


User Manual

# Guidelines for the Evaluation of HOS of Biologics

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By 1D and 2D NMR

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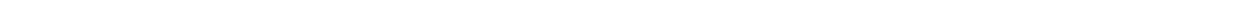
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## 1 Introduction

Biologics (mAbs, proteins, etc.) are large complex molecules that are made by living cells. Biologics not only have a primary sequence, but also have secondary, tertiary, and quaternary structures, collectively called higher order structures (HOS), making their complete characterization difficult. NMR is one of the key analytical methods that can characterize such HOS.

This Guideline is intended to support the acquisition and analysis of NMR data for the evaluation of the HOS of biologics. HOS is a Critical Quality Attribute (CQA) for most compounds of this class, and it can be assessed using different approaches. In this document, some of the typical NMR experiments (both 1D and 2D) used for that purpose are discussed. The standard HOS parameter sets are presented which are included in TopSpin version 4.1.4 and higher.

The NMR analysis is designed to compare statistically a reference population (originators or reference compounds for example) to a test population (biosimilars or stressed samples).

The 1D PROFILE<sup>1</sup> analysis implies a statistical approach, which compares not only the spectra as such, but also the intragroup (reference-reference and test-test) variability with the intergroup (reference-test) variability. One of the key points is that the reference spectra should reflect all the variability naturally occurring in the reference samples. The similarities between the spectra of the reference material and those of the test samples are then measured. In case the variability within the reference samples is the same as that between the reference and the test samples, the test sample is considered “the same as” the reference sample. In case the intra-group variability of the reference samples is significantly lower than the inter-group variability of the reference and test samples, then the test sample is considered “different from” the reference sample. Principal Component Analysis (PCA), Soft Independent Modeling by Class Analogy (SIMCA) and Projection to Latent Structures (PLS) methods use classical multivariate statistical methods to achieve a similar goal.

The 2D <sup>13</sup>C methyl fingerprint method uses a similar methodology but compares 2D <sup>1</sup>H-<sup>13</sup>C heteronuclear spectra acquired from reference and test materials.<sup>2</sup> The statistical analysis for comparing reference and test spectra include Easy Comparability of HOS (ECHOS), Combined Chemical Shift Difference (CCSD), Principal Component Analysis (PCA), Soft Independent Modeling by Class Analogy (SIMCA) and Projection to Latent Structures (PLS).<sup>2,3</sup> ECHOS compares two different 2D NMR spectra pointwise, one obtained from the reference sample and the other from the test sample. CCSD, on the other hand, analyzes the variation of peak positions and intensities between two spectra. PCA, SIMCA and PLS methods compute new latent variables that allow to separate the different groups.

For more information, you can watch the following webinar:

<https://mestrelab.com/software/mnova/mnova-biohos/>

For all these approaches, a comparison between spectra of reference materials and test materials is required. Therefore, all spectra must be measured on samples prepared under identical conditions and acquired using identical standardized procedures.

### 1.1 Instrument and software requirements

- Bruker Avance NEO with a proton frequency of 600 MHz or higher.
- Cryoprobe (TCI or TCI-F or QCI-F/QCI-P)
- TopSpin 4.3.0 or higher
- Mnova™ version 15.1 or higher that includes the BioHOS plugin  
See <https://mestrelab.com/>

<sup>1</sup>Poppe, L., Jordan, J.B., Rogers, G., Schnier, P.D., Anal. Chem., 2015, 87, 5539–5545

<sup>2</sup>Arbogast, L.W., Delaglio, F., Schiel, J.E., Marino, J.P., Anal. Chem., 2017, 89, 11839–11845

<sup>3</sup>Brinson, R.G. et al., mAbs., 2019, 11, 94–105

## 1.2 System suitability test

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Before running valuable samples, it is recommended to perform a system suitability test (for example using AssureSST) to ensure that the instrument is running under optimal conditions. An important parameter for measuring HOS data is the exact temperature of the sample. As data can be recorded over a long period of times on large batches of samples, it is important to calibrate the exact temperature of the Cryoprobe prior to data acquisition (or on a routine basis) and to make sure that the same temperature is used throughout the study. The procedure to measure and calibrate the temperature of the Cryoprobe is described in Appendix I.

## 1.3 Sample preparation

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The standard formulation for commercialized, formulated, therapeutic mAbs is classically either 200, 100, or 50 mg mAb/mL. Since the analysis is performed on unlabeled samples, 10 to 15 mg of mAbs need to be used in the NMR tube for a combined 1D and 2D NMR analysis. The samples are prepared by simply adding a small amount (ca. 5% v/v) of a D<sub>2</sub>O solution containing 0.03% w/w of TSP to the biologics sample in its natural buffer solution. The buffer and the excipients stabilize the mAb by preventing aggregation and degradation. Excipients will however give rise to extra signals in the spectrum.

Typically, the following NMR tubes can be used:

- 5 mm tube (total volume 600 µl)
- 5 mm Shigemi tubes (total volume 260 µl)
- 4 mm Match tube (total volume 315 µl)
- 3 mm tube (total volume 200 µl)

Since mAbs are expensive products that may be available only in limited quantity, having the choice between different tubes is important.

Please, note that using 3 mm tubes will make the acquisition of 2D <sup>1</sup>H-<sup>13</sup>C XLAFHMQC experiments longer.

The mAb solution can be completely recovered after the NMR measurements.

If possible, an NMR tube containing the pure buffer solution prepared under identical conditions is strongly recommended to allow the identification of the chemical shifts of buffer and excipient components.

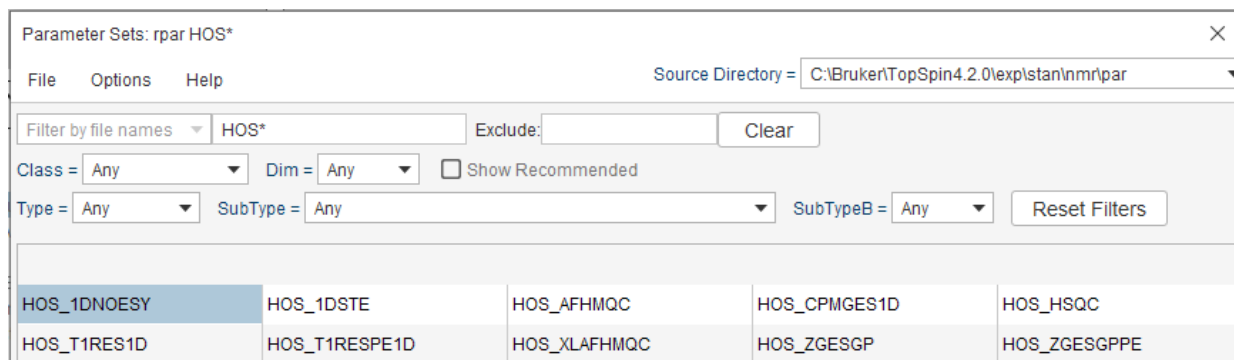
## 1.4 Applicability

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These Guidelines are applicable only when reference samples exist where the correct form of the HOS is confirmed. Replicates of a single lot of reference sample (e.g., the time zero sample in stability studies) or reference samples from different lots (e.g., batch comparability) should reflect the natural variability of the reference samples.

## 2 NMR data acquisition and processing

A suite of NMR experiments and parameter sets for these analyses is available from TopSpin 4.1.4. They are readily accessible by filtering the parameter sets with the key *HOS\** (i.e., “rpar *HOS\**”).



|             |               |              |              |              |
|-------------|---------------|--------------|--------------|--------------|
| HOS_1DNOESY | HOS_1DSTE     | HOS_AFHMQC   | HOS_CPMGES1D | HOS_HSQC     |
| HOS_T1RES1D | HOS_T1RESPE1D | HOS_XLAFHMQC | HOS_ZGESGP   | HOS_ZGESGPPE |

Additional adjustments of sample specific parameters may be required and will be discussed in the next pages. Given the high reproducibility of NMR, reference spectra can be stored and reused for future data analysis. Throughout the measurements, the conditions used for the test samples must be identical to those of the reference samples.

For antibodies, the temperature of the measurements is typically set between 35°C and 45°C in order to narrow the linewidth of the signals without compromising the stability of the mAb. In all cases, the temperature should be at least 10 degrees below the melting temperature of the protein.

### 2.1 Acquisition and processing of 1D NMR data

Measurements should be performed at high or very high field (600 MHz–1 GHz) in order to achieve enough sensitivity and dispersion.

The parameter sets recommended for 1D NMR are:

- HOS\_1DNOESY: with noesy1d scheme to suppress the solvent signal
- HOS\_ZGESGP: with excitation sculpting to suppress the solvent signal
- HOS\_ZGESGPPE: with excitation sculpting and perfect echo to suppress the solvent signal
- HOS\_1DSTE: with diffusion filter to suppress the signals from the buffer and protonated excipient
- HOS\_CPMGES1D: with T2 filter to obtain the spectrum of the excipients alone
- HOS\_T1RES1D: with T1rho filter to obtain the spectrum of the excipients alone
- HOS\_T1RESPE1D: with T1rho filter and perfect echo to obtain the spectrum of the excipients alone

In case the sample contains no protonated buffer or excipients, HOS\_1DNOESY or HOS\_ZGESGP can be used to remove only the residual water signal. HOS\_ZGESGPPE is recommended when the spectra from HOS\_ZGESGP gives some lineshape distortion due to the excitation sculpting.

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For these spectra, besides the parameters indicated in the parameter sets, the following entries should be optimized

|   |  |
|---|--|
| Field Strength                                | ≥600 MHz   |
| Parameter set                                 | HOS_1DNOESY or HOS_ZGESGP or HOS_ZGESGPPE  |
| Temperature (TE)                              | 310–323 K or lower if sample does not allow it   |
| Number of scans (NS)                          | Enough to allow good sensitivity – 128 or higher   |
| Offset frequency <sup>1</sup> H channel (O1P) | 4.7 ppm on resonance on the water signal, or use the AU program <i>o1calib</i> . Keep this O1p for all the subsequent experiments. |

In case the sample contains protonated buffer, the HOS\_1DSTE parameter set is suggested. The protonated buffer typically consists of molecules with sizes smaller than that of the protein, which therefore diffuse faster. This procedure is recommended to allow the comparison of the spectra, that otherwise would be dominated by the excipients' sharp peaks. For this experiments the diffusion parameters, d20 and p30, should be adjusted to optimize the removal of the unwanted peaks while retaining maximum sensitivity.

|   |   |
|---|---|
| Field Strength                            | ≥600 MHz  |
| Parameter set                             | HOS_1DSTE   |
| Temperature (TE)                          | 310–323 K or lower if sample does not allow it  |
| Number of scans (NS)                      | Enough to allow a good sensitivity – 128 or higher  |
| diffusion time (D20)                      | Starting value is 100 ms, but it may be increased for stronger suppression of the excipient peaks   |
| Gradient strength (GPZ6)                  | 95–100%   |
| gradient pulse - little DELTA * 0.5 (P30) | Starting value of P30 is 1200 ms but can be increased for better suppression of the excipient peaks up to 1500 ms. Larger values lead to substantial loss in sensitivity. |

In case the peaks of the buffer or the excipients cannot be fully removed, a software subtraction of the buffer can be performed. This procedure requires having the spectrum of the buffer alone. However, if it is not possible to obtain a 1D NMR spectrum of the buffer components alone, T2 or T1rho edited experiments can be used for that purpose. Note however that the result of the subtraction can generate artefacts in the spectrum.

|                        |   |
|------------------------|---|
| Field Strength         | ≥600 MHz  |
| Parameter set          | HOS_CPMGES1D  |
| Temperature (TE)       | 310–323 K or lower if sample does not allow it  |
| Number of scans (NS)   | Enough to allow a good sensitivity – 128 or higher  |
| Interpulse delay (D20) | 100 μs. It does not need to be changed. Shorter values may damage the probe!  |
| Total CPMG time (D21)  | Starting value is 50 ms. Although not usually needed d21 can be increased up to 200 ms for stronger suppression of the protein peaks. |

|   |   |
|---|---|
| Field Strength                                | ≥600 MHz  |
| Parameter set                                 | HOS_T1RES1D and HOS_T1RESPE1D   |
| Temperature (TE)                              | 310–323 K or lower if sample does not allow   |
| Number of scans (NS)                          | Enough to allow a good sensitivity  |
| RF field strength for T1rho spinlock (CNST62) | 5000 Hz. Does not need to be changed as higher values may damage the probe!   |
| length of T1rho spinlock (D21)                | Starting value is 50 ms. Although not usually needed d21 can be increased up to 200 ms for stronger suppression of the protein peaks. |

All these spectra can be processed with the *xaup* command.

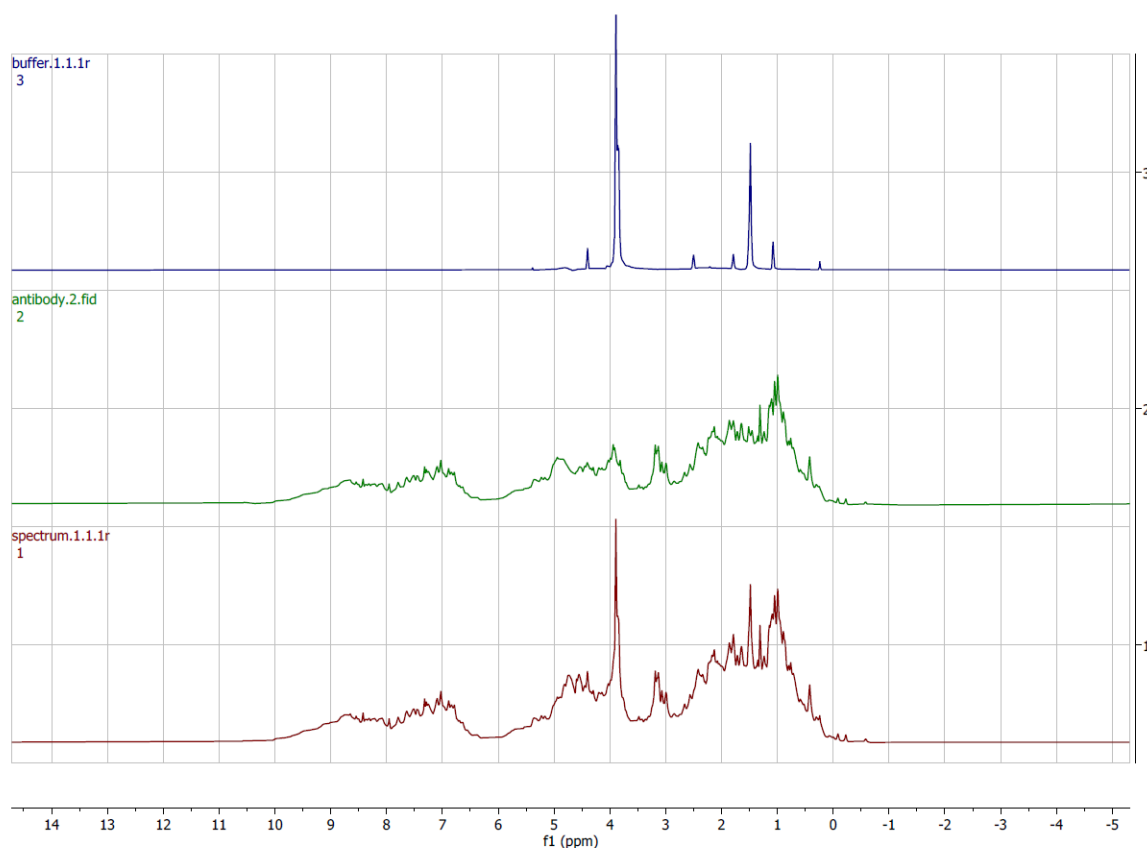


Figure 2.1: 1D  $^1\text{H}$  spectra of buffer alone (in blue), of the antibody with the buffer (in red) and of the antibody alone (in green). The spectrum in green was obtained by buffer SW subtraction with Mnova.

## 2.2 Acquisition and processing of 2D NMR <sup>1</sup>H-<sup>13</sup>C Methyl correlation

The parameter sets recommended for a 2D NMR analysis are:

- HOS\_HSQC: the 2D <sup>1</sup>H-<sup>13</sup>C HSQC experiment
- HOS\_AFMQC: the 2D <sup>1</sup>H-<sup>13</sup>C ALSOFAST HMQC optimized for large, protonated proteins, e.g., 100 kDa–150 kDa<sup>4</sup>
- HOS\_XLAFMQC: the 2D <sup>1</sup>H-<sup>13</sup>C XL-ALSOFAST HMQC optimized for large, protonated proteins, e.g., 100 kDa–150 kDa<sup>5</sup>

For samples in formulation buffers with high concentrations of excipients, giving rise to a few sharp spectral signals which are much higher (>>10x) than sample signals, a “*Selective Excipient Reduction and Removal*” (SIERRA) filter<sup>6</sup> prepended to a 2D <sup>1</sup>H-<sup>13</sup>C methyl correlation experiment could be acquired. This experiment should however only be used to remove a maximum of two signals. Details are found in [Appendix II: Experiment parameters for <sup>1</sup>H-<sup>13</sup>C ALSOFAST HMQC with optional SIERRA filter \[ 15\]](#). Note that the gradient selected XL-ALSOFAST experiment produces good quality 2D NMR spectra even in the presence of excipients and that a SIERRA filter is usually not required.

All experiments are implemented with Non-Uniform-Sampling<sup>7</sup> with a NusAMOUNT of 50%, however we advise switching to the regular acquisition mode (“FnTYPE Traditional(Planes)” in AcqPars) and using the “AWS” option. The typical spectral window for <sup>13</sup>C is 30 ppm centered at 18 ppm. This should cover all of the methyl region, but in case of special need, it can be adjusted. Since these proteins display rather short T2<sup>8</sup>, there is no reason to extend the AQ in F1 beyond 15–25 ms. To ensure adequate signal-to-noise for 2D NMR methyl fingerprint spectra, the number of scans should be chosen to ensure a 40:1 S/N in the first FID (row) of the 2D spectrum. The signal region should be 2.8 ppm to –0.5 ppm, excluding strong excipient signals, and use a noise region of 1 ppm. The TopSpin AU program *sinocal* can be used for this measurement. The o1 position of the HOD peak can be set automatically using the TopSpin AU program *o1calib*.

The parameters that can be adjusted for each specific protein are listed below.

|   |   |
|---|---|
| Field Strength                                | ≥600 MHz  |
| Parameter set                                 | HOS_HSQC  |
| Temperature (TE)                              | 310–323 K or lower if sample does not allow   |
| Number of scans (NS)                          | 512 is the starting value but it should be large enough to allow a good sensitivity, as described in the text |
| Offset frequency <sup>1</sup> H channel (O1P) | 4.7 ppm on resonance on the water signal. Use the same value previously used for 1D experiments.              |
| ZGOPTNS                                       | -DAWS (apodization weighted sampling) for molecule with short T2. <sup>8</sup> It replaces the NUS.           |
| Number of increments TD (F1)                  | Set to allow an AQ in F1 of 25 ms at most which will depend on the magnetic field strength.                   |

<sup>4</sup> Mueller, L., J. Biomol. NMR, 2008, 42, 129–137

<sup>5</sup> Roessler, P., Mathieu, D., Gossert, A.D., Angew. Chem. Int. Ed., 2020, 59, 19329–19337

<sup>6</sup> Arbogast, L.W. et al., J. Biomol. NMR, 2018, 72, 149–161

<sup>7</sup> Kazimierczuk, K., Orekhov, V.Y., Angew. Chem. Int. Ed., 2011, 50, 5556–5559

<sup>8</sup> Simon, B., Koestler, H., J. Biomol. NMR, 2019, 73, 155–165

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In case the protein is very large:

|   |  |
|---|--|
| Field Strength                                | ≥600 MHz   |
| Parameter set                                 | HOS_AFHMQC   |
| Temperature (TE)                              | 310–323 K or lower if sample does not allow  |
| Number of scans (NS)                          | 512 is the starting value but it should be large enough to allow a good sensitivity, as described in the text  |
| Offset frequency <sup>1</sup> H channel (O1P) | 4.7 ppm on resonance on the water signal Use the same value previously used for 1D experiments.  |
| ZGOPTNS                                       | -DCALC_SP as default to allow the calculation of the selective <sup>13</sup> C pulses<br>With -DSIERRA It allows for SIERRA filtering to remove the observable peaks of the excipient (see <a href="#">Appendix II: Experiment parameters for <sup>1</sup>H-<sup>13</sup>C ALSOFAST HMQC with optional SIERRA filter</a> ▶ 15])<br>With -DAWS (apodization weighted sampling) for molecule with short T <sub>2</sub> . <sup>8</sup> It replaces the NUS. |
| Number of increments TD (F1)                  | Set to allow an AQ in F1 of 25 ms at most which will depend on the magnetic field strength.  |

|   |  |
|---|--|
| Field Strength                                | ≥600 MHz   |
| Parameter set                                 | HOS_XLAFHMQC   |
| Temperature (TE)                              | 310–323 K or lower if sample does not allow  |
| Number of scans (NS)                          | 512 is the starting value but it should be large enough to allow a good sensitivity, as described in the text  |
| Offset frequency <sup>1</sup> H channel (O1P) | 4.7 ppm on resonance on the water signal   |
| ZGOPTNS                                       | -DCALC_SP as default to allow the calculation of the selective <sup>13</sup> C pulses<br>With -DAWS (apodization weighted sampling) for molecule with short T <sub>2</sub> |
| TD (F1)                                       | Set to allow an AQ in F1 of 25 ms at most which will depend on the magnetic field strength.  |

# Guidelines for the Evaluation of HOS of Biologics

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The XL version should be processed using “*proc\_dd*”, for the other experiments the processing parameters are typically as below

| Parameter                  | F2       | F1                               |
|----------------------------|----------|----------------------------------|
| Window function (WDW)      | QSINE    | QSINE                            |
| Sine bell shift (SSB)      | 3        | 2                                |
| Filter width for bc (BCFW) | 0.2 ppm  | n.a.                             |
| Fid baseline mode (BC_mod) | qfil     | no                               |
| ME_mod                     | no       | LPfc or ‘no’ in case AWS is used |
| <b>NCOEF</b>               | <b>0</b> | <b>60</b>                        |

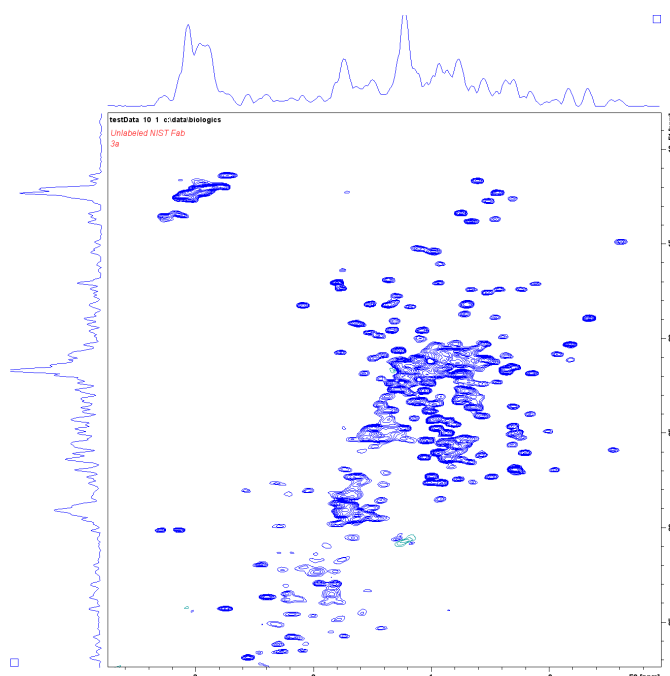


Figure 2.2: 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC of 429  $\mu\text{M}$  unlabeled NIST Fab fragments (50 kDa ca.) at 37°C, uniformly sampled with AWS, measuring time 3.25 h, field strength 600 MHz.

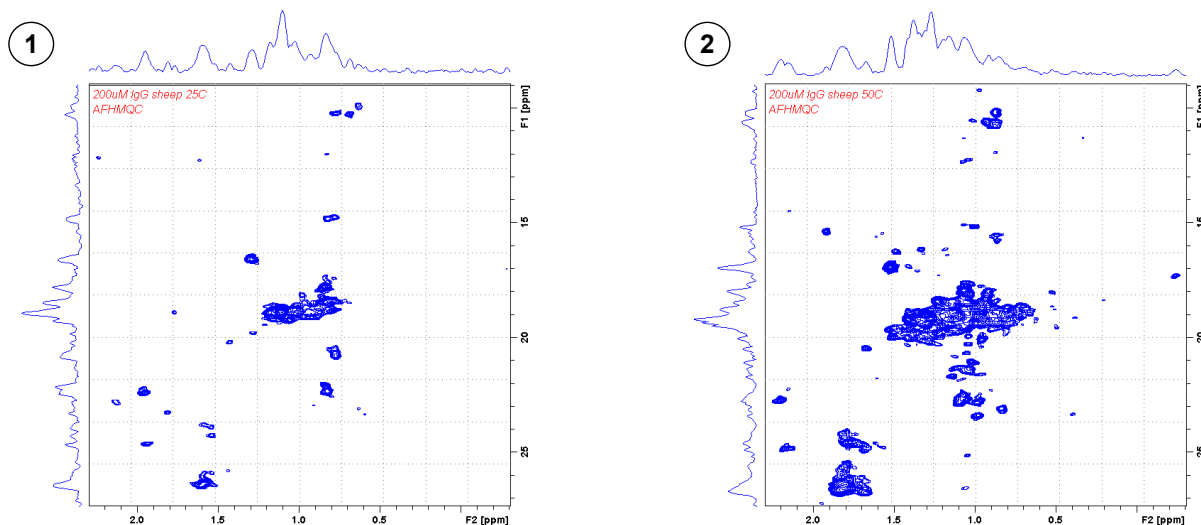


Figure 2.3: 2D  $^1\text{H}$ - $^{13}\text{C}$  AFHMQC of 200  $\mu\text{M}$  IgG, field strength 600 MHz, (1) at 25°C, (2) at 50°C.

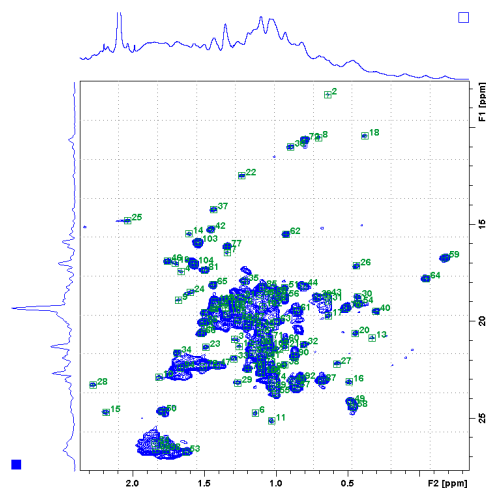


Figure 2.4: 2D  $^1\text{H}$ - $^{13}\text{C}$  ALSOFAS-T-HMQC with SIERRA filter on  $\sim 300 \mu\text{M}$  intact mAb (150 kDa ca.) at 50°C, measuring time 15 h.

## 3 Data analysis with Mnova BioHOS

Data analysis is performed with the BioHOS plugin of Mnova. Only a brief outline of the data analysis procedure will be given here. For further information, please refer to the BioHOS manual of Mestrelab at the following address [Mnova BioHOS – Mestrelab](#).

### 3.1 Analysis based on 1D NMR spectra

The following analysis are available under BioHOS for 1D spectra: PCA, PLS, SIMCA and PROFILE

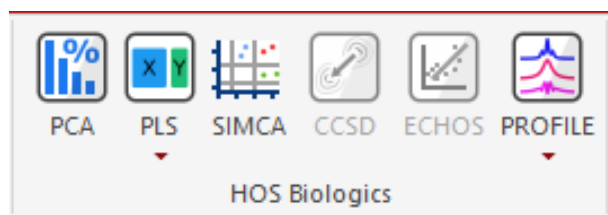


Figure 3.1: Analysis available under BioHOS for 1D NMR spectra

The most typical situation is the case where the data to be analyzed have been recorded with a diffusion filter (“HOS\_1DSTE” parameter set) to remove the low MW compounds. The data can be analyzed directly without any further treatment.

The PCA, PLS and the Profile methods are the two most important methods to identify differences between two sets of spectra. If numerical Y data are also associated with the data, then it is also possible to perform a PLS regression on the data.

If buffer signals need to be removed, the Profile method can be run with a buffer subtraction option.

### 3.2 Analysis based on 2D <sup>1</sup>H-<sup>13</sup>C NMR spectra

The following analysis are available under BioHOS for 1D spectra: PCA, PLS, SIMCA, CCSD and ECHOS.

Note that CCSD requires a preliminary peak picking of the cross peaks in the 2D spectrum.

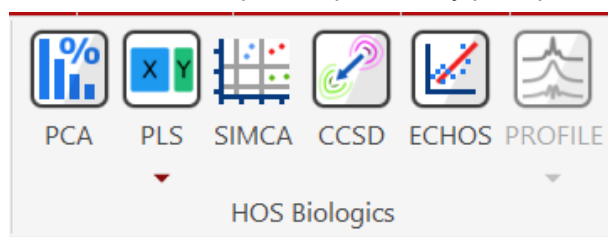


Figure 3.2: Analysis available under BioHOS for 2D NMR spectra

The processed 2D <sup>13</sup>C methyl fingerprint spectra are typically analyzed using the PCA and the ECHOS methods. If it is possible to peak pick reliably the cross-peaks, the CCSD method is also a good option.

If numerical Y data are also associated with the data, then it is also possible to perform a PLS regression on the data.

## 4 Appendix I: Temperature calibration of the CryoProbe



For more information, also check the Bruker manual *Variable Temperature Control for NMR Probes*. It is available in the TopSpin help menu.

### Sample

- **99.8% Methanol-d<sub>4</sub>, 5 mm** for Temperature [282 K-330 K] Calibration – NMR Thermometer (P/N Z10627)

### Acquisition parameters

|                       |   |
|-----------------------|---|
| Field Strength        | ≥ 600 MHz                                   |
| Parameter set         | PROTON                                      |
| Temperature (TE)      | 310–323 K or lower if sample does not allow |
| Dummy scans (DS)      | 0   |
| Number of scans (NS)  | 1   |
| Digital mode (DIGMOD) | baseopt                                     |

### Processing parameters

|                       |      |
|-----------------------|------|
| Window function (WDW) | EM   |
| Line broadening (LB)  | 2 Hz |

Type *calctemp* on the TopSpin command line and then type *D* in the calctemp pop-up window. The measured temperature is then reported for the given TopSpin TE value. The TopSpin TE value that corresponds to the target measuring temperature should be the TE value set in the 2D <sup>13</sup>C methyl fingerprinting dataset. To ensure tight temperature control during 2D data acquisition, a Self tune of the temperature unit should be run at that TopSpin TE value. For this, type *edte* on the TopSpin command line to open the temperature unit display. In the vtudisp display window, click on the 'Set' button under Target Temperature in the Temperature tab window of the Temperature Control Suite. In the Set target temperature pop-up window, enter the TopSpin TE value that corresponds to the target temperature for measuring the 2D methyl fingerprint of all HOS datasets. After clicking 'OK', wait for the Sample Temperature value to turn green. Then click on the Self tune tab and click on the Start button and wait until the Self tune routine completes. Then click on Store to store the Self tune parameters so that they can be recalled for subsequent measurements. It is recommended to include the TE value in degrees Kelvin and the gas flow rate in lph used for the Self tune in naming the stored self-tune parameters.

## 5 Appendix II: Experiment parameters for $^1\text{H}$ - $^{13}\text{C}$ ALSOFAST HMQC with optional SIERRA filter

### Acquisition parameters

|                      |   |
|----------------------|---|
| Field Strength       | ≥600 MHz  |
| Nucleus              | $^1\text{H}$  |
| Parameter set        | AFHMQCGPPHSF*   |
| Temperature (TE)     | Determined from procedure in <a href="#">Appendix I: Temperature calibration of the CryoProbe [ 14]</a> |
| Number of scans (NS) | Sufficient to produce S/N of 40:1 in first FID (row)  |
| SPNAM11              | Squa100.1000 for CryoProbes   |

The Python program, *afh\_prep.py*\*, is used to set up experimental parameters for the AFHMQCGPPHSF dataset. The program accepts several arguments:

```
[water] [sierra # [right_limit] [left_limit]] [silent]
```

where and for example,

```
afh_prep sierra 2 (suppress the 2 strongest signals in the region of ~4 ppm to ~-1 ppm in  $^1\text{H}$  and ~60 ppm to ~0 ppm in  $^{13}\text{C}$ )
```

```
afh_prep sierra 2 -1 2.5 (suppress the 2 strongest signals between -1 ppm and 2.5 ppm)
```

```
afh_prep water (optimize water suppression only)
```

```
afh_prep (determine the 90 degree pulse only)
```

```
afh_prep water sierra 2 -1 2.5 silent (all of the above)
```



## 6 Contact

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### Manufacturer

Bruker BioSpin GmbH & Co. KG

Rudolf-Plank-Str. 23

D-76275 Ettlingen

Germany

Website: [www.bruker.com](http://www.bruker.com)

Contact: [www.bruker.com/en/services/service/magnetic-resonance.html](http://www.bruker.com/en/services/service/magnetic-resonance.html)

WEEE DE43181702

### Hotlines

Contact our Bruker BioSpin service centers.

Bruker BioSpin provides dedicated hotlines and service centers, so that our specialists can respond as quickly as possible to all your service requests, application questions, software or technical needs.

Please select the service center or hotline you wish to contact from our list available at:

[www.bruker.com/en/services/support.html](http://www.bruker.com/en/services/support.html)

To contact customer support directly, select the email address according to your region:

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