturation of the solvent signal) and set the following acquisition parameters:

General acquisition parameters:

- PARMODE = 2D

Thanks to the *edasp* command, check the settings of the channels: if pre-saturation must be performed on a second channel, open the F2 channel (^1H). (Note: this step should be performed before setting the parameters associated with pre-saturation, otherwise they will be reinitialized when doing so).

- PULPROG = *zTOCSY-em-3*. Use one of the other sequences listed in the paragraph "4.3Need for acqt0" if the configuration of the spectrometer requires it, if the acqt0 is needed or if the pre-saturation must be performed on the same channel as the detection (See 4.2).

- SW = identical in both dimensions, value chosen in order to include all ^1H signals of interest (Default value is 12x12 ppm, but it is preferable to avoid using a window wider than necessary, in order to obtain the best compromise between experiment time and resolution in the indirect dimension)

- FnMODE = TPPI

- AQ(F2) = 1.5 s (in order to avoid truncation of the signal in F2)

- TD(F1) = 256 at least (more if time allows it and better resolution in F1 is necessary)

- TD(F2) = adjusted automatically when Q is set

- DS = 2

- NS = 2 (or a multiple of 2 if SNR needs to be increased)

- DIGMOD: baseopt

- O1 = central frequency of interest

- O2 = frequency of the solvent signal (if using pre-saturation on F2 channel – see above).

- d9 = 80 ms (spin-lock duration)

- d1 = 3.5 s (to obtain a TR equal to 5 s, a typical value for small molecules mixtures)

- DIGMOD : baseopt

Pulse powers and durations:

Adjust the power and the pulse durations with the previously calibrated values:

- p1 et pl1 for the 90° ^1H hard pulse

- p6 et pl10 for the 90° ^1H low-power pulse

- pl9 for pre-saturation signal of the solvent on F2 channel (set it to maximal attenuation value or minimal power if the pre-saturation is not necessary)

Parameters specific to the zero quantum filter:

Set the following parameters:

- GPZ0 = as calibrated in 1.3 paragraph
- GPZ5 = 50%
- p11 = 50 ms
- sp1 = as calibrated in 1.2 paragraph
- spnam1 = Crp20,50,10.1-2
- p12 = 30 ms
- sp2 = as calibrated in 1.2 paragraph
- spnam2 = Crp20,30,10.1-2
- d6 = 3 ms
- d7 = 200 μ s

NUS parameters:

If NUS is used, set the following parameters:


- FnType = non-uniform sampling
- NUSAmount[%] = 50 (smaller values are possible but can lead to the apparition of artefacts along the F1 dimension)
- NUST2[sec] = 1 in both dimensions

All the other parameters are adjusted automatically.

Once the acquisition parameters are set, just before launching acquisition, open the edasp (*edasp* command) and, without touching any parameters, save using the “save and close” button. This allows the parameters (O1, O2...) to be correctly set and prevent the acquisition of spectra with an offset of the chemical shift along the F1 dimension when using the two-channel sequence. For more details, please read section 3.

Start the experiment:

Once the parameters above have been adjusted, set the gain of the receptor and start the acquisition. For experiments including NUS, the direct use of the *rga* command is not recommended. Instead, use the RG value optimized on the 1D version of the 2D experiment: the ZQF-TOCSY experiment should be copied entirely, then in the new file, click on the following icon, in order to change the dimensions of the experiment from 2D to 1D

 , the *rga* can thus be applied. After calculation of the gain on this experiment, the obtained RG value can be divided by 2 and transferred in the original 2D experiment.

Note: The gain value should be optimized on the most concentrated sample. The number of scans must be adapted depending on the concentration of the sample (the most diluted sample will be the limiting one) and the configuration of the hardware.

Once the parameters are correctly configured for a defined matrix, they can be saved in a parameter file to be used later on (`wpar commnd`).

1.5 Processing

Standard parameters are described below and recalled in a table at the end of this section.

- $SI(F2) \geq 2 * TD(F2)$ and multiple of 2^n
- $SI(F1) \geq 4 * TD(F1)$ and multiple of 2^n (if linear prediction is used). $SI = 1024$ if $TD(F1) = 256$
- $WDW(F2) = EM$
- $LB(F2) = 0.3$ Hz
- $WDW(F1) = QSINE$ (if truncation artefacts are too visible, then EM apodisation can be used instead with a high LB value)
- $SSB(F1) = 2$
- $PHMOD = pk$ in both dimensions
- $ABSG \leq 3$ in both dimensions. Advised value: $ABSG = 3$.
- $ABSF1 =$ Highest frequency in both dimensions of the spectrum. fréquence la plus élevée dans les deux dimensions sur le spectre. Advised value = 1000 ppm (the upper limit will then be used by default)
- $ABSF2 =$ lowest frequency in both dimensions of the spectrum. Advised value = -1000 ppm (the lower limit will then be used by default)
- $MC2 = TPPI$
- $Mdd_mod = cs$ (compressed sensing algorithm for the processing of NUS data – the most relevant algorithm for processing 2D NMR experiments is the CS/IRLS, which requires the purchase of a license. The CS/IST is freely available on recent versions of Topspin)

For the spectra generated with 2D, it is advised to perform “forward” linear prediction (LPfr / LPfc) in the indirect dimension (F1), in order to compensate the truncation of the FID, which is generally significant:

- ME_MOD : “LPfr” in F1 dimension
- $TDeff$: this box can be left empty and the parameter will be calculated automatically.
- $LPBIN = 0$.

This parameter corresponds to the number of points that will be calculated at the end of the FID by linear prediction. This are final FID points but different from the Si point since Si points are equal to 0 as opposed to linear prediction points. When $LPBIN = 0$, Topspin automatically calculate a value equal to TD, hence the initial number of point (TD) will be doubled.

Warning: in the case where linear prediction is used in the direct dimension (F2), if the AQ (hence, TD) is high, doubling the number of points can lower the S/N: Indeed, if too many points are added, this is equivalent to adding noise.

- $NCOEFF (F1)$: 32.

Typically 2-3 times the number of expected points should be used (number of signals in a column when linear prediction is performed in the indirect dimension).

Summary of the processing parameters used by the MetaboHUB working group:

	F2	F1
SI	65536 (TD=28844)	1024 (TD = 256)
WDW	EM	QSINE
LB	0,3	
SSB		2
ABSG	3	3
ABSF1	1000	1000
ABSF2	-1000	-1000
PHMOD	pk	pk
BCMOD	quad	no
MC2		TPPI
Mdd_mod	cs	
Mdd_CsVE	TRUE	
Mdd_Csalg	IRLS	
ME_mod		LPfr
NCOEF		32

Recall the first FID with *rser 1* command. Type *efp*, then adjust the phase as 1D spectrum and click on the “save as 2D” icon before leaving the phase correction interface.

Close the window in order to come back on the 2D spectrum.

Type *xfb*.

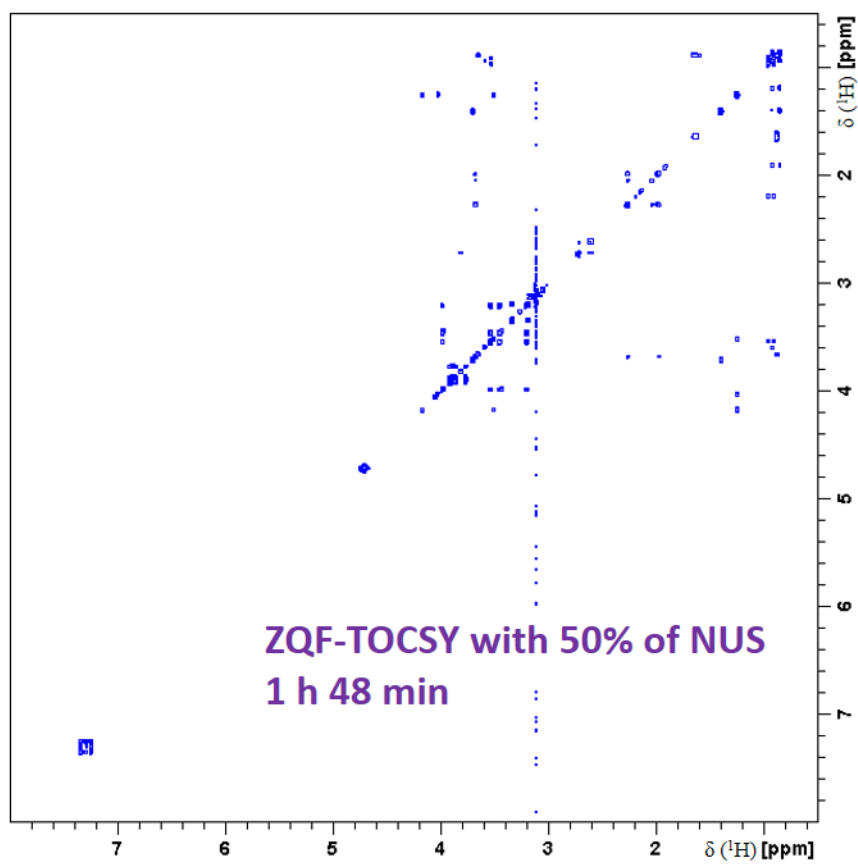
If the data were acquired with NUS, type *xht2* (a Hilbert transformation in F2 recreates the imaginary data for phasing).

Use the 2D phase correction tool in order to adjust the phase on rows and columns (the phase should be close to the optimum in the F2 dimension since it was optimized on the first row).

For optimal results, type *xfb* again. This will minimize reconstruction artefacts and improve the overall quality of the spectrum.

Apply baseline correction with *abs2* then *abs1* commands.

A typical 2D spectrum from a metabolite mixture is depicted on the following figure:



2 Parameters to be optimized for a new sample

After implementation, the following parameters must be adjusted for each new matrix or set of samples:

2.1 General parameters

- Parameters to be adjusted in routine

For each new matrix or set of samples, the following parameters must be systematically adjusted, as usual for metabolomic experiments:

P1, p11 ; P6, pl10 ; O1 ; SW ; O2, pl9

- D1 / TR delay

This can be adapted depending on the needs, matrix and instrumental time availability.

In order to optimize the experiment time, it is advised to keep a low D1, typically 3,5 s (equivalent to TR = 5 s when the AQ is set to 1.5 s). As opposed to a classical quantitative 1D NMR experiment, there is no need to use a repetition time (TR = D1 + AQ) greater than $5 \cdot T1_{\max}$. Although this creates partial saturation conditions, this is acceptable for 2D metabolomics profiling. In the case of absolute quantification, this is also acceptable when standard addition or a calibration curve is used, on the condition that the concentrations of the calibration points are close to the target.

In the particular case where ZQF-TOCSY sequence is used to assess isotopic enrichments with a ^{13}C decoupling on F2, then TR > $5 \times T1_{\max}$ must be chosen (the ^1H linked to ^{12}C are characterized by T1 values that are different from the ^1H linked to ^{13}C), in order to keep quantitative experimental conditions (TR around 30 s). A dedicated sequence should then be used (including ^{13}C decoupling).

- AQ: Acquisition time

Can be adapted depending on the needs, matrix and instrumental time availability.

- NS

In a classical way, this parameter can be changed depending on the needs in terms of sensitivity.

2.2 Parameters associated with 2D and NUS

- Adiabatic pulses

Sp1 and sp2: depending on sample type and when others parameters are changed (such as the 90° hard pulse P1), the power of these pulses should be adapted.

- Gradients

The gpz0 is theoretically associated with a type of probe and it is not necessary to adjust it for each new matrix or sample group. Ideally, this should be double-checked.

- TD (F1)

This parameter can be adapted, if the SW value is changed and if there is a need to adapt resolution or experiment time. Processing parameters should then be adjusted accordingly.

- Percentage of NUS

It is recommended to avoid using a rate of NUS that is too high (*i.e.* decreasing % NUS), in order to prevent reconstruction artefacts on the final spectrum.

- NUS-list – Usage of the "*calculate*" function

Ideally, the *calculate* function from Topspin must be used for each new spectrum involving a change of parameter or a new type of sample. It should be noted that when certain parameters are modified (TD F1 for instance), Topspin automatically performs a calculation of the points to be acquired.

Warning: However, for samples from a single study (cohort for instance), this function should **not** be used, in order to make sure that the NUS-list stays the same for each acquisition *i.e.* the acquisition of the same points, thus ensuring optimal comparability of the samples. Alternatively, the NUS-list can be manually set, to make sure the same list is used across the study (see below).

- NUS-list – manual insertion for ensuring comparability of the spectra (*e.g.* in the case of a multi-sample study)

In order to ensure that the same experimental points are acquired from one experiment to another, it is possible to manually set the NUS-list that will be used. For this, a reference experiment must be created first (experiment number 320 in the following example) where the NUS-list is calculated automatically (*calculate* function) after all other parameters of the experiments have been set. Then, the "*nuslist*" file can be retrieved in the files of the associated experiment (typically, under Windows environment: "*C:\Users\marchand-j-3\topspin_data\Jeremy\data_400C\20221221-Metabomix-MTH\320*"); it should be copied and pasted in the Topspin lists, that can be found in the relevant directory (typically "*C:\Bruker\TopSpin4.1.4\exp\stan\nmr\lists\vc*") with a new recognizable file name (such as "*CohortMTH_Feb_2023*"). Then, for each experiment of the study, this reference NUS-list can be recalled directly, by entering its name in the topspin acquisition parameters (in this case "*NUSLIST = CohorteMTH_Fev_2023*")

3 Miscellaneous warnings

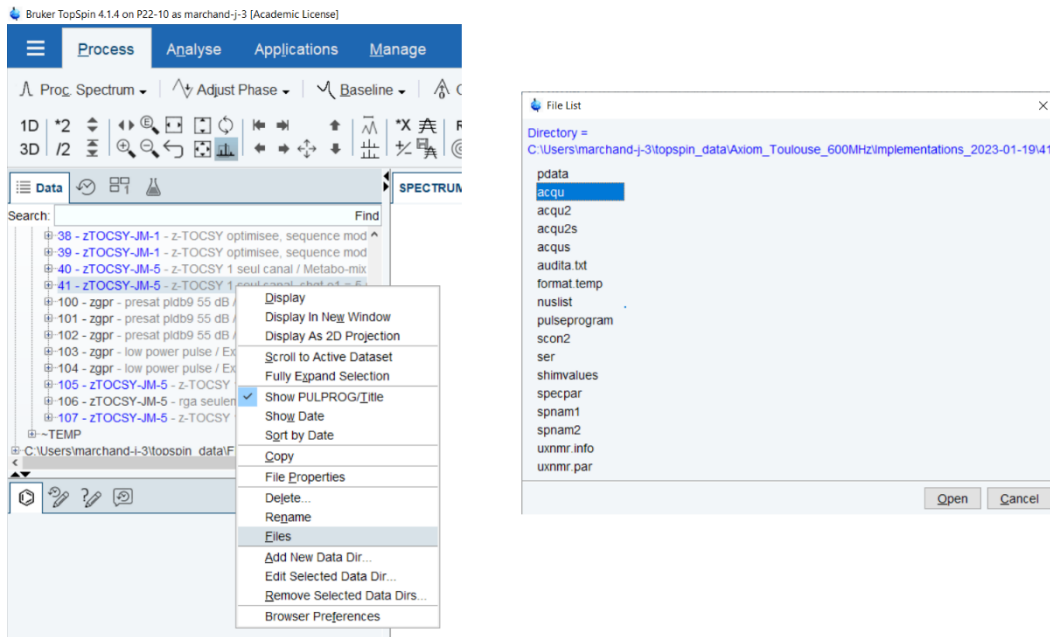
3.1 Potentials issues

3.1.1 Offset of the spectra along the indirect dimension/settings of O1 and O2

It is advised to be careful with the O1 and O2 parameters that are used. In Topspin, the desired values might not be actually taken into account by the experiment, thus generating spectra with an offset along the indirect dimension (in most cases, the O1 value in F1 used by the experiment is different from what was originally set, thus creating this offset). Although the exact origin of this issue is still unknown (which appears to be software-related), several solutions were found to prevent it or correct it.

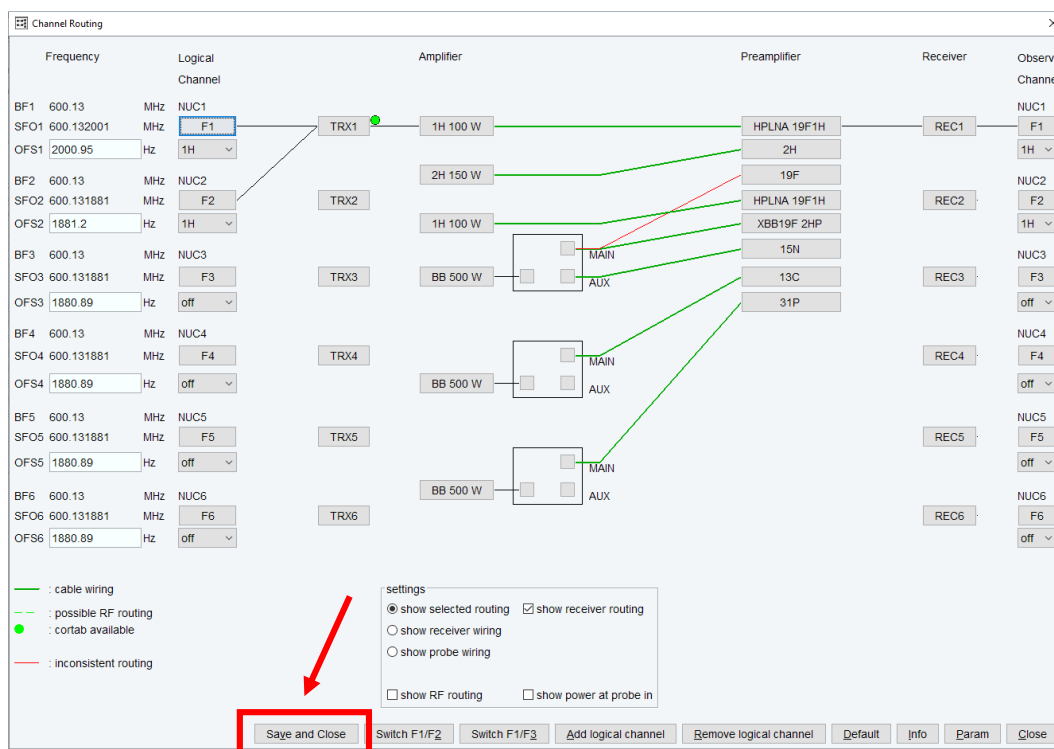
- Ensuring that the desired values are used for O1 and O2:

To achieve this, after setting all parameters of the experiment, it is advised to directly verify inside the experiment files, and not just the Topspin interface, which might be deceiving. This verification should be carried out when programming the experiment. This can be done by opening the *acq* and *acq2* files which can be found in the file explorer of the computer (for instance, for the files associated with the experiment n° "41", they can be found inside the following folder: *\topspin_data\Utilisateur\20230213_zqfTOCSY_MTH\41*) or, alternatively through Topspin, by right-clicking on the relevant ZQF-TOCSY experiment, then clicking on "files" and selecting "acq" and "acq2" (see screenshots below). If the parameters match the ones that are desired, the spectrum offset will not appear.



- Prevent the issue by ensuring the right configuration of O1 and O2:

To ensure that the O1 and O2 are correctly updated in the *acqu* and *acqu2* acquisition parameters, the *edasp* should just be opened (*edasp* command) then, without changing anything in the window, save through the “*save and close*” button (see screenshot below). The correct values can then be checked in the acquisition parameters files (*acqu* and *acqu2*).



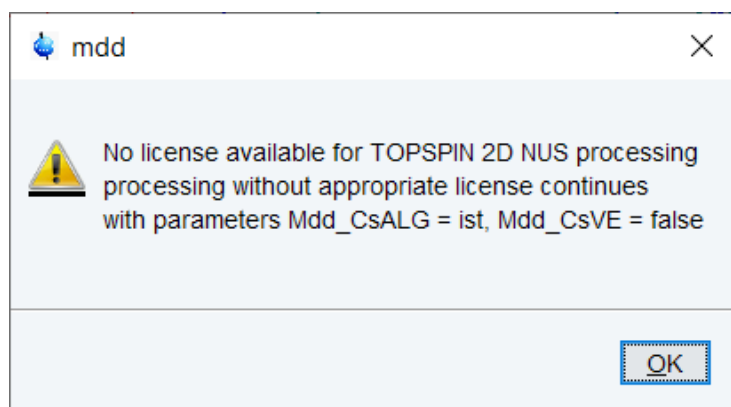
- Correct a spectrum with an offset

A dedicated script called *corr_hscale.py* can be used in order to correct the offset (script to be saved in “C:\Bruker\TopSpin4.1.4\exp\stan\nmr\py\user”): Once the spectrum is fully processed (x_{fb}, x_{ht2}, phasing, baseline correction), type the “*edpy*” command, then select the *corr_hscale.py* script and click on “execute”. The

spectrum can then be processed again (*xfb* command). However, it should be noted that this script only corrects the offset for an existing spectrum but does not correct the original acquisition issue.

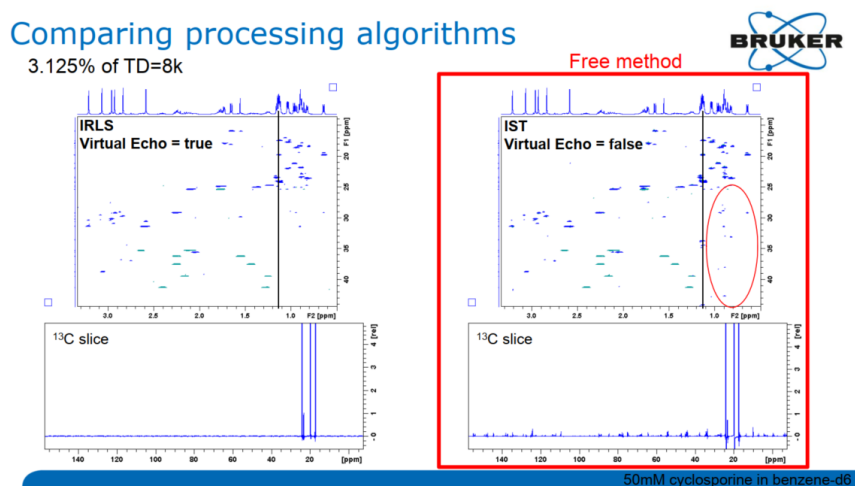
3.1.2 Missing NUS processing license (*cs*)

There are two versions of the *cs* spectrum reconstruction algorithm: a free version named IST (available from Topspin 3.5) and another one requiring a license named IRLS. If the latter is not available when the *cs* parameter has been set in the *Mdd_mod* parameter, the following error message will appear:



The free version of *cs* (IST) will then be used by default.

According to Bruker (*“Getting the most of your NMR” presentation, Eric Johnson, Bruker User Meeting, PANIC, 2018*), for a high percentage of NUS (e.g. 50%), the difference on the spectrum is negligible between the two algorithms. However, for low percentages (e.g. 3%) where reconstruction is more challenging, differences might appear:



3.2 Change of parameters

- SW

In order to make sure that a squared spectrum is obtained, check that the same SW values (in Hz) are used in the F1 and F2 dimensions.

- Pre-saturation

In the case where the pre-saturation is used, it is advised not to use an excessive D1 value in order to avoid damage for the probe or heating of the sample. Moreover, the power used for p19 should be cautiously set. The latter should be adjusted to the intensity of the solvent signal. If a longer RT is necessary (*i.e.* a longer D1), a dedicated sequence (*zTOCSY-em-3_D1*) can be used, where D1 is split in two parts: One part including pre-saturation (parameter defined as “D1”) and one part without pre-saturation (new parameter defined as “D2”, corresponding to a new relaxation delay = D1 + D2).

- Phasing when NUS is carried out

For optimal results with NUS, a first adjustment of the phase should be performed using the *rser 1* command **before** generating the spectrum with the *xfb* command since the phasing will directly influence the quality of the spectrum reconstruction. It is also possible to generate the spectrum (*xfb*, then *xht2*), perform a first phase adjustment, then generate the spectrum again and readjust the phasing.

3.3 Use of automation/getprosol

By default, the use of the *getprosol* command **will change the chirp pulses that are necessary for the sequence** (sp1 and sp2 associated with P11 and P12 respectively) with different pulses (gaussian pulses). Therefore, it is **strongly advised not to use *getprosol***. If this command is used nonetheless (*e.g.* by the automation routine), multiple solutions can be employed:

- After *getprosol*, manually check the parameters or use a parameter file thanks to *rpar* command
- In the case of a systematic use, a different and adapted edprosol file can be of interest:
 - For instance, modify the *default* file in
`C:\Bruker\TopSpin4.1.4\exp\stan\nmr\lists\prosol\pulseassign`
It is then advised to assign the chirps in P11 and P12 to unused lines (P47 and P48 for instance) and then modify the pulse sequence accordingly (using P47 and P48)
 - Recall a different file (with the chirp pulse correctly assigned), instead of *default* (for instance *default_modify-TOCSY*) and modify the sequence so that it will use the new modified file. **This procedure is not recommended**, because in the case where the *default* file is modified (after maintenance for instance), the other file needs to be updated in the same way, to ensure that the correct values (hard pulse power for instance) are always used.

Similarly, when using the “*edc*” command in order to create a new ZQF-TOCSY experiment, always make sure that the boxes for the “**read parameters**” and “**getprosol**” options are unticked.

4 Specificities on certain configurations

4.1 Field strength, magnet

Depending on the magnetic field strength or the probe (for instance), sensitivity parameters should be adjusted (*e.g.* NS).

4.2 Pulse sequence to be used: two channel sequence versus one channel sequence

The ZQF-TOCSY pulse sequence described in this protocol makes use of two channels, channel F2 being dedicated for solvent pre-saturation. **It is advised to use the latter** (*zTOCSY-em-3*, or *zTOCSY-JM-1* if acqt0 is necessary). However, depending on the needs, a pulse sequence using only one channel, for both acquisition and pre-saturation could be used (“*z-TOCSY-JM-5-2*” or “*z-TOCSY-JM-5*” if acqt0 is necessary), even though it should be

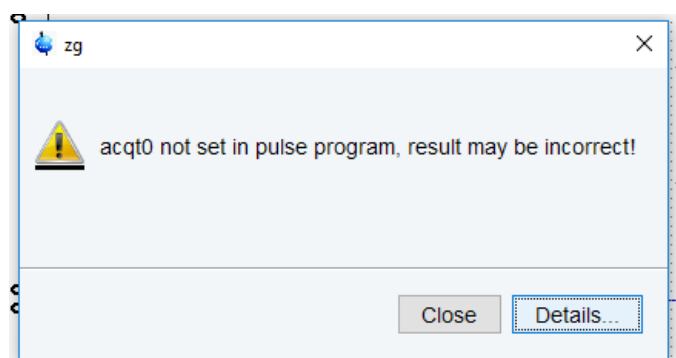
noted that these sequences have not been fully put to the test yet. With this version, the pre-saturation is performed thanks to an offset of the O1 frequency during D1 so that it matches the solvent frequency to be pre-saturated. Hence, the solvent pre-saturation frequency to be used can be set (in Hz) as the “*cnst2*” parameter (instead of O2 for the two-channel pulse sequence) and can be different from the carrier frequency O1.

This one-channel version can be of interest in particular when the channel F2 is required for a different use (*e.g.* for ¹³C decoupling).

However, it should be noted that, as opposed to the two-channel pulse sequence, this version of the ZQF-TOCSY has not been validated for a global metabolomic study yet. Hence, this should be done before use on an actual set of samples.

4.3 Need for acqt0

On certain configurations, the *zTOCSY-JM-1* sequence should be used instead of *zTOCSY-EM-3*. Indeed, in the latter, the acqt0 parameter is not included although it might be expected by the instrument when *baseopt* is used. When missing, the following error message then appears (the experiment can be run nonetheless) when DIGMOD is set to *baseopt*:



In *zTOCSY-JM-1*, a line was added to correct this error: "acqt0=-p1*2/3.1416".

This is the only change compared to the original pulse sequence (z-TOCSY-EM-3).

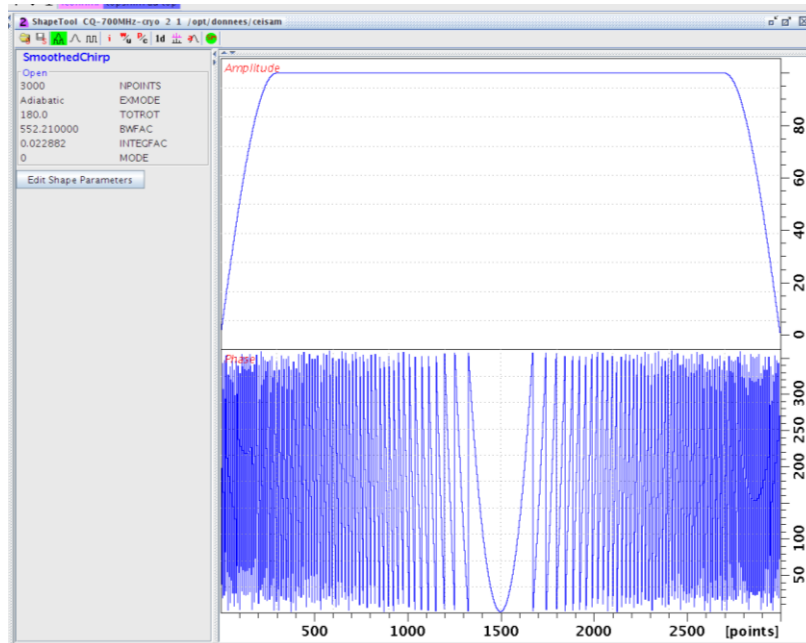
For the **one-channel sequence**, “*z-TOCSY-JM-5*” should be used when acqt0 is required.

4.4 Version of the Topspin software

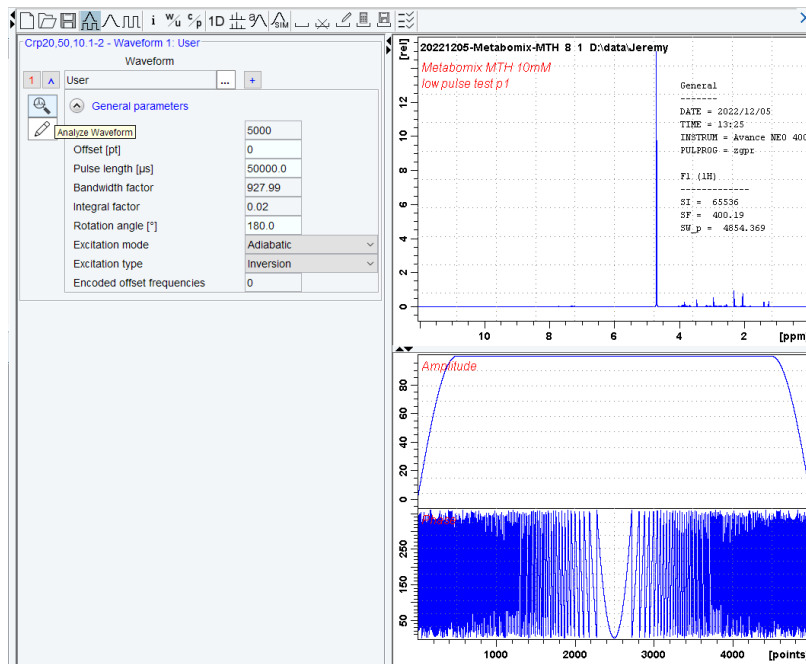
- Shapetool

Depending on the Topspin version used, the shapetool can appear slightly different.

For instance, here in Topspin 3.2 under CentOS environment:



And here in Topspin 4.0.5 under Windows 10 environment:



- Processing du NUS

Pour les versions inférieures à la 3.5, une licence est nécessaire pour retraiter les spectres en cs (voir 3).

5 Integration of the 2D signals

When performing integration of 2D signals in Topspin, multiple sets of values are available:

- **I+** (positive signal integration only)
- **I-** (negative signal integration only)
- **Ia** (positive signal integration + negative signal integration partie intégrée en négatif)

Regarding the data obtained thanks to this protocol, it is advised to work with $\mathbf{I}+$, since signals from ZQF-TOCSY are in theory entirely positive (*i.e.* pure absorption. As opposed to a NOESY for instance).