Chemical Shift Referencing for Biomolecular NMR

By

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The Need for Proper Referencing

NMR chemical shifts need to be described accurately fro two reasons. First, it is difficult or impossible to evaluate and compare NMR data between instruments, samples, and conditions without knowing exactly where the chemical shifts of the resonances are. Because NMR data are reported in units of ppm to make such comparisons possible, they require agreement on where the chemical shifts should be. This is best handled by establishing which frequency, in MHz, corresponds to 0.000 ppm and reckoning all other ppm values from that point. Second, the chemical shift contains meaning, but it must be known both precisely and accurately to capitalize on its value. Importantly, secondary structure can be inferred from a peak's position relative to the value tabulate for that residue when it is in a random coil; but these "secondary" chemical shifts are small, so peak positions must be known confidently within approximately 0.010 ppm for these secondary measurement to be meaningful.

The Reference Sample

To begin referencing, you need to have a spectrum with one peak whose chemical shift will be assumed accurate. Normally, we reference everything to the 0.000 ppm peak of DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate). It would be nice if we could reference H_2O or HDO, but its chemical shift varies strongly with temperature, and, to some extent, pH and ionic strength, so water is too unpredictable for precise referencing. It is perfectly legitimate to simply add DSS to your sample of interest, but DSS may interact your molecule, which will probably alter the reference compound's chemical shift and potentially compromise the accuracy of your results. DSS' long T_1 is also likely to give problems with t_1 ridges in multidimensional datasets.

Our normal strategy is therefore to measure the chemical shift of water relative to DSS in a "blank" sample, and assume its value is the same in the sample of interest. Thus, you need two samples for precise NMR: one containing your molecule of interest in an appropriate buffer, and one with DSS dissolved in that same buffer.

Collecting the Buffer+DSS Spectrum

At the spectrometer, with your buffer+DSS sample in the magnet, proceed normally with locking, shimming, tuning, and autocalibrating to find the tof value that confers optimal presat water suppression. Once you have good water suppression and a good ^{1}H 1D spectrum, record your tof value.

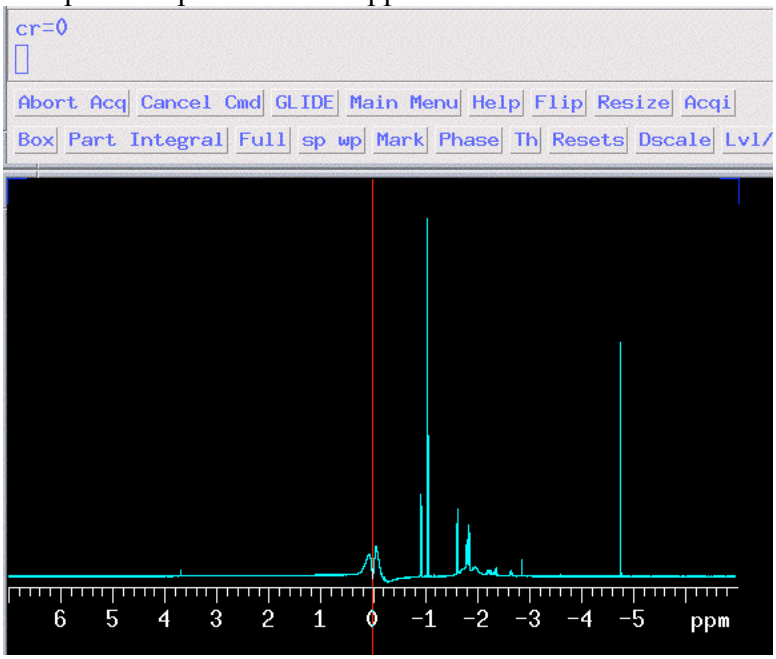
QUICK CALCULATION:

You can capitalize on a feature of the AutocalibrateNow routine to get a quick determination of water's chemical shift. When that procedure is complete and provides a good-looking ^{1}H 1D spectrum, it sets the frequency at the exact center of the spectrum to 0.000.

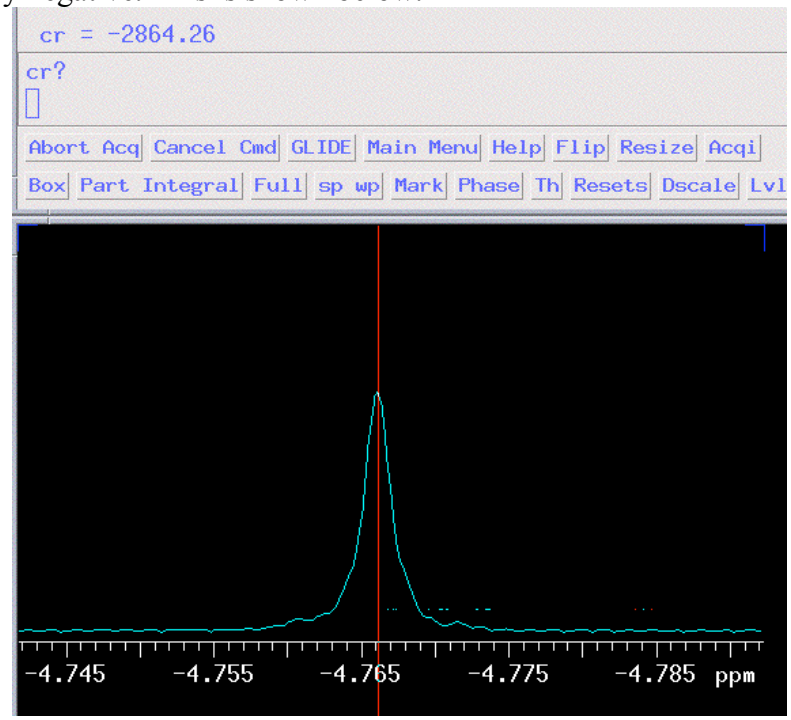
1) Expand on the most upfield DSS peak – it should be at approximately -4.6 – -5.0 ppm, depending on your sample's temperature.
2) Place your left cursor exactly in the center of the peak.
3) Type the following: r4=cr/sfrq r4?
4) The chemical shift of water, multiplied by -1, will then be displayed in the VNMR text display window.

EXPLANATION & ILLUSTRATION:

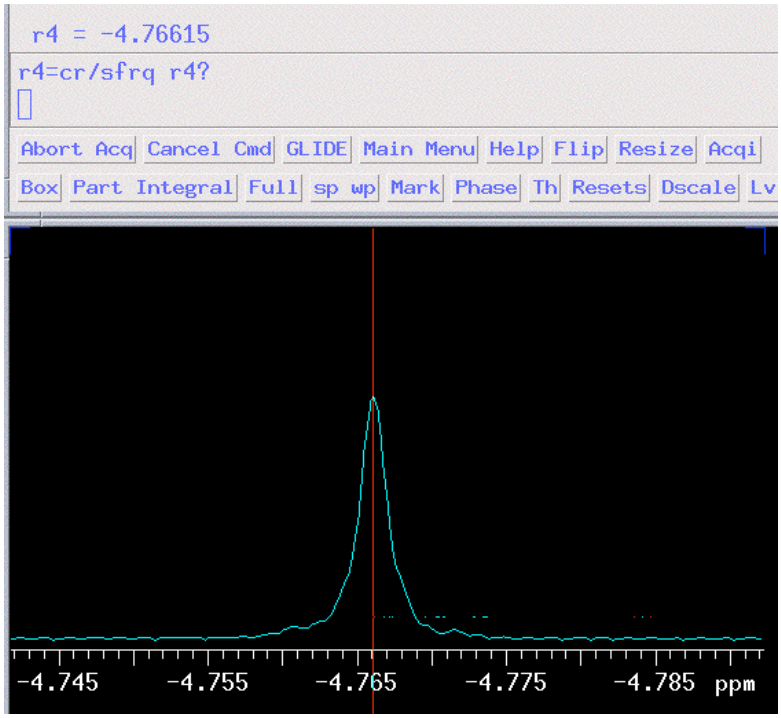
Consider the position of your cursor. “cr” is a variable describing the frequency at which you’ve placed the left cursor. If you type “cr?”, you’ll see the frequency corresponding to the cursor’s position appear in the VNMR text window. Also, if you type “cr=0” immediately after autocalibrating, your cursor will move to the exact center of the spectrum. This is shown below for a typical buffer sample. The peak near -4.8 ppm is the DSS line of interest.



If you query “cr?” when the cursor is on the reference DSS line, you get a frequency value that assumes water is at 0.000 ppm, which is just the same as the frequency of water when DSS is set to 0.000 ppm, only negative. This is shown below.



"cr" is given in units of Hz, so to convert to ppm, you simply divide the cr value by the spectrometer frequency in MHz (approximately 600 Hz/ppm on the 600s). "r4" is one of the handy scratch variables available on the VNMR command line. The phrase "r4=cr/sfrq" calculates water's chemical shift, and the query "r4?" displays it in the VNMR display. In the example illustrated below, the center of the spectrum remains at 0.000, the cursor is placed on the DSS line, and the typed instruction reveals that the chemical shift of water is +4.766 ppm. WRITE THIS NUMBER DOWN ONCE YOU GET IT!

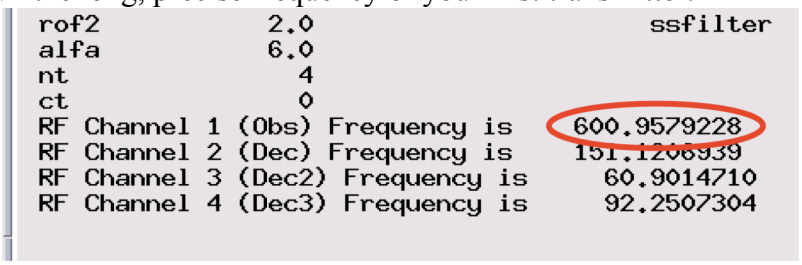


FAIL-SAFE CALCULATION

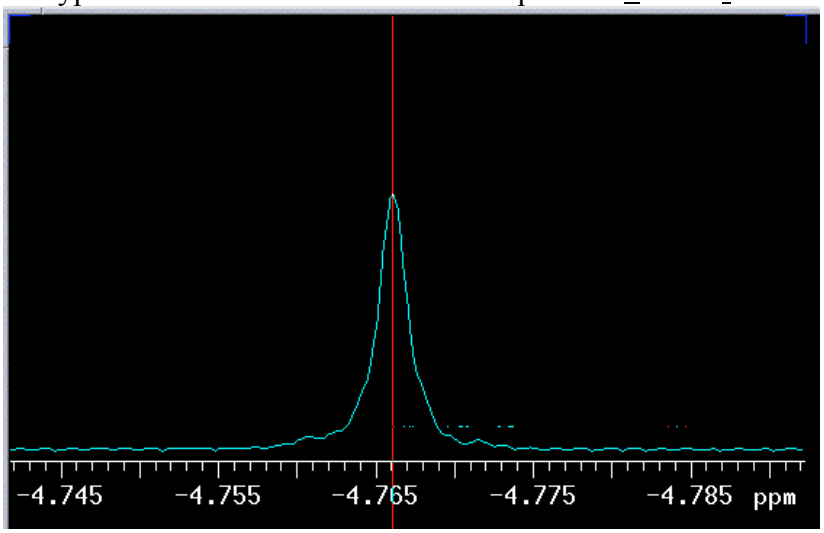
If you did something to change the chemical shift at the center of your spectrum before you did your computation, e.g. setting the reference ppm value with "rl" or simply changing the value of sw, you'll need a more extensive routine, described here:

Let's assume you acquired a spectrum of your buffer+DSS sample and you've loaded it into VNMR; this can be done from a saved spectrum as well as a freshly acquired one.

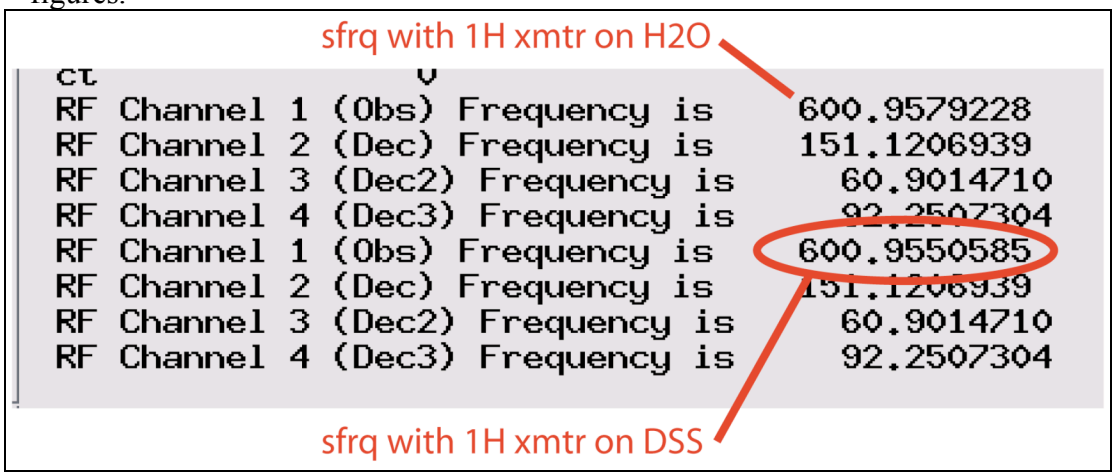
1) When examining that spectrum, type "spcfrq" to get the precise frequency settings for all four transmitters on your spectrometer. You'll only be interested in the first one, but typing "sfrq" doesn't provide enough significant figures for the precision you require. Write down the long, precise frequency of your first transmitter.



2) Next, expand on the DSS 0.000 ppm line. Place your cursor precisely on this line. You may wish to type "nl" to move the cursor to the tip of the nearest line.



3) Type "movetof". This sets the transmitter frequency to that line.
4) Now type spcfrq again and record the value of sfrq to the proper number of significant figures.



5) It would be prudent at this point to reset the tof to its original, optimized, value.
6) The difference between the two ^{1}H frequency values gives you the separation between the water and DSS frequencies, in MHz. Calculate this using a calculator or the Sun desktop calculator tool, because VNMR's "r"-variables cannot handle these long numbers unless you describe them in Hz instead of MHz. In the example figures shown here, the difference is

   600.9579228 – 600.9550585 = 0.0028643 MHz, or 2864.3 Hz.

7) Divide the difference number, in Hz, by water's frequency, in MHz.

   2864.3 (Hz) / 600.9579228 (Hz/ppm) = 4.766 ppm

8) The resulting number is water's chemical shift, in ppm! You're done!

Indirect Referencing of ^{13}C and ^{15}N

Determining accurate ^{13}C and ^{15}N chemical shifts for biological molecules is tricky because you generally don't use chemical shift standards like DSS for this purpose. Fortunately, math and physics will come to the rescue! For proper referencing, we'll use the fact that the gyromagnetic ratios of ^{1}H, ^{13}C, and ^{15}N (and other NMR-active nuclei) are known to large numbers of significant figures, therefore the ratios of their gyromagnetic ratios will be accurate and sufficiently precise.

For example, the gyromagnetic ratios ($\gamma/2\pi$) for ^{1}H and ^{15}N are 42.577481337 MHz/T (*CRC Handbook of Chemistry & Physics*, Online) and -4.314338631 MHz/T, respectively, so the ratio of the ^{15}N to ^{1}H constants is 0.101329118. (For these purposes, the sign of this ratio is unimportant.) Therefore, if we multiply the frequency corresponding to 0.000 ppm in the ^{1}H spectrum by 0.101329118, we get the frequency corresponding to 0.000 ppm in ^{15}N. EZ!

Here's a table of ratios of gyromagnetic ratios relative to ^{1}H for various nuclei, courtesy of the BioMagResBank (BMRB) at the University of Wisconsin and National Magnetic Resonance Facility at Madison (NMRFAM); see http://www.bmrb.wisc.edu/ref_info/cshift.html

	$\gamma(X)/\gamma(^{1}H)$	Reference 1	Reference 2
$^{1}H/^{1}H$	1.000000000	*J. Biol. NMR 6*, 135 (1995)	*Pure & Appl. Chem. 70*, 117 (1998)
$^{13}C/^{1}H$	0.251449530	*J. Biol. NMR 6*, 135 (1995)	*Pure & Appl. Chem. 70*, 117 (1998)
$^{15}N/^{1}H$	0.101329118	*J. Biol. NMR 6*, 135 (1995)	*Pure & Appl. Chem. 70*, 117 (1998)
$^{2}H/^{1}H$	0.153506088	- NMRFAM -	*Pure & Appl. Chem. 70*, 117 (1998)
$^{31}P/^{1}H$	0.404808636	- NMRFAM -	*Pure & Appl. Chem. 70*, 117 (1998)

Once we know the X-nucleus frequency at its 0.000 ppm and the frequency of the X-nucleus transmitter (obtainable by typing "spcfrq" or looking up "dfrq", "dfrq2", or "dfrq3" in the spectrum's procpar file), we simply compute the difference in frequencies, then divide by the transmitter frequency to yield the chemical shift at the center of that axis.

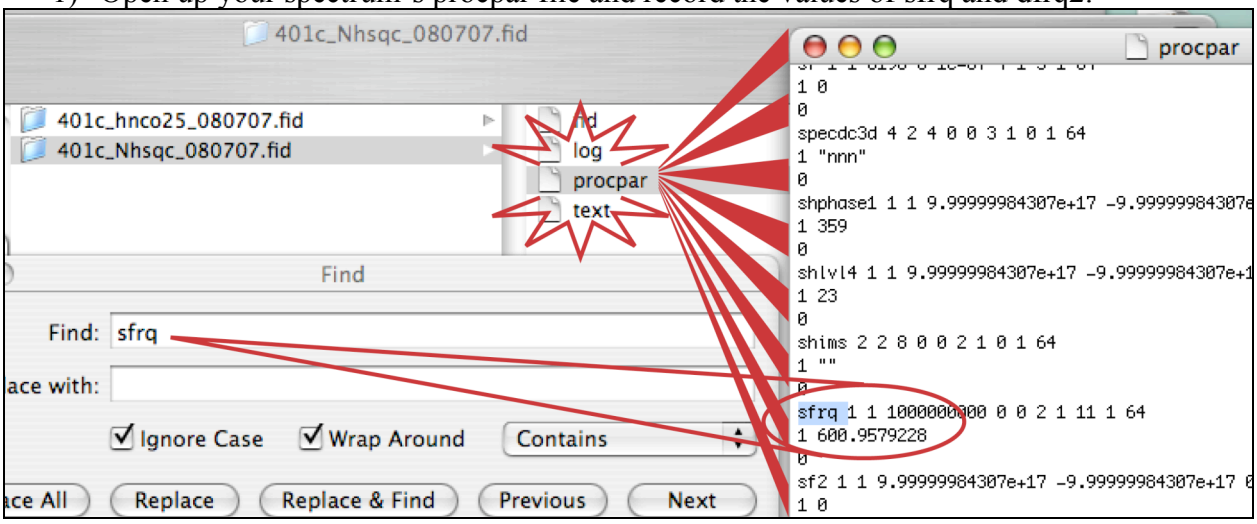
So here's the short list of tasks for indirect referencing (using ^{15}N for an example):

1) Obtain the long versions of the transmitter frequencies. If you're on the spectrometer, and have the spectrum loaded, type "spcfrq" to get the values for ^{1}H, ^{13}C, ^{15}N, and whatever frequency the 4th channel is set to, in that order. If you're using a different computer, open the spectrum's procpar file (recall every raw dataset, whose title ends in ".fid", is a folder/directory comprising four files: fid, log, procpar, and text) and find the values for "sfrq" and "dfrq2" ("dfrq" for ^{13}C).
2) Calculate the frequency, in MHz, of 0.000 ppm in ^{1}H.
3) Calculate the frequency, in MHz, of 0.000 ppm in ^{15}N by multiplying the 0.000 ppm ^{1}H frequency by 0.101329118.
4) Calculate the difference between dfrq2 and the 0.000 ppm ^{15}N frequency, in MHz.
5) Multiply by 10^{6} to convert MHz to Hz.
6) Divide by dfrq2 to convert Hz to ppm. This is the ppm value at the center of your ^{15}N dimension!

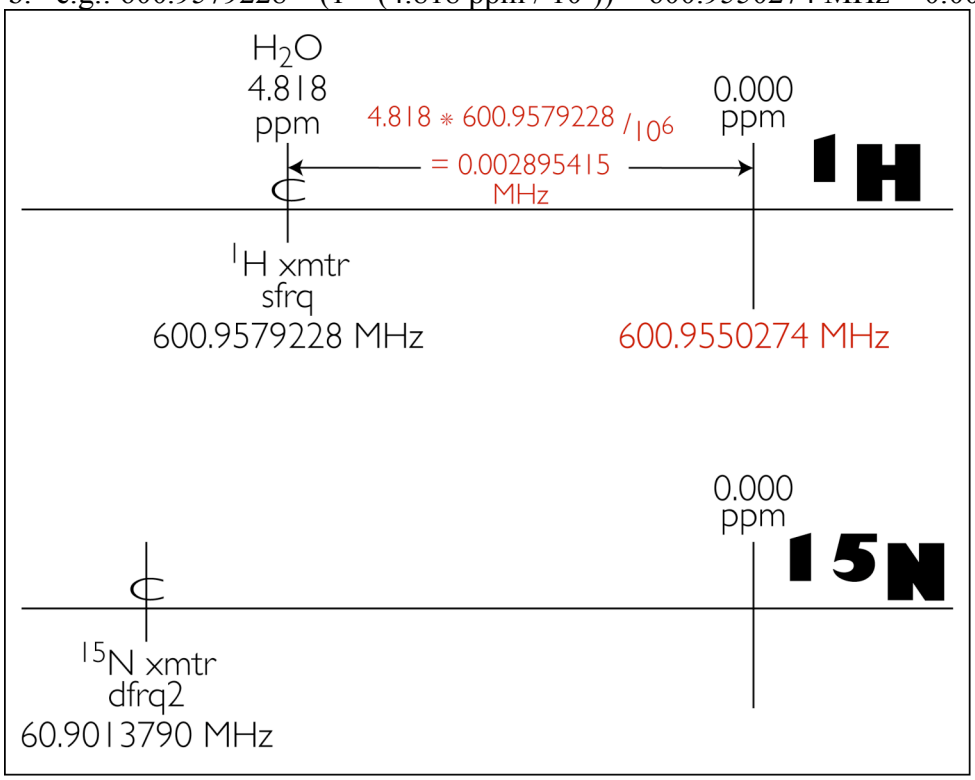
Illustrated Indirect Referencing Procedure

I like to think of this visually, so here's a redundant explanation with added illustration.

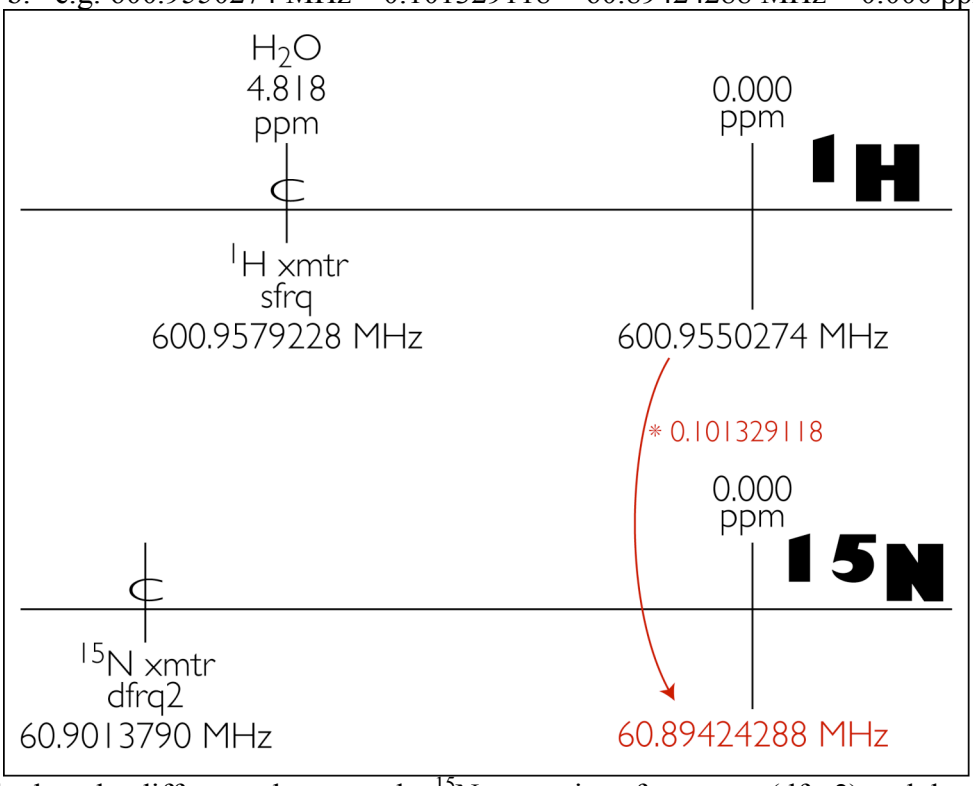
1) Open up your spectrum's procpar file and record the values of sfrq and dfrq2.



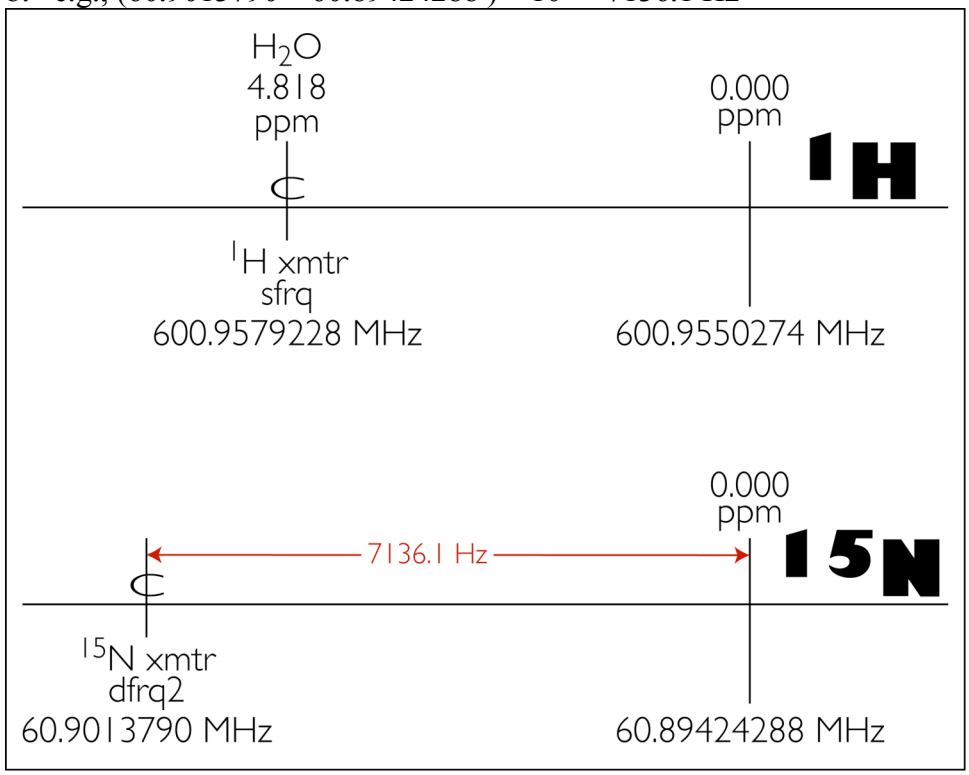
2) Calculate the frequency of 0.000 ppm in ^{1}H (0freqH) in units of MHz; this assumes you already know what the proper chemical shift of water is in your sample, in ppm (H2Oppm).
   a. 0freqH (MHz) = sfrq – (H2Oppm*sfrq/10^6) = sfrq * (1 – H2Oppm/10^6)
   b. e.g.: 600.9579228 * (1 – (4.818 ppm / 10^6)) = 600.9550274 MHz = 0.000 ppm



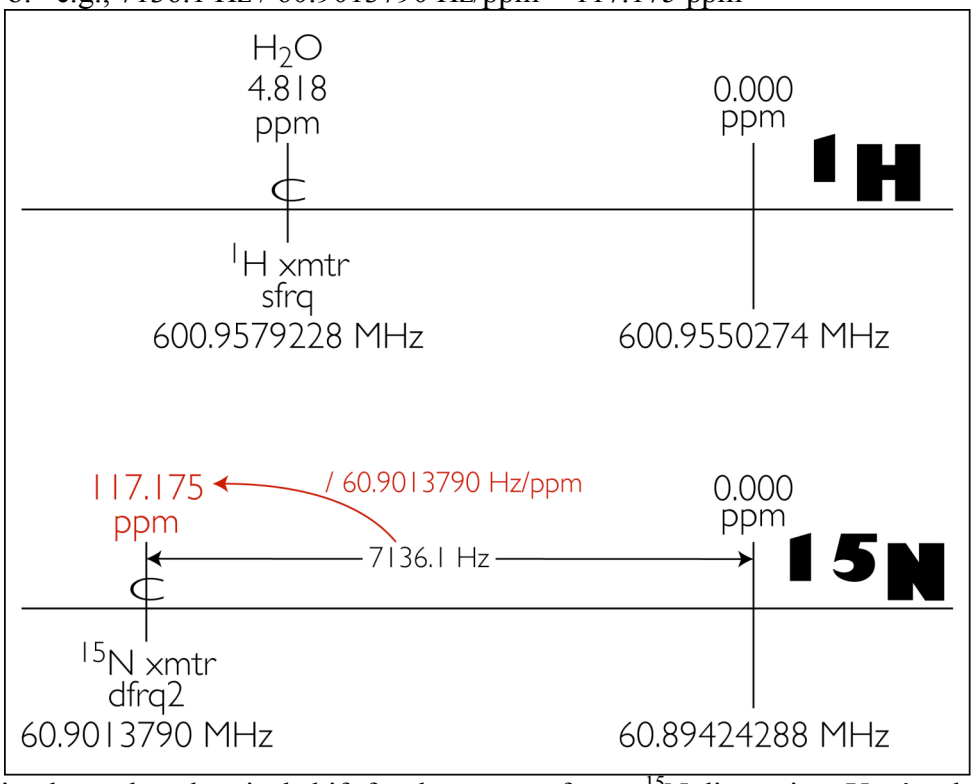
3) Calculate the frequency, in MHz, of 0.000 ppm in ^{15}N using the ratio of gyromagnetic ratios
   a. 0freqN (MHz) = 0freqH * 0.101329118
   b. e.g. 600.9550274 MHz * 0.101329118 = 60.89424288 MHz = 0.000 ppm in ^{15}N



4) Calculate the difference between the ^{15}N transmitter frequency (dfrq2) and the 0.000 ppm ^{15}N frequency; multiply by 10^6 to obtain in units of Hz.
   a. DN (Hz) = 10^6 * (dfrq2 – 0freqN)
   b. e.g., (60.9013790 – 60.89424288) * 10^6 = 7136.1 Hz



5) Convert that frequency value to ppm (ctrN) by dividing by dfrq2:
   a. ctrN = DN / dfrq2
   b. e.g., 7136.1 Hz / 60.9013790 Hz/ppm = 117.175 ppm



6) Write down that chemical shift for the center of your ^{15}N dimension. You're done!

Beware of Varian ^{1}H-^{13}C HSQCs!

Varian's BioPack hsqc for ^{1}H-^{13}C correlation, gChsqc.c, moves the center of the ^{13}C dimension around depending on which acquisition options are selected, yet the dof and dfrq values are the same in all circumstances. To know what the real center of your spectrum is, you must be mindful of the frequency offsets the pulse program imposes on the fly. By default, gChsqc assumes the center of the spectrum will be at 35 ppm; whether or not this is precisely true is irrelevant, but the real-time offsets the pulse sequence imposes are set with this number in mind.

Acquisition mode	Intended offset	Difference from apparent chem.. shift
aliph='y'	35 ppm	0
alphaC='y'	56 ppm	21 ppm, 21*dfrq Hz
arom='y'	125 ppm	90 ppm, 90*dfrq Hz
allC='y'	70 ppm	35 ppm, 35*dfrq Hz

So if you've acquired an "arom='y'" gChsqc spectrum, and your dfrq value is, say, 150.8697180 MHz, you need to add 90 ppm to this value to know the real frequency at the center of your spectrum: 150.8697180 * (1 + (90/10^{6})) = 150.8832963 MHz.