

Practical guide for quantitative 1D NMR integration

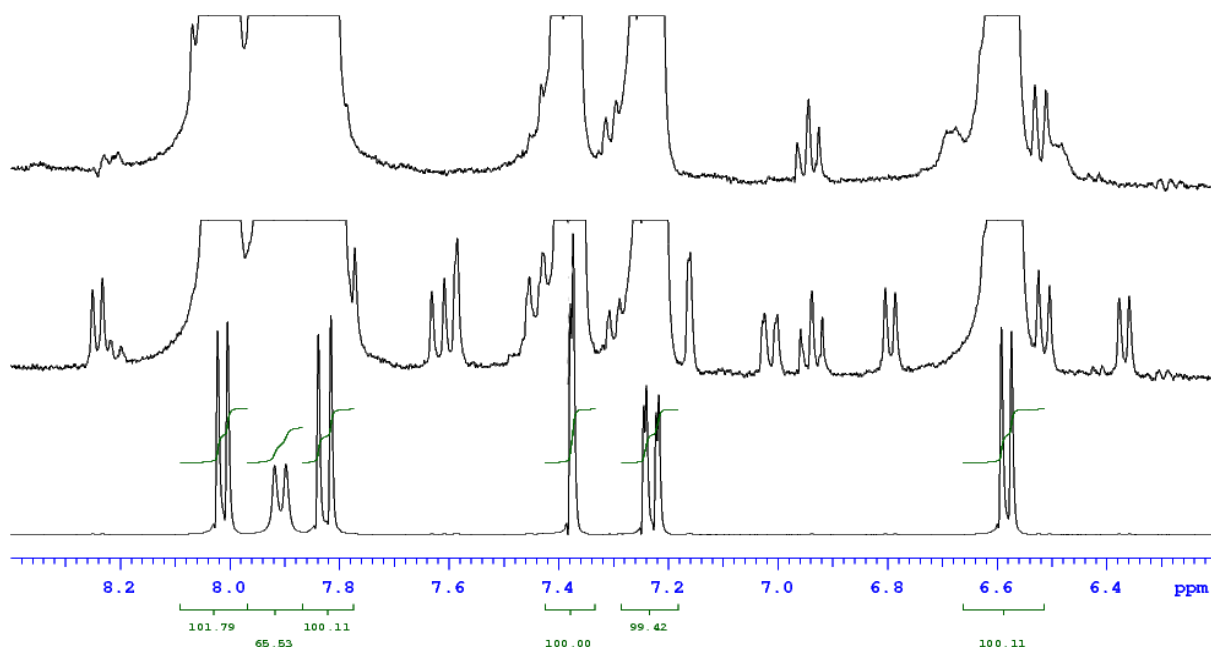
Eugenio Alvarado, University of Michigan, 05/10/10

The purpose of this manuscript is not to present a discussion about quantitative NMR, but to offer practical advice on how to set up appropriate parameters for acquisition and processing of 1D spectra to accurately measure integrals. Detailed information can be found in the references and in standard NMR textbooks. These notes refer mainly to proton spectra; quantitative analysis of spectra of other nuclei may require different settings, but these notes can be used as a guide.

It is important to keep in mind that the protocol used depends on the level of accuracy that you want to achieve. The following steps are aimed at getting about a 1% accuracy. If more is needed, you will need a more rigorous control of the parameters.

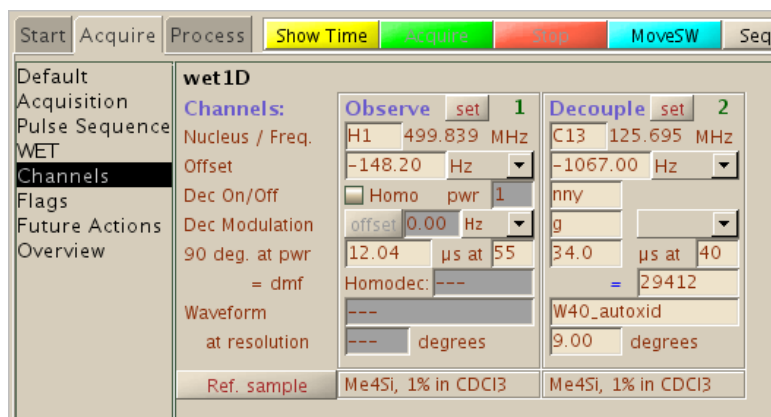
Acquisition

- 1. Shim the sample carefully and turn spinning off.** Accurate shimming is important for integration and may be crucial if signals overlap and deconvolution is needed. If spinning side bands are visible, it is advisable to turn spinning off.
- 2. If measuring ^1H , use ^{13}C decoupling during acquisition.** Carbon decoupling helps clean up the region around the base of the peaks. The ^{13}C satellites around each hydrogen peak account for 1.07% of the integration and should be taken into account in each integral region if an accuracy of 1% or less is required. The figure below shows the aromatic region of chloroquine; the lower trace is plotted at an usual intensity and the ^{13}C satellites cannot be seen; in the middle trace the intensity is 100 times higher and the satellites (and impurities) can now be clearly seen; the upper spectrum was acquired with ^{13}C decoupling (the small signals that remain are impurities). Notice that the satellites of a signal, when present, may overlap the integral regions of other signals and interfere with the measurements. Also, by using ^{13}C decoupling, the



integral regions do not have to be so wide to include the satellites and they will be easier to set up in crowded regions. It will also be easier to exclude impurities or determine their presence in the integrals.

To enable ^{13}C decoupling, go to the Acquire, Channels parameter panel and in the “Dec On/Off” field of the Decouple channel type nny, and in “Dec Modulation” type g. This turns on GARP decoupling during acquisition.



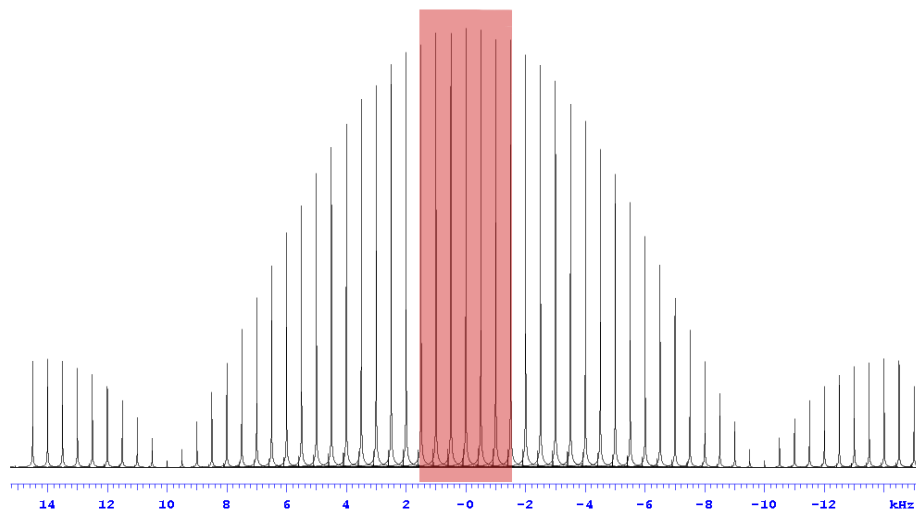
On the other hand, **if you are measuring any other nucleus (not ^1H or ^{19}F) and need proton decoupling, NOE effects from neighboring hydrogen atoms should be avoided** as these will affect your integrals. If you don't need proton decoupling, set “Dec On/Off” in the Decouple channel (H1) to nnn. If you need proton decoupling, use inverse gated decoupling by setting “Dec On/Off” to nny and “Dec Modulation” to w (waltz decoupling). If you are measuring fluorine, proton decoupling is off by default (there is no way of doing it except in our new vnmrs-500 spectrometer with two high frequency channels).

- Choose the spectral window so that all peaks of interest fall within 60% of its center.** Take a spectrum and choose a spectral window with about 20% of “empty” space on both ends of the spectrum. This is recommended in order to compensate for the signal attenuation that occurs at the spectral edges due to the receiver filters.
- Choose an appropriate digital resolution.** Each peak should be described by at least 5 points above its half width in order to have a reliable integration. The digital resolution (the spacing between data points) is defined by the spectral width (sw) divided by half the number of acquired points (np). Having previously defined an appropriate spectral width, the digital resolution is then determined by the number of points which in turn is assigned by the acquisition time (at). Assuming a half width of 1 Hz (typical for a medium size organic molecule), that means a digital resolution of 0.2 Hz. At 500 MHz with a 10 ppm spectra width, an acquisition time of about 5 seconds gives a digital resolution of 0.2 Hz. In general, for proton spectra, use an acquisition time of 4 to 5 seconds. When in doubt, take a survey spectrum, put the cursor in one of the peaks of interest and type dres to get the digital resolution and linewidth of the peak; if necessary, adjust the acquisition time and repeat the procedure. Keep in mind that highly symmetric compounds and some nuclei (fluorine comes to mind) may have very narrow peaks and long relaxation times.
- Use a 90° pulse with a long relaxation delay (d1).** All signals of interest must have relaxed

completely before each pulse. If this condition is not met, the next pulse will produce signals that will not show their full intensities and their relative areas will be incorrect. After a 90° pulse, the magnetization relaxes with a rate that is a function of the longitudinal relaxation time, T_1 , of the nucleus. Thus, after $3T_1$ the magnetization has recovered to 95.0% of its original size; after $5T_1$ the recovery is 99.3% and after $7T_1$ it is 99.91%. Therefore, to obtain an accuracy of 1% or less, the recycle time (the time between pulses) must be at least five times the T_1 of the slowest relaxing signal of interest in the spectrum. The major problem here is that usually the relaxation times of the nuclei in the sample are not known (although they can be quickly estimated with an inversion recovery experiment) and therefore some guessing may be required. Relaxation times for hydrogen nuclei in medium size molecules are *usually* from 0.5 to 3 or 4 seconds; while those for ^{13}C can go from 0.1 to tens of seconds for quaternary carbons. For example, for atropine (MW=289, $\text{C}_{17}\text{H}_{23}\text{NO}_3$) in non degassed chloroform, proton T_1 s go from 0.6 to 3.0 seconds; and ^{13}C T_1 s go from 0.6 to 11.7 s. So, assuming the longest ^1H T_1 in the sample is 3 seconds, the recycle time should be 15 s. As the recycle time in a 1D spectrum is the sum of the relaxation time d_1 and the acquisition time a_t , if the acquisition time is 5 s, the relaxation delay d_1 should be set to 10 s.

If you added an “integration standard” to you solution, keep in mind that the signals from the small molecules usually chosen as standards may relax much more slowly than typical organic molecules and can be ten seconds or more. If you use an standard and it doesn't relax completely, your calibration will be wrong. Also, carbon-13 and other nuclei can relax extremely slowly (tens of seconds). Use of relaxing agents in these cases may be very useful to speed up the measurements.

6. **Verify that the spectral width is 30% or less of the inverse of the pulse width: $sw \leq 0.3/pw$.** If it isn't, decrease the pulse width. This is not an issue with proton spectra because the spectral widths and pulse widths are short, but it may be important for other nuclei where spectral widths and pulses are large. A radio-frequency pulse doesn't excite all regions equally but has an excitation profile that has a shape similar to the one shown below for a $pw=100\ \mu\text{s}$. For quantitative integration, it is important that all signals be located within the middle 15% of the profile (shaded area), where their intensity variation will be less than 1% of the maximum intensity. The parameter pw is in units of microseconds; convert it to seconds to calculate the maximum sw . If you must know, the excitation profile function is: $f(\nu) = \sin(pw \cdot \pi \cdot \nu) / (pw \cdot \pi \cdot \nu)$.



For example, fluorine spectra have default pulse widths of about 5 μ s and spectral widths of 230 ppm, or more than 90 kHz. With those values, peaks near the left and right edges of the spectrum will not show their maximum intensity. The solution would be to change the pulse width to 3 μ s or to make sure that all the peaks of interest are located near the center of the spectrum in an area not larger than 60 kHz (0.3/0.000005).

7. **Collect enough scans to get a good signal to noise ratio.** The signal to noise should be higher than 1000:1 for integration errors of less than 1%. This is easy to do with proton spectra on spectrometers with indirect probes, but may not be so easy for other nuclei. Use solvent suppression if the sample contains non deuterated solvents. To calculate the signal to noise ratio of a peak, expand a section of the spectrum containing both a region with only noise and the peak of interest; then use both cursors to select a flat region containing with only noise, and type the command `dsnmax`. That command calculates the s/n of the tallest peak displayed relative to the average noise in the selected region.

Processing

1. **Use zero-filling and exponential weighting.** Zero filling means increasing the number of data points before Fourier transformation. This has been found to incorporate information from the dispersive part of the transformed spectrum into the absorptive part resulting in increased integral precision. To do a one time zero filling, type on the command line `fn=np*2` or to do a double zero filling type `fn=np*4`, then do the Fourier transformation as usual.

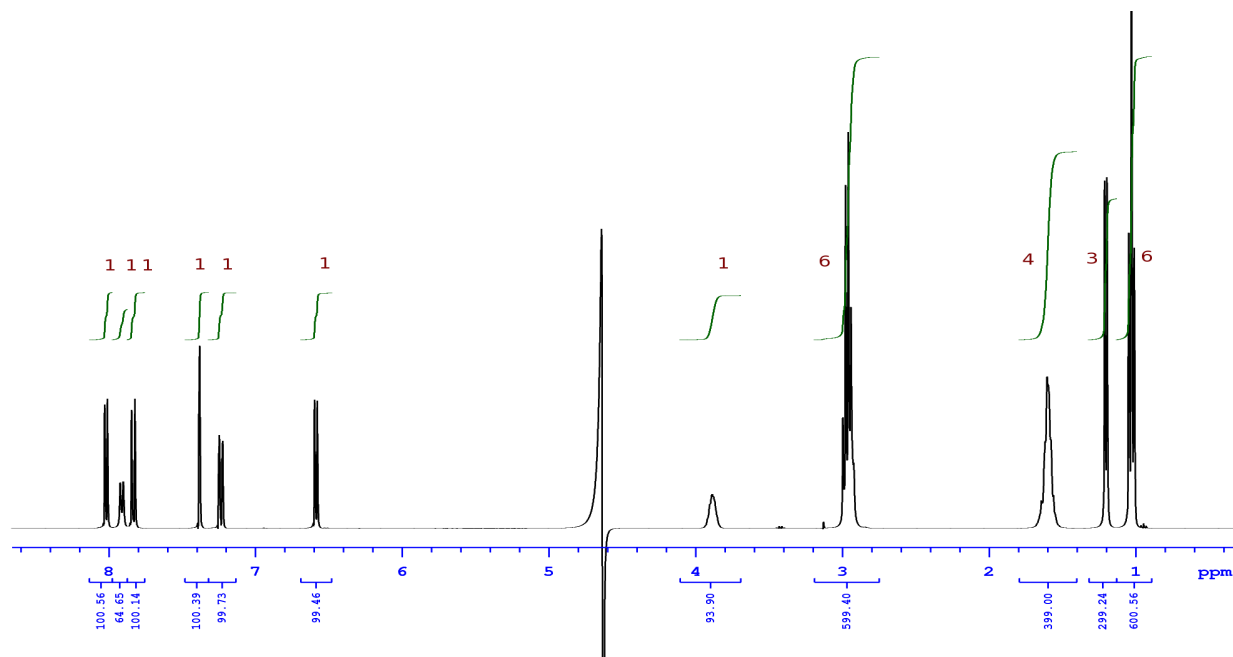
If the spectrum is noisy, you can also increase the amount of line broadening that is usually applied to most spectra to increase the signal to noise. Normally, an exponential weighting function is applied to the FID before transformation to increase signal to noise. The parameter `lb` controls the amount of s/n enhancement or line broadening applied; the higher the number, the higher the noise will be suppressed, but the more the peaks will be broadened. Usually, values from 0.2 to 1.0 are adequate for ^1H and ^{19}F . Other nuclei may benefit from much higher values (1-5 for ^{13}C , 10-100 for others). In theory, the best value is that which approximates the linewidth of the peaks in Hz. Enter a value for `lb` and Fourier transform with `wft`.

2. **Accurately correct the phase of the spectrum and the bias and slope of the integral.** You will obtain better results if you do it manually instead of relying on the macros that try to do it automatically for you.
3. **Use baseline correction to ensure a very flat baseline.** It is critical to have a very flat baseline in order to obtain accurate integrals. In order to do it in `vnmrj`, you must first indicate what points in the spectrum belong only to the baseline and should be flattened, and what points belong to peaks. This is done with integration regions. The software will know that the points outside those regions belong to the baseline and will try to synthesize a polynomial function that reproduces the shape of all those points. The function will then be subtracted from the spectrum to give a flat baseline. Display the integral trace and cut it in pieces; not too narrow, allow at least 0.1 or 0.3 ppm on either side of the peaks. Don't worry if the regions are not selective; these are only to define the baseline correction and we will define accurate regions later. All peaks must be inside a region, even the solvent and those peaks you don't want to measure. Then, apply a baseline correction with the command `bc (5)`. If for any reason, you

later need to re-transform the spectrum, this step must be repeated. Refer to the “Commands and Parameters Reference Manual” (Help menu in vnmrj) for more information about the `bc` command. The *MestreNova* software has a very impressive baseline correction routine that you can try.

- 4. Define appropriate integration regions.** Now that the spectrum is flat, we can reset the previous integration regions and define new ones with better precision. At this point, it is very important to realize that the peaks do not start to grow from the baseline at a defined distance from the tip like a pyramid, but they grow slowly from the infinite. The shape of an NMR peak is given by the Lorentzian function: $L(x)=h/(1+(x/w)^2)$, where h is the peak's height and w is its line width at half height. Obviously, we can't integrate every peak from $-\infty$ to $+\infty$, so we must set a practical limit. Griffiths and Irving have shown that in order to cover 99.0% of the area of any peak, the region must spread over a range that is at least 25 times the line width of the peak in both directions. For example, with a line width of 1Hz, the region should be 50 Hz wide (0.125 ppm at 400 MHz or 0.1 ppm at 500 MHz). To get an accuracy of 0.1% the regions must cover about 75 times the linewidth in both directions. Therefore, get a good estimation of your peaks' widths (command `dres`) and select your integral regions accordingly.
- 5. Use deconvolution if the peaks of interest overlap.** Integral regions should not overlap. For example, two singlets 1 Hz wide should be separated by at least 50 Hz (0.1 ppm at 500 MHz) to be able to be integrated individually with an accuracy of at least 1%. If they are not, a different method called Line Fitting or Deconvolution can be used. Essentially, this method attempts to reproduce the individual peaks in your spectrum thereby knowing their areas. Although vnmrj can do deconvolution, it is not easy to use. *MestreNova* on the other hand has a very nice implementation that you can try. Advanced Chemistry Development's *ACD/NMR Processor Academic Edition* can also do deconvolution and it is free for academic users. It can be downloaded from: http://www.acdlabs.com/resources/freeware/nmr_proc/index.php

As an example, the spectrum of chloroquine diphosphate in 90% H₂O is shown below. The spectrum



was acquired at 400 MHz using WET water suppression, with a relaxation delay of 20 s, acquisition time of 3.7 s, and $pw=6.6 \mu s$. The second signal from left to right, at 7.9 ppm, shows a lower than expected integration due to saturation exchange with the solvent (it is an NH). The signal at 3.9 ppm also shows a reduced integration due to partial saturation because of its proximity to the water signal being saturated. The expected normalized integrations are shown on red for each multiplet. The compound was used from the manufacturer's bottle without further purification (small amounts of impurities are clearly seen when the spectrum's intensity is increased). Except for the signals at 7.9 and 3.9 ppm, all integrals are within $\pm 1\%$ of the expected values even though the 3 regions between 7.8 and 8.1 ppm partially overlap each other.

References:

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