MNova: Guide to Processing Absolute-value and Phase-Sensitive 2D NMR Spectra

Absolute-value 2D spectra are not phase sensitive – all peaks are positive and phase correction is not required (COSY and HMBC). The phase-sensitive experiments have positive and negative peaks, and phase correction is extremely important.

Loading Your Data and Selecting a Window Function

1. Open the 2D fid file in MNova. MNova will process and display the spectrum (or multiple spectra, if your folder contains more than one spectrum.

2. You will most likely want to use the regular 1D proton spectrum (and carbon spectrum, if HSQC, or HMQC) as the "traces", plotted along the two dimensions of the 2D spectrum. If not already loaded, open these in the same MNova document as your 2D spectrum. This should give you multiple "pages" (in the Pages window to the left of the spectrum) in your MNova document.

3. Process your 1D spectra first. Make sure they are referenced properly (i.e. to TMS or other appropriate peak).

4. 2D spectra can be displayed/plotted via a number of methods, but Contour Plot is the default, and is what you want to use. [FYI: all the options are Bitmap Plot, Contour Plot, Stacked Plot, and Whitewashed Stacked Plot. To switch between the methods use the View menu: *View > 2D Plotting Method >* select *Bitmap Plot, Contour Plot, Stacked Plot, or Whitewashed Stacked Plot.* You can also use the 2D Plotting button on the vertical tool bar to find these options. Be sure to display the spectrum as a Contour plot before printing.

   Feel free to play with all, to see what they do, but the two most useful are Bitmap and Contour. Bitmap is faster to respond to changes you make, in terms of the computations needed for updating the display as you manipulate the spectrum. In the days of slow computers, this was important, but, today, computing power is not an issue, so just use the default Contour Plot mode.

4. ***Setting the proper window functions.*** MNova provides default window function settings (based on the Bruker file) for the different experiments, and they should be fine. But, FYI, you can check these and change them if you want….

   Remember that there are two dimensions, f1 and f2, and you have to set the window functions in both of them. You can switch between the two as shown in the Apodization figure dialog box. To access the Apodization dialog box, click on the *Apodization* button (right) or from the Processing menu, select: *Processing > Apodization.* Here are proper window functions for common experiments.

   - Absolute-value COSY: **Sine Square 0.00 in both dimensions** (this is default, probably the best). Also used is Sine Bell 0.00 (in both dimensions).
   - Phase-sensitive NOESY: **Sine Square 90.0 along both dimensions**. Alternatively, Sine Square 90.0 along f2 and Sine Bell 90.0 along f1 has been recommended, or Sine Bell 45.0 in the f2 dimension (but only if the S/N is very good).
   - Phase-sensitive HSQC: **Sine Square 90.0, in both dimensions**. Sometimes a Sine Bell 45.0, in both dimensions, is used.
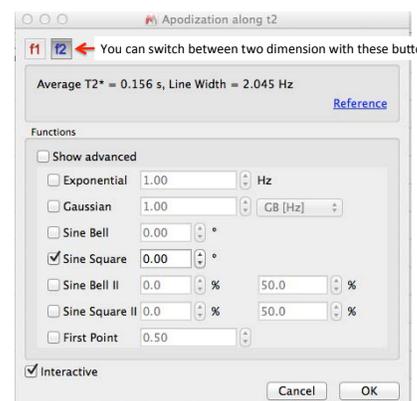
   Start with the suggestions in bold, first, and you can try the others if desired.

Once you set the proper window function, make sure all other functions are unchecked. I'm not entirely sure about the significance of the *"First Point"* box, but it has an effect on baseline noise. Check/uncheck it (try it in both dimensions) to see the affect on the noise in the spectrum. Choose, checked or unchecked, depending on how things look. Make sure the Interactive box is checked, so you can see the effect of your changes as you make them.

Examples of Processing Various 2D datasets (COSY, NOESY, and HSQC)

I: Absolute-value gradientCOSY.

1. To the right is a look at the initial autoprocessed COSY, after the proper window functions have been set. The noise stripes that run in the f1 (t1) dimension are called t1 noise and are associated with all peaks, and can be seen if the vertical scale is low enough. Tall peaks have more intense t1 noise levels. You can use the middle scroll wheel to adjust the spectrum vertical scale. Note that all peaks are red (or of the same color, if you've chosen a your own coloring scheme) because in an absolute-value (or magnitude mode) experiment, all peaks have the same phase (all are projecting out from the paper).

2. Apply baseline correction along both dimensions. Use the Baseline correct button to select baseline correction.
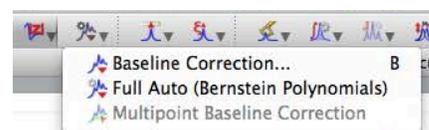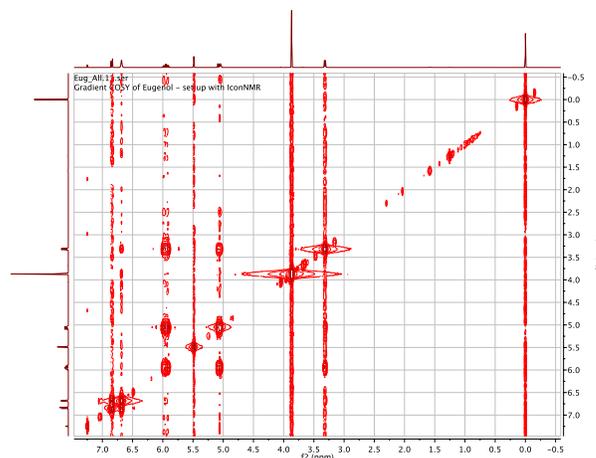
   In the dialog box, select *Full Auto (Bernstein Polynomials)*.

3. At this point, you can adjust the vertical scale of the spectrum appropriately, expand regions, as desired, and plot. Alternatively there are two other manipulations you can apply to the data, to clean up the spectrum, but I recommend you be wary of these unless noise is real problem. Note that there is no "danger" in trying these manipulations. You can turn them on and off at will.
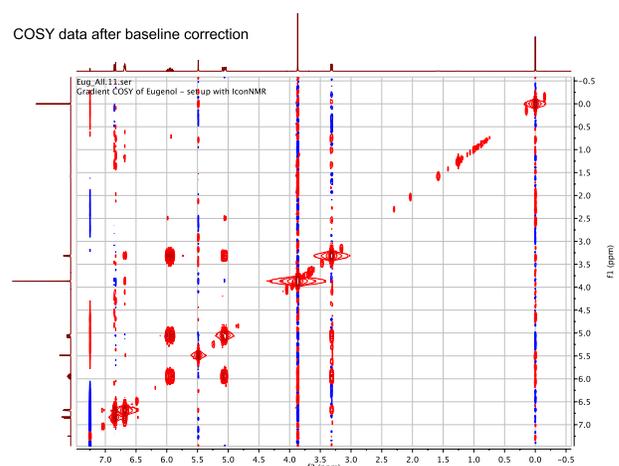
   For a magnitude mode COSY and related "symmetrical" spectra, you can *symmetrize*, to remove t1 noise. To *symmetrize*, use the menu combination: *Processing > Symmetrize > COSY-like*. For *symmetrize* to be possible, both dimensions need to have the same digital resolution (same number of points), so you may need to zero-fill the t1/f1dimension up to the same number of points as the t2/f2 dimension. In MNova usage, you must have a "square matrix" spectrum. If your data is not "square matrix", MNova will tell you so (with a dialog box that pops up) and it will offer to "convert" your data to a square matrix, i.e. it will zero-fill for you. Just accept by clicking on the *yes* button.

Note: MNova also has another routine that reduces t1 noise. To apply this function select from the Processing menu: *Processing > Reduce t1 Noise*. Like the symmetrization routine, you can toggle this setting on and off. With either *symmetrize* or *Reduce t1 Noise*, you need to be a bit careful. With *symmetrize*, for example, if there happens to be noise in the same place across the diagonal, symmetrization can make this noise look like a cross peak! Note that symmetrization is not recommended for phase sensitive data, due to possible distortions of the cross peaks.

FYI: you can display the acquisition and processing parameters if you like (*View > Tables > Parameters*).


II. Phase-sensitive gradientNOESY

1.   First check the window functions (Sine Square 90.0 in f2 and Sine Bell 90.0 in F1). As with the COSY, you'll see t1 noise. In the *Apodization* dialog box, in the f1 dimension, uncheck the *First Point* box, if it's checked. This should reduce the t1 noise significantly. As the spectrum shows, this phase sensitive experiment will have both positive (red) and negative peaks (blue). To the right is a spectrum after setting the window functions, and unchecking the *First Point* box (in f1).

2.   This phase-sensitive experiment needs to be phased. First, select *Automatic* from the *Phase Correction* button.
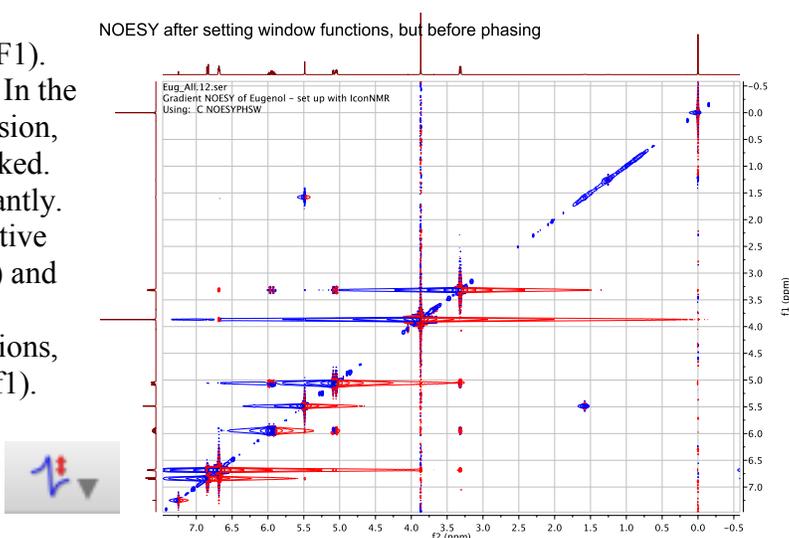
The automatic routine does a good job, but you often need to touch-up the phase manually, in both the f1 and f2 dimension. You might want to lower the threshold in order to see a bit of the noise, where it is easier to see the effects as you manually phase. First select *Manual Correction* from the *Phase Correction* button.
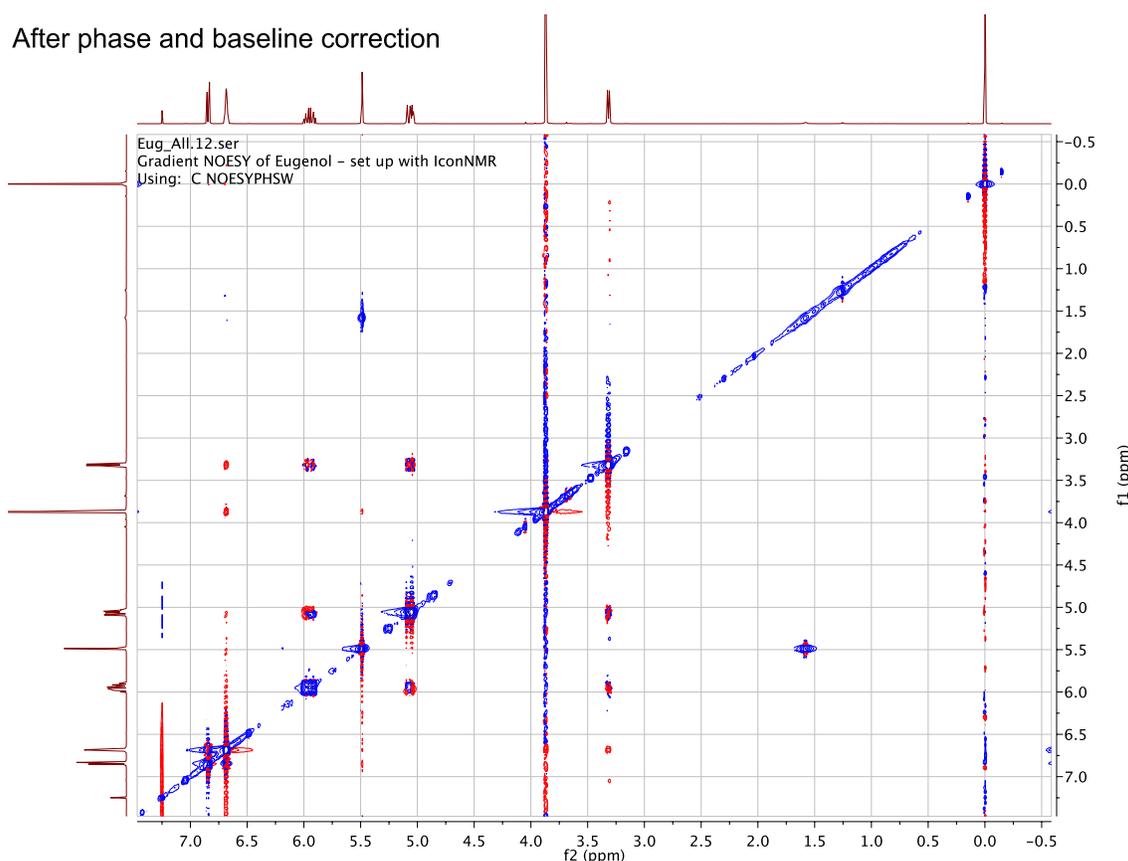
The f1 and f2 buttons in the phase dialog box allow you to move between the two dimensions. Start with f2. Set the Pivot to a peak near the right (or left) of the spectrum and do the zero-order phase (PH0) correction by holding the left mouse button and sliding the mouse up or down in the blue are of the dialog box, so as to remove as much of the red and blue noise that flanks the peak in the f2 dimension – basically, sharpening the peak along the f2 dimension. Next, leaving the Pivot alone, focus on a peak at the opposite end and apply the first-order (PH1) correction (hold the right mouse button as you slide the mouse up and down in the blue area of the dialog box), again trying to sharpen the peak by removing the flanking noise.

Next, click the f1 button in the Phase dialog box and phase in the f1 dimension, using the same procedure.you used to phase in the f2 dimension. Due to t1 noise, it is not as easy to phase in the f1 dimension. You may want to set the threshold to a higher level, to where you don't see too much noise. Once you are satisfied, exit the phase routine.
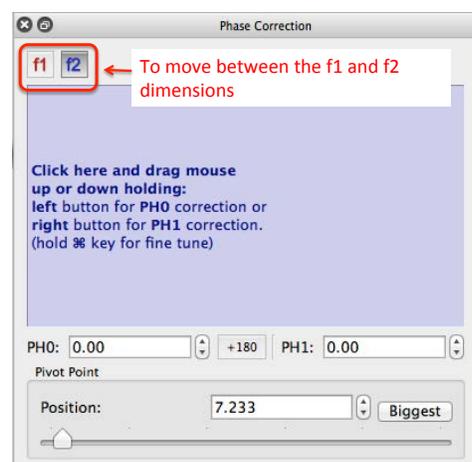
3. Baseline correction. You can lower the spectrum threshold to see the baseline defects. Apply baseline correction along both dimensions. Use the Baseline correct button to select *Full Auto (Bernstein Polynomials)*.

   After phasing and baseline correction, your spectrum should look something like the following, if you have a fairly low threshold – "down" in the noise, a bit.

After phase and baseline correction

Eug_All.12.ser
Gradient NOESY of Eugenol – set up with IconNMR
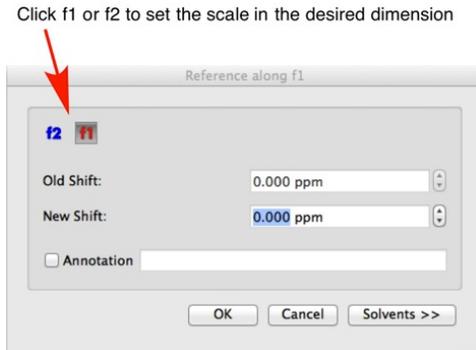Using: C NOESYPHSW

4. At first blush, the "square" 2D spectrum of a NOESY is analogous to the absolute-value COSY and symmetrization can be applied to remove t1 noise. However, the phase-sensitive nature of the spectrum makes symmetrization less desirable and can introduce undesirable distortions in cross peaks. I recommend that you stay away from the *symmetrize* function. In general, for "square" phase-sensitive experiments, perhaps the *Reduce T1 Noise* function in MNova is a better solution to t1 noise (see step 3 in the COSY processing section, above). It might be best, if the noise is not too bad, to simply accept the noise to see the weaker peaks.

5. Your spectrum is likely to already have the 1D spectra automatically placed along each dimension of the 2D spectrum, but, if not, you can simply drag the 1D spectrum page (from the "Pages" panel) on to the appropriate axis (on top and on the left side) of the 2D spectrum and they will appear.

   When you have vertical and horizontal traces displayed about a 2D spectrum, you simply "hover" over the trace or 2D spectrum of interest, to activate that spectrum for manipulation. If you have a middle-mouse-scroll wheel, it serves to increase or decrease the vertical scale on the active spectrum.

III. Phase-sensitive HSQC

1.   Process this phase sensitive experiment in the same way as is done for the phase-sensitive NOESY. For window functions, use Sine Square 90.0, in both dimensions. CH_2 carbons will appear with positive phase (red) and CH and CH_3 with negative phase (blue), if you see the opposite, you can switch the phase by 180˚, by clicking the *+180* button in the manual phase dialog box.

For all types of 2D spectra…

1.   Expanding and basic spectrum manipulations on the 2D spectrum are analogous to what you do with a regular 1D spectrum. For example, to expand on a region of the 2D spectrum, select the zoom button, then drag a box over the portion of the spectrum you want to expand.

2.   Your spectrum is likely to appear with proper scale referencing, but if not, follow these instructions. First, set the reference peaks correctly in your 1D spectra. You may have to then drag these spectra onto the appropriate dimension along the 2D spectra, to replace incorrectly referenced spectra. To manually reference your 2D spectrum, click on the 'Reference ' toolbar button (select "Reference"). [Make sure the scale of your 1D trace spectrum has been set.] You'll get a crosshair, which you can use to set the select a peak in the spectrum (e.g. TMS). Place the crosshair on the peak and click. A dialog box appears where you can set the scale. Of course, in a 2D spectrum you have two axes to set, f2 and f1. You can use the f2 and f1 buttons in the box to set the scale in the two dimensions.

3.   As with regular 1D spectra, you can do all sorts of formatting. You can change the colors of peaks, remove the background grid, annotate, etc. Play around to make it look as you want it to look.

4.   Plotting is analogous to plotting for 1D spectra.

Notes on the Interpretation of Phase-sensitive NOESY spectra

The NOESY is phase-sensitive experiment, meaning peaks can be positive or negative, depending on what causes the peak. The diagonal peaks are, by convention, phased positive (blue is the default color for positive peaks in MNova). The phase of crosspeaks due to an NOE depend on the size of the molecule. Crosspeaks due to NOEs for small molecules (MW < ca. 1000) give a purely negative peak (red, opposite of the diagonal). For large molecules (MW> ca. 2000), the NOE cross peaks are positive (same phase as the diagonal). Molecules of intermediate size (MW ca. 1000-2000) generally give no observable NOE (a ROESY experiment must be used for such molecules).

Positive crosspeaks (blue, same phase as the diagonal) also arise due to chemical exchange. For instance, if your molecule has two –OH groups, where the two alcohol protons give rise to separate peaks in the spectrum and the alcohol protons undergo slow exchange with one another, a crosspeak between them will occur due to *chemical exchange*. This cross peak (blue) will have opposite phase of a true NOE crosspeak (for a small molecule). Chemical exchange crosspeaks also occur when a molecule exists in solution in two or more slowly (on the NMR timescale) interconverting conformations. For instance, if a molecule exists in two slowly interconverting conformations, all of the signals in the regular 1D spectrum will be doubled – each type of proton gives rise two separate peaks. Since these conformations do slowly

interconvert, a cross peak between the peaks for the identical proton in each of the conformations will arise due to this *chemical exchange*. This cross peak (blue) will have opposite phase of a true NOE crosspeak (for a small molecule).

A more subtle artifact to be on guard for is due to zero-quantum coherence (ZQC). This results in COSY-like crosspeaks due to *J*-coupling. These cross peaks are *anti-phase*, meaning they are dispersive in nature (both positive - blue - and negative - red -phase). They tend to be more prominent and likely for crosspeaks that are close to the diagonal. Note that peaks that are *J*-coupled are *also* likely to show an NOE. Thus, the presence of an anti-phase peak doesn't rule out an NOE.

Even more subtle are artifacts that result from spin diffusion. Long NOE mixing times can lead to spin diffusion, in which the perturbation of one proton leads to perturbation of a second (the regular NOE), and the now non-equilibrium-perturbed second proton causes perturbation in a third. This results in an NOE cross peak between the first and third proton, even though they may not be close in space (< 5 Å). It is as if the NOE is relayed from the first proton to the third proton, via the second proton.

Getting a good NOESY depends on good calibration of the 90˚ pulse and on a good choice of the mixing time. Mixing times of about $2/3*T_1$ generally provide maximum NOE enhancements, but, of course, different protons have different T_1 values, so a compromise value for the mixing time must be chosen. The COSY-like anti-phase peaks tend to be more of a problem if a mixing time is too short, while one that is too long can result in spin diffusion. In practice, a number of NOESY spectra are often acquired, with different mixing times used.