NOVA

Mnova BioHOS
Application Note

Mestrelab Research
chemistry software solutions

Mnova BioHOS Application Note

Therapeutic pharmaceuticals (drugs) have witnessed a sea change in recent years. The large, dominant group of drugs derived from synthetic, small molecules has been joined by a new type of drug that has been very effective with diseases that were previously untreatable. These biopharmaceuticals include monoclonal antibodies (mAbs), proteins that are produced (cloned) in large quantities using recombinant DNA methods.

mAb therapeutics are hugely profitable and useful. Their high cost to the patient in part reflects the technical challenges in their design and manufacture. And there are significant analytical challenges: how can one ensure such a large, complex protein was produced correctly and is biologically active? Whilst mass determination using MS might seem the obvious answer, it only answers part of the question because the biological activity is influenced by the protein's overall "higher order structure" (HOS): its 3D shape.

NMR has an excellent track record with protein studies, and much has been done to develop the instrumentation and pulse sequences to measure their spectra. Whilst the full spectral assignment of mAbs is very difficult and not yet reported in mid-2019, we can still achieve a lot by using "fingerprint" methods. Small but significant changes to the protein are reflected in the resonance positions – even if we do not know their assignments. This has been proven to be an excellent approach to the QA of mAb therapeutics.

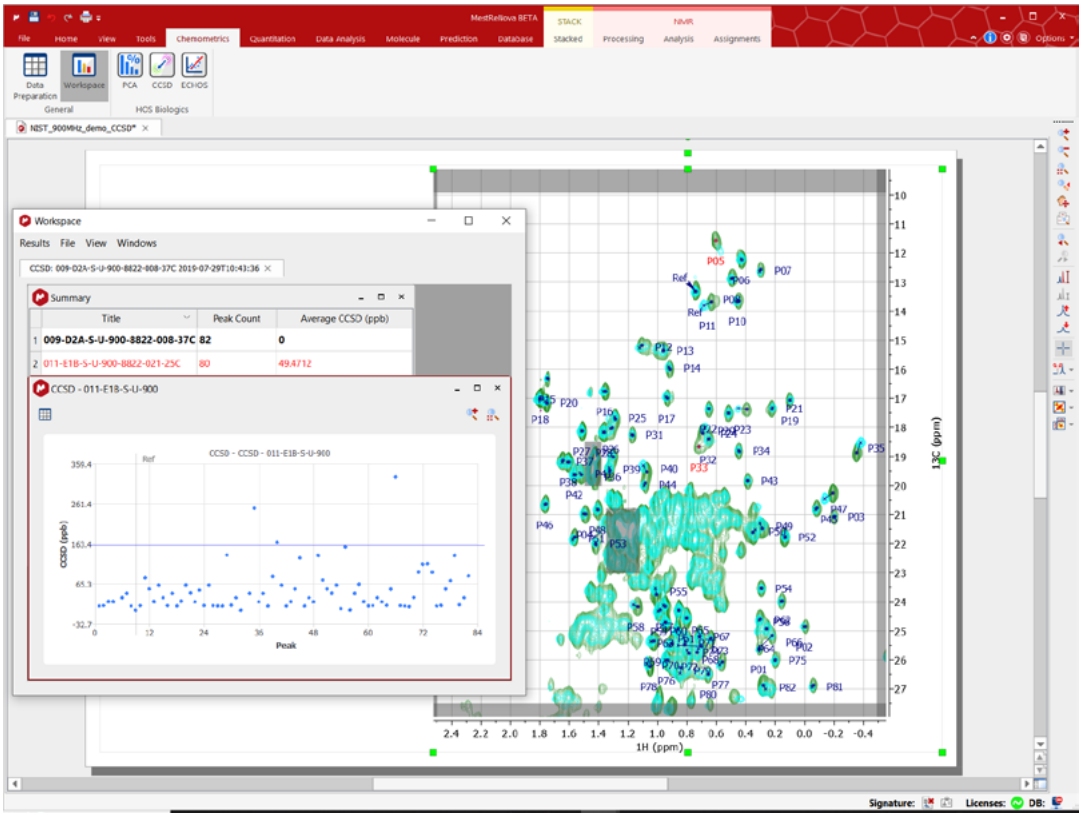
A considerable body of literature supports the notion of NMR fingerprinting for QA purposes. NIST has been influential, first with production of a primary standard mAb (NISTmAb, RM8671) and leading a global "round robin" study that amply demonstrated the NMR method's generality and robustness. The data from this study are available to the public.

MestReNova, scientific processing and analysis software produced by Mestrelab, has a long reputation for ease-of-use, combined with powerful processing and analysis capabilities for NMR, UV-IR, and LC-MS. In collaboration with key individuals from Bruker Biospin, we set out in early 2019 to produce a suite of new capabilities that will meet many of the analytical needs for mAb analysis using 2D NMR spectral data.

Getting the “basic” procedures right: validation

An analysis cannot start without a lot of preparation. A sample preparation should be validated, including temperature tolerance and sample conditions such as concentration and excipients. Next, experimental acquisition should be optimised for the common ^{1}H-^{13}C 2D NMR heterocorrelation map of the protein side-chain methyl signals. The challenge is further exacerbated by the molar mass of the protein and signal overlap. Finally, the ^{13}C isotope, required for NMR, is relatively insensitive and present only at natural abundance. Sample material is usually not a limiting factor, and these problems are often not insurmountable. mAbs are usually stable to elevated temperatures, which assists signal acquisition. So, let’s start by assuming that NMR signal acquisition of validated material was successful: now what?

Other software “basics” are needed before considering use of the 3 major approaches to mAb fingerprinting (see below). NMR signal processing of large sets of 2D spectra requires quite specific capabilities, including NUS support and noise reduction. You need flexible tools to set analysis regions, and other tools that just facilitate the analysis, such as setting the class and display colour of each spectrum. Everything should be easy to use, intuitive, and a good starting point for automated analysis.



What makes Mnova BioHOS uniquely valuable and useful

- Capitalise on a powerful, intuitive user interface

- Excellent, fundamental capabilities and algorithms

- Interactivity between the analysis results and spectral data

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The “3 pillars”: ECHOS, CCSD, and PCA

Any fingerprint method is based on having material that is authentic and represents the “pass” condition – reference material. All tests will be a measure of how similar the new compound’s spectrum is to that of *reference material*. Let us look at the available analytical methods.

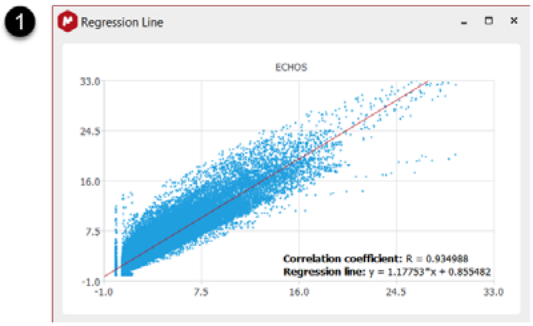
ECHOS

*E*asy *C*omparability of *H*igher *O*rder *S*tructure (ECHOS) is a fast and simple analysis. The same point in the test- and reference spectra are compared, and a scatter plot made of significant point amplitudes. So, the more similar spectra are, the more the points will cluster around the line of equality, where x=y. The axes for the ECHOS plot are point intensities, meaningless unless used for comparison.

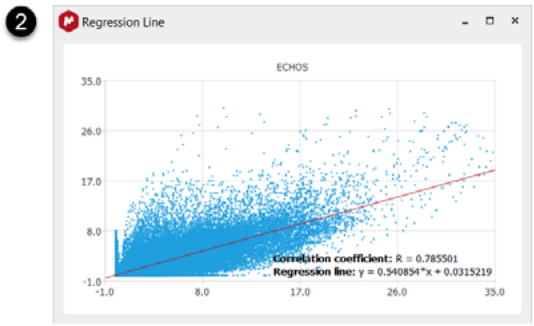
We then fit a straight line to the data and report the regression results. The literature suggests that the coefficient of correlation, R, is a simple bellwether of spectral similarity: the closer this value is to 1.0, the more similar the spectra.

We compared 2 spectra from the NIST study dataset that were acquired on the same hardware (900 MHz NMR):

1. Both spectra recorded at 37 °C: **R = 0.935**
2. One at 37 °C and the second at 25 °C: **R = 0.785**



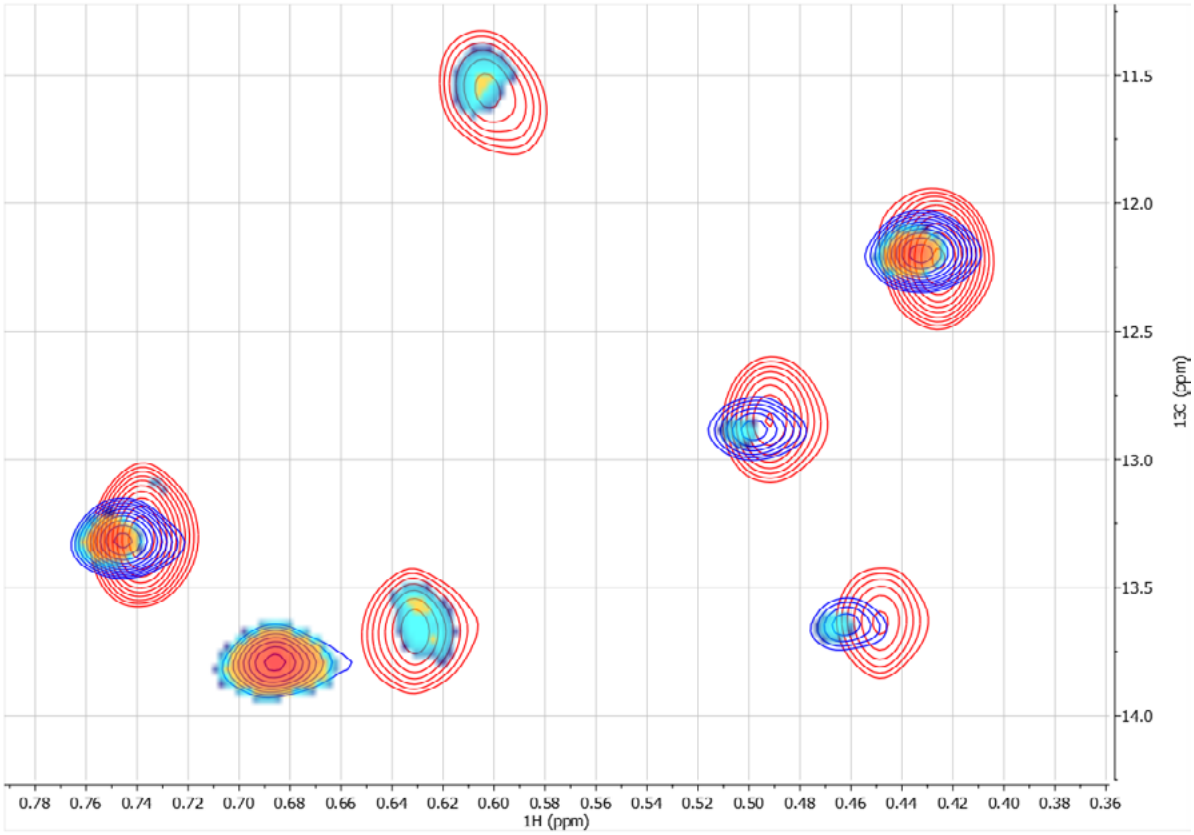
37 °C compared with 37 °C



37 °C compared with 25 °C

But, wouldn’t it be nice to see what parts of the spectrum have the biggest differences? Mnova allows this by plotting the points furthest from the fitted line as a heat map, together with the spectra. The threshold can be easily changed.

Here we see the 37 °C (blue) and the 25 °C spectrum (red) contours. Using the ECHOS analysis, the hot and cold splodges show where the spectra differ the most. For this small part of the spectrum it is easy to see that the software found the expected changes from peak shifts and broadening at lower temperature.



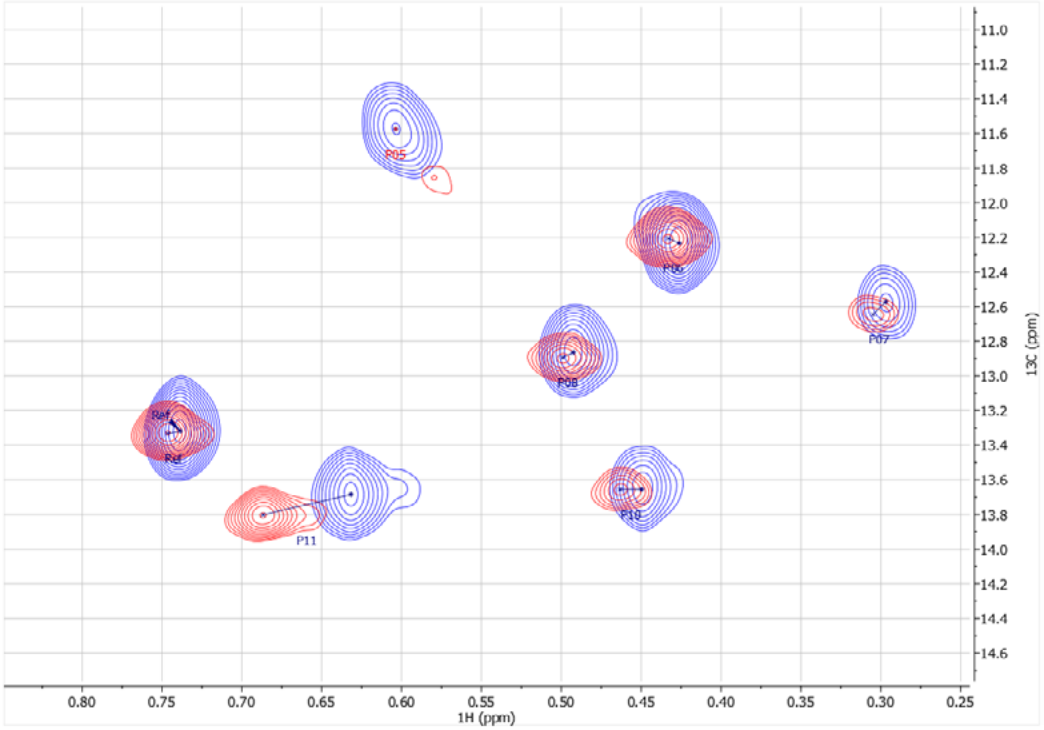
CCSD

The *C*ombined *C*hemical *S*hift *D*ifference (CCSD) is a proven measure of peak movements in a spectrum. Starting with the reference spectrum where the interesting peaks have been picked, Mnova finds the associated peak in the test spectrum and calculates the CCSD for each peak. We also determine the change in amplitude of each peak pair, because stressed material can also show broader peaks.
When we compare the spectra recorded at different temperatures the combined result is as follows:

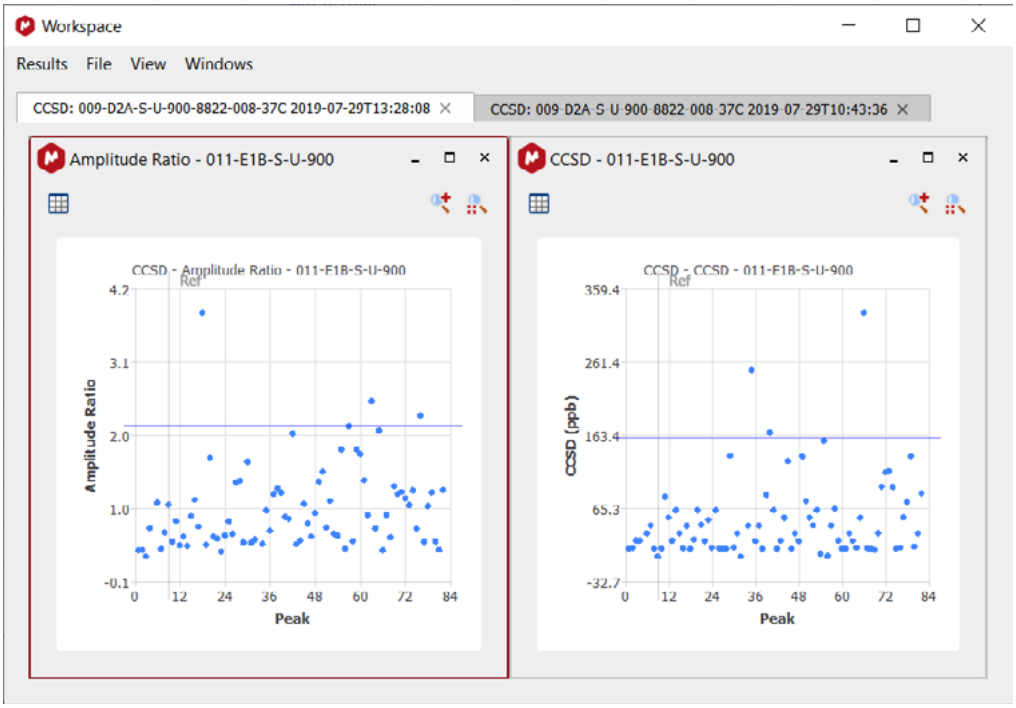
Summary

	Title	Peak Count	Average CCSD (ppb)
1	**009-D2A-S-U-900-8822-008-37C**	**82**	**0**
2	011-E1B-S-U-900-8822-021-25C	80	49.4712

The average CCSD for the 80 matched peaks is shown. Two peaks were not matched.
A spectral region shows the automatically calculated peak pairs. For P05 the matching partner peak was below the threshold and ignored.



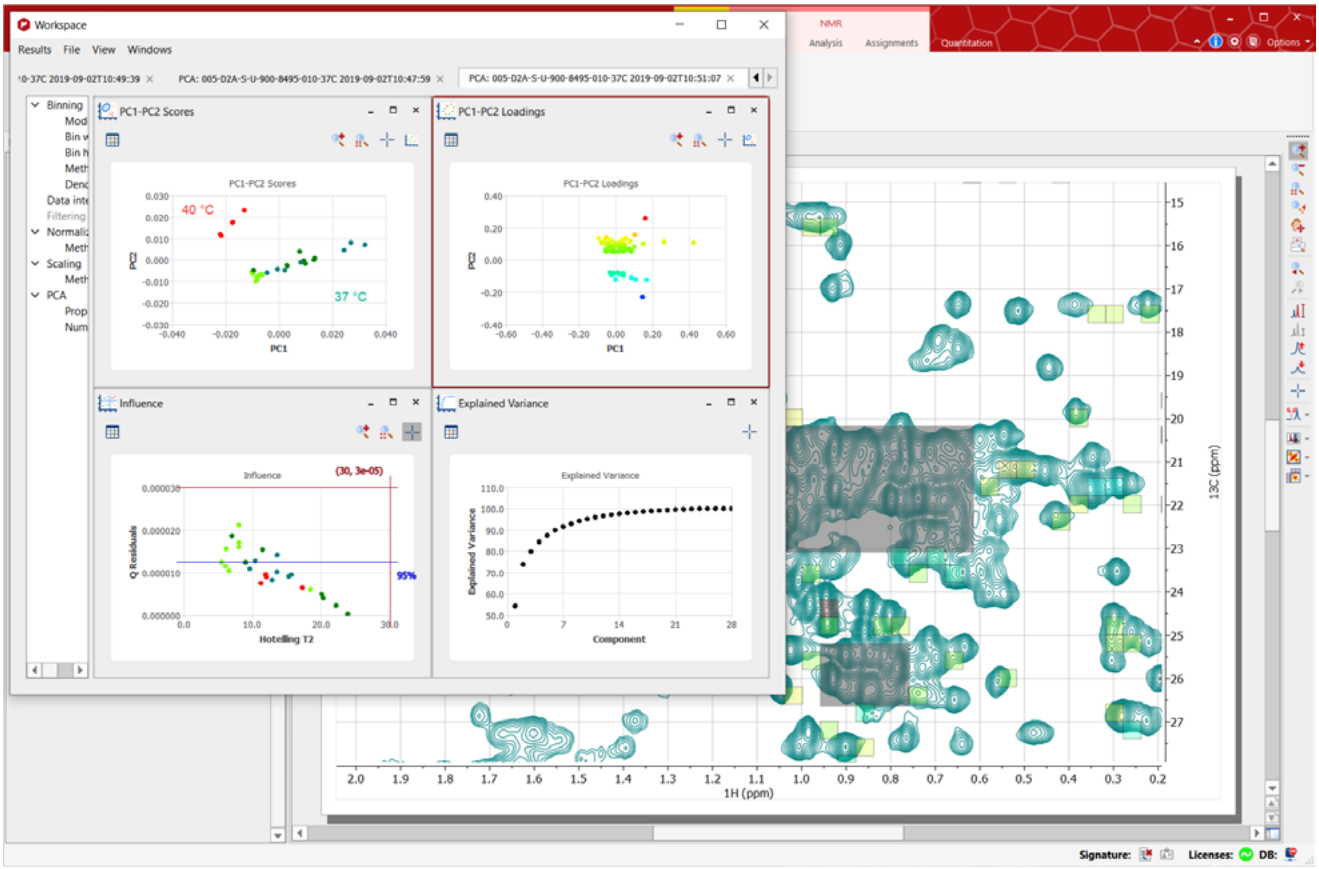
We can also see the peaks that have shifted and broadened the most. It is very useful, too, to be able to click on a "peak" in the Workspace plots that looks interesting, and the corresponding spectral region will be shown.
We see here the CCSD and Amplitude Ratios of all peak pairs, sequentially numbered:



It is easy to inspect the peaks that have either moved or broadened the most because there is interactivity between the plots and the NMR data.

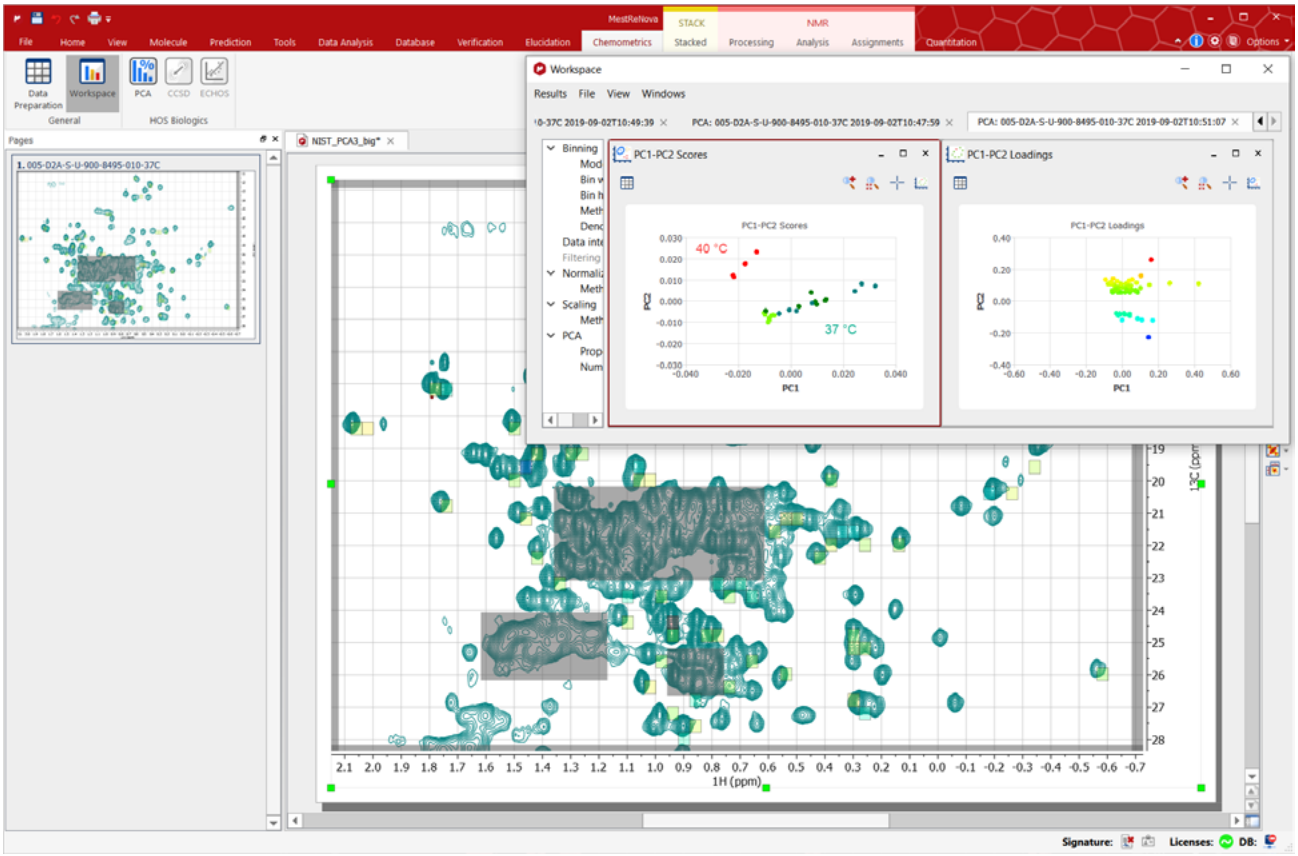
PCA

*P*rincipal *C*omponent *A*nalysis is a well-known statistical method which is widely used in metabonomics studies with NMR. The analysis relies on having a set of spectra of reference materials – ideally, more than 8. PCA uses intensities in spectral buckets to find similarity and difference between datasets.
In this example we used 18, 700 and 900 MHz ^{1}H-^{13}C HSQC spectra recorded at 37 °C as reference spectra, and the test spectra were recorded at 45 and 50 °C. Colour coding helps to distinguish the spectra.



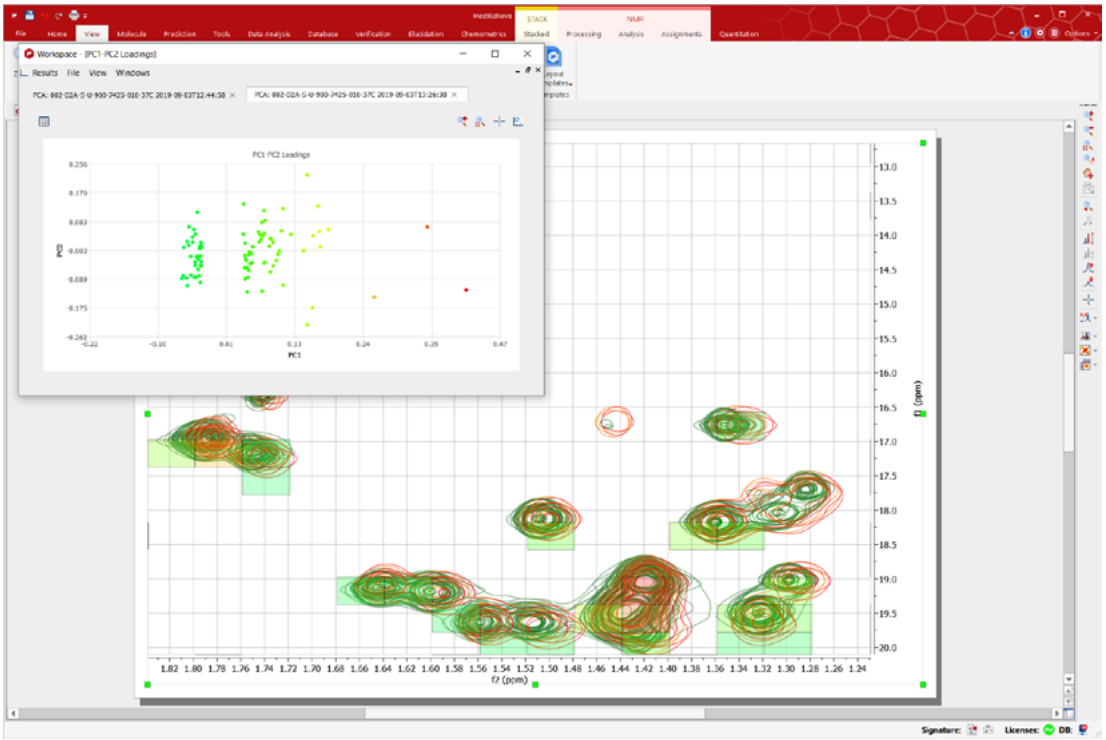
Scores plots. Now we can use PCA to reduce each spectrum to a point on the scores plot. The closer the points, the more similar they are. The reference spectra, blue, "cluster" close to one another, and the more similar a test spectrum is, the closer it will be to the cluster of reference spectra.
In this example we see that the higher-temperature spectra are very close to each other on the PC1 axis, and the high-temperature spectra are separated to the left (red).

Scores plots. Now we can use PCA to reduce each spectrum to a point on the scores plot. The closer the points, the more similar they are. The reference spectra, blue, "cluster" close to one another, and the more similar a test spectrum is, the closer it will be to the cluster of reference spectra.
In this example we see that the room temperature spectra are very close to each other on the PC1 axis, and the higher-temperature spectrum is separated to the left (red).



Loadings plots. These help us find the spectral regions that allow discrimination of these spectra. Here we again make full use of interactivity between the analysis results and spectral data.
First, we can accentuate the loadings (buckets) that are most important. Those that are furthest from the axis PC1=0 are the most important and are coloured with the hottest and coldest colours.

Now we can visualise only the most important buckets on the spectrum and plot. We see that PCA used the obvious spectral differences, shown in this small region of the spectrum.



Conclusions

NMR is an effective method for fingerprint comparison of protein 2D spectra, and this is an excellent measure of HOS. The tools for the comparison (and processing) are available in the Mnova BioHOS package.

Future outlook

Mnova BioHOS software can be used to effectively process and analyse 2D NMR data from mAbs. The plan is to test this in more end users' laboratories and further improve the software usability. New capabilities will be created to facilitate testing against a "model" of the reference spectra.

Further studies will demonstrate the general suitability of the fingerprinting analysis for other, complex materials.

Selected bibliography

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