

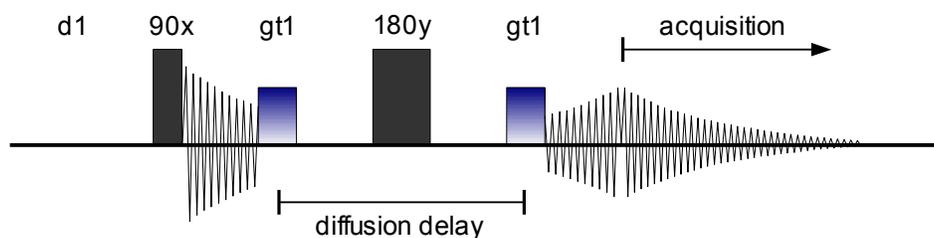
## Diffusion experiments with Vnmrj 2.2C and 2.2D.

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The diffusion coefficient of a molecule in solution depends on its effective molecular weight, size and shape, and can be used to estimate its relative molecular size (its hydrodynamic radius). It has applications in organic and inorganic chemistry<sup>3,5</sup>, for example to determine molecular weight in polymers, biopolymers and other aggregated materials. It can also be used to study molecular interactions and complexation processes in chemistry and to determine association constants. Recently, a method to estimate the molecular mass of small molecules in dilute aqueous and organic solutions was developed<sup>8</sup>.

The present manuscript refers to diffusion experiments with Vnmrj 2.2C and 2.2D and Chempack 4.1. The software contains several pulse sequences for different experiments. Check the Vnmr manual for details. Here we will use the pulse sequence DgsteSL\_cc (DOSY gradient compensated stimulated echo with spin lock and convection compensation), which is an enhancement of the classical PGSE (Pulsed Gradient Spin-Echo) pulse sequence.

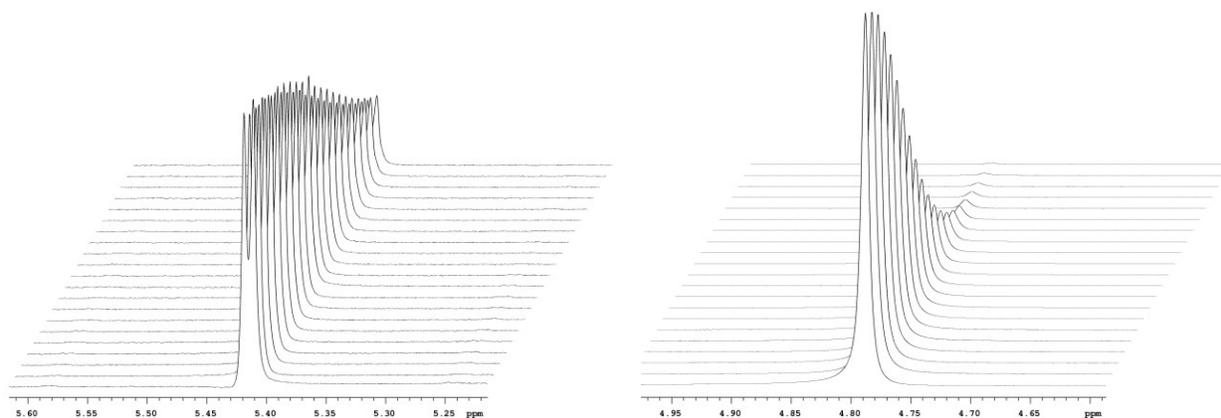
The PGSE pulse sequence, as originally proposed by Stejskal and Tanner 40 years ago is perhaps the easiest to understand, see the Figure 1 below. Briefly, after applying a  $90^\circ$  pulse, the nuclear spins of the sample will start precessing along the main magnetic field and dephase according to their absorption frequencies at this field. Then, a magnetic field gradient of strength  $g_{z1}$  and duration  $gt1$  is applied along the z axis of the tube and the spins will now be dephased due to their location in the gradient. After a short delay, a  $180^\circ$  pulse is applied that has the effect of inverting the precession direction. If the spins have not undergone translational motion along the z axis, the second applied gradient will be identical to the first, canceling its effect and the spins refocus (produce a spin-echo). However, if there was motion the effective magnetic field experienced by the spins during the second gradient will be different to the first and the spins will not completely refocus. The resulting signal will have a decreased intensity. The amount of attenuation is proportional to the displacement along the z axis, the gradient strength and the diffusion delay. Many other, more complex pulse sequences are available<sup>6,10,11</sup>, with portions aimed at increasing sensitivity and reducing artifacts, but they all follow the same principle.



**Figure 1.** Classic PGSE pulse sequence.

To measure molecular diffusion in a solution, a series of spectra with increasing gradient strengths must be recorded, Figure 2. By fitting the intensity (or integration) of the peaks to the Stejskal-Tanner function as described later, the diffusion constants can be calculated. And from the diffusion coefficients it is possible to calculate the hydrodynamic radius via the Stokes-Einstein equation<sup>2</sup>.

This document contains only enough information to setup a basic experiment with Varian's Vnmrj software and analyze the results. Extensive discussions of diffusion experiments and its applications can be found in the chemical literature, in the references below and in advanced NMR texts.



**Figure 2.** Sections of a DgsteSL\_cc experiment of a solution of sucrose in D<sub>2</sub>O. The anomeric hydrogen of sucrose at 5.42 ppm is shown on the left while the residual HDO signal at 4.79 ppm is shown on the right. Notice the sigmoidal shape of the decay and how the HDO signal decays faster.

## Experiment setup

*Some recommendations:* If your solvent is organic, it is advisable to have TMS in solution so it can be used as a reference in the calculation<sup>8</sup>. If it is D<sub>2</sub>O, the residual solvent signal (HDO) can be used as the reference. It is very important to regulate and have a very stable and homogeneous temperature. Extreme (low and high) temperatures may produce convection currents inside the tube that are difficult to avoid and lead to errors. During the experiment, the parameters should be chosen so that the intensities of the signals of interest decay from 100% to about 20%.

After inserting the sample and loading the shims, shim the magnet quickly (you will need to reshim later) and take a quick spectrum. Reduce the spectral width to your sample's needs and take a new spectrum. Display the optimized spectral width, transmitter offset, and gain (type `sw?` `tof?` `gain?`) and write down the results. When measuring diffusion on nuclei other than proton, the following parameters are also reset by the setup macro and the correct values will need to be reentered: `tn`, `dn`, `tpwr` and `pw` (set `pw` to the 90° value of the nucleus you want to measure). From the **Experiments** menu or from the **Experiment Panel** (on the **Holding** tab at the left side of `vnmrj`) select `DgsteSL_cc`. Set `sw`, `tof` and `gain` to the values you just found (and do the same for the other parameters if the nucleus is not H1). Set the desired temperature, for example by typing `"temp=25 su"` on the command line, and while the temperature is stabilizing, adjust the remaining parameters that follow. Most of the parameters can be set from the **Acquire, Pulse Sequence** panel shown below.

Of particular importance for this experiment are three parameters. The **Diffusion delay** (`de1`) is the amount of time allowed for the molecules to diffuse. Larger molecules will move slower and may require long periods of time for an accurate measurement, while small molecules move faster and require short diffusion delays. If the diffusion delay is set too short, the peaks will not have enough time to decay sufficiently for an accurate determination; and if it is too long, the signals will decrease in intensity to zero well before the end of the experiment and the last few spectra will contain only noise. Additionally, the diffusion delay should not be too long because signal intensity also decreases due to relaxation during this delay and thermal convection processes have more time to interfere with the experiment. It is for those reasons that in general the diffusion delay should be between 50 and 200 ms. If more time is required, it is better to increase the **diffusion gradient length** (`gt1`) instead. For example, a gradient length of 2 ms with a delay of 400 ms is equivalent to a gradient of 4 ms with a delay of 200 ms. Good values to start for medium size organic molecules in low viscosity solvents are: diffusion gradient length = 2 ms, diffusion delay = 200 ms. Double the gradient length for water and other viscous

solvents. Change the values of these parameters in the parameter panel as shown.

The screenshot shows the 'DgcsteSL\_cc' parameter panel with the following settings:

DOSY Parameters	
Diffusion gradient level	array
Diffusion gradient length	2.0 ms
Diffusion delay	200.0 ms
Off-center delay (del2)	0.0 ms
Grad. stab. delay	0.5 ms
1st HS gradient level	849
1st HS gradient duration	3.00 ms
2nd HS gradient level	1274
2nd HS gradient duration	3.00 ms
DAC to G 0.009419 Gauss/cm/DAC	
Probe in use id	

SEQUENCE parameters	
<input type="checkbox"/> Activate purge TRIM pulse	
Purge pulse power level	51
Purge pulse duration	2.00 ms

DOSY Setup	
Setup coarse gradient array	
Number of increments	15
Lowest gradient value	50
Highest gradient value	2040
Setup DOSY using conditions above	

DOSY Options			
<input checked="" type="checkbox"/> DOSY on/off			
<input checked="" type="checkbox"/> Alternate gradient sign on odd scans			
<input checked="" type="checkbox"/> Lock gating during gradients			
<input type="checkbox"/> Triax grad.			
<input checked="" type="checkbox"/> GRD-90-GRD Steady State Block			
Gradient strength	1380		
Gradient time	1.00 ms		
Solvent presaturation			
<input type="radio"/> never	<input type="radio"/> d1 only	<input type="radio"/> del only	<input type="radio"/> both
Power	0	dB	
Delay	2.000	s	
Frequency	599.539		

Buttons: Clear Subexp., Display Pulse Sequence

For this and other diffusion experiments we have to collect a series of spectra where the diffusion gradient (whose length we just defined above) has increasing gradient strengths. This is accomplished by setting up in an array the values of the Diffusion gradient level (`gzlv11`) from 0 to the maximum value allowed by the gradient amplifier. In our Inovas and 400MRs the maximum value is 2048. These numbers are in an arbitrary scale without units provided by a digital-to-analog converter (DAC). The calibration for these DAC numbers to gauss/cm was performed by the NMR Facility staff and is shown in the parameter panel as “DAC to G”. For example, in our Inova 500 with the “id” probe, `DAC_to_G` has been calibrated with D<sub>2</sub>O at 25 °C and has a value of 0.00961 gauss/cm·DAC. Thus the maximum gradient for this probe/instrument is 19.7 gauss/cm or 1.97 T/m. To setup the array you can either click on [Setup coarse gradient array] or on [Setup DOSY using conditions above]. The first sets up an array of only 8 gradients while the second sets up an array using the Number of increments shown above (15 is the default). The coarse array is useful for running a quick experiment to determine if the diffusion delay and diffusion length are appropriate to the sample.

Before starting the experiment select **Alternate gradient signs on odd scans** and **Lock gating during gradients**. If solvent presaturation is needed, check one of the options **d1 only**, **del only** or **both** and set the saturation frequency (it has to be determined before this experiment in a presaturation experiment). Do not increase the power to more than 5. Set the number of scans desired (`nt`), the relaxation delay (`d1`) and check the experimental time. When everything is what you want, click on [Acquire] to start the acquisition.

## Processing

Most of the processing can be done from the Process, DOSY process panel shown below. First, enable a line broadening of 0.3 or 0.5 and click on [Process all spectra]. Carefully phase the first spectrum and verify that the phasing is consistent for all spectra in the array using the buttons. Select integral mode and manually cut the integral into regions. Apply a baseline correction to all the spectra in the array with [Baseline correct all spectra]. This step is very important to obtain more accurate peak intensities or integrals. Select the peaks to use in the calculation with the minimum threshold tool . When you click on [Calculate full DOSY] the calculations will be performed and the results displayed in tabular form and a 2D dosy spectrum will be shown.

Notice that the calculation is always done with the value of `DAC_to_G` that was read from the configuration files when the spectrum was acquired. If you run your own calibration and want to re-calculate the diffusion

coefficients using your own value, click on [Recall original NMR spectra] and then, on the command line type: `setvalue('DAC_to_G', your_gcal, 'processed')` where *your\_gcal* is your DAC\_to\_G calibration. Then click on [Calculate Full DOSY] again.

The screenshot shows the Vnmrj software interface for DOSY analysis. It is organized into three main sections: Data, DOSY, and Misc.

- Data:**
  - 1D FT Size: fn [dropdown]
  - Acquired Points: np 16,384
  - Interactive Weighting:
    - line broadening: 0.5 [checkbox checked]
    - sinebell: 0 [checkbox]
    - shift: 0 [checkbox]
    - gaussian: 0.1 [checkbox]
    - shift: 0 [checkbox]
  - Process All Spectra [button]
  - Baseline Correct All Spectra [button]
  - Fiddle (TMS) [button] | Fiddle (no TMS) [button]
- DOSY:**
  - 2D Display Size in F2: fn2D 16k [dropdown] | fn1 256 [dropdown]
  - Calibration Flag [checkbox]
  - Use peak heights [radio checked] | Use integral values [radio]
  - Calculate Full DOSY [button]
  - Calculate Full DOSY with Dialogue [button]
  - Calculate Partial DOSY Spectrum [button]
  - Calculate Partial DOSY with Dialogue [button]
  - Lower Diffusion Limit: 0.0 m<sup>2</sup>/s [input]
  - Upper Diffusion Limit: 25.0 m<sup>2</sup>/s [input]
- Misc:**
  - Peak #: 1 [input] | Fit multiplier: 10.0 [input]
  - Auto Increment peak # [checkbox]
  - Show fit for Peak # above [button]
  - Show residual for Peak # above [button]
  - Recall DOSY Display [button]
  - Recall original NMR spectra [button]
  - Plot DOSY [button]
  - Clear Subexperiment [button]

Also notice that if the 2D display is being shown and you click on [Calculate Full DOSY] again, the data gets corrupted and you will have to reload and reprocess you spectrum again. If you want to recalculate a different section of the spectrum or use different parameters, click on [Recall original NMR spectra] first.

After the calculation, you can display the line fitting of an individual peak in the spectrum to the Stejskal-Tanner equation by entering a peak number in **Peak #** and clicking on [Show fit for Peak # above]. The actual data will be shown along with the fitted curve and a curve showing the differentials between the two. This is useful to determine if one or more of the individual spectra contains data that deviates considerably from the trend and may need to be discarded. When this happens, the [Calculate Full DOSY with dialog] can be useful. This button allows individual spectra to be omitted from the analysis. Remember to click on [Recall original NMR spectra] first.

According to the manual, the **Calibration Flag** option corrects systematic errors in the experiment. Unfortunately, the manual doesn't mention how these errors are corrected.

In general, the interface to this one and other diffusion experiments is very buggy; be cautious. For example, the "Fiddle" buttons (for deconvolution) do not work. The **Use Integral Values** option does not work. And do not click on the [Plot DOSY] button; not only it doesn't work, but it also freezes Vnmrj.

## Manual analysis

While Vnmrj's analysis routines give you a quick and easy way to calculate diffusion coefficients from the spectra, you will get better control of the analysis by measuring peak heights or integrals and doing your own calculations. Stejskal and Tanner have shown that the intensity of the signals in diffusion experiments is described by the following equation:

$$\ln(I/I_0) = -\gamma^2 \delta^2 G^2 (\Delta - \delta/3) D$$

where:

$I$  = intensity or integral of the peak at a given  $G$

$I_0$  = intensity or integral of the peak at  $G = 0$

$\gamma$  = magnetogyric constant of the nucleus (for  $^1\text{H}$ ,  $\gamma = 2.675 \times 10^8 \text{ T}^{-1} \text{ s}^{-1}$ )

$\delta$  = diffusion gradient length (parameter  $gt1$ )

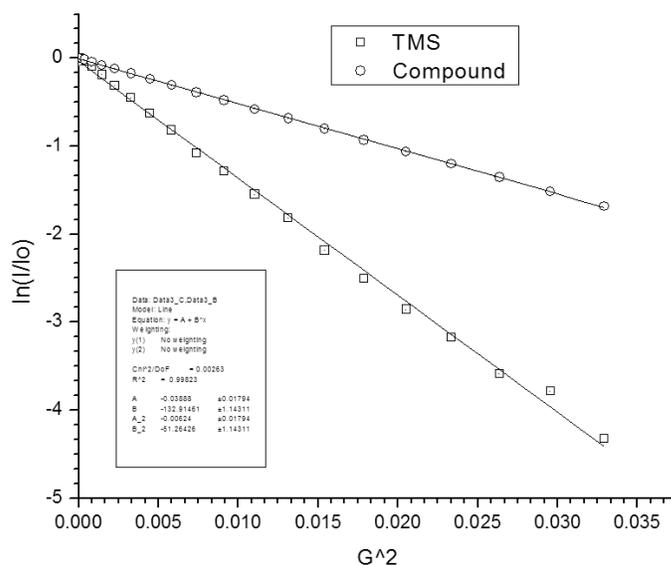
$\Delta$  = diffusion delay (parameter  $del$ )

$G$  = gradient field strength ( $gzlvl1[n] * \text{DAC\_to\_G}$ )

$D$  = diffusion coefficient

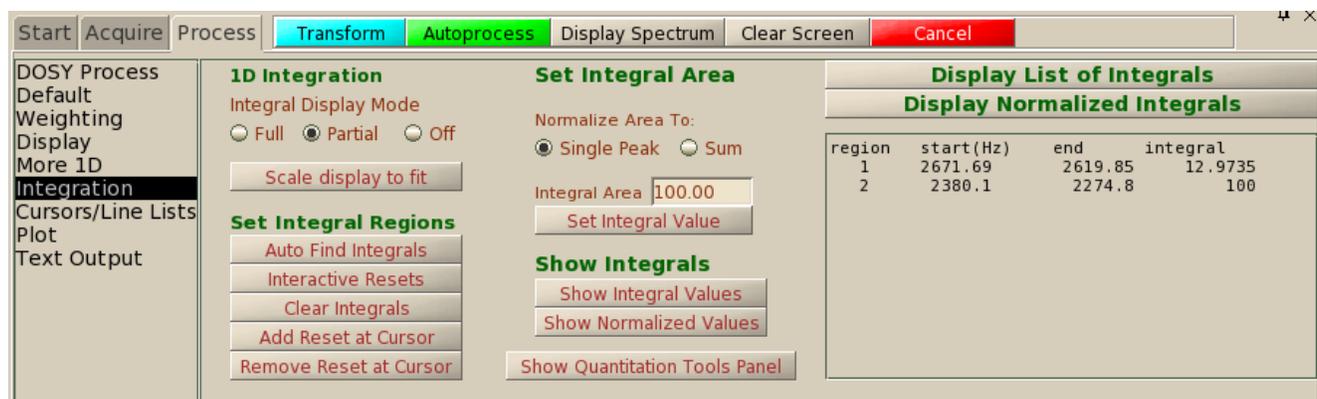
With the list of integrals or intensities, a Stejskal-Tanner attenuation plot can be constructed (see Figure 3). This is a plot of  $\ln(I/I_0)$  vs.  $G^2$ , and from the slope of this plot the diffusion coefficient  $D$  can be extracted.

Alternatively, the data can also be fitted directly to the equation  $I = I_0 \exp[-(\gamma \delta g)^2 (\Delta - \delta/3) D]$  using line fitting programs like QtiPlot, Origin, Scientist, SigmaPlot, etc.



**Figure 3.** Stejskal-Tanner plot of a solution of a ruthenium coordination complex in  $\text{CD}_2\text{Cl}_2$  at 25 °C. From the plot, the diffusion coefficients of TMS and of the compound were measured as  $23.3 \times 10^{-10}$  and  $9.0 \times 10^{-10} \text{ m}^2/\text{s}$  respectively. Having TMS in the solution allows the determination of the solution's viscosity and of the compound's hydrodynamic radius<sup>8</sup>. The measured compound's radius gives an insight into its dimerization process ( $A \leftrightarrow AA$ ).

To produce a list of integrals, transform the spectra, select integral regions, carefully correct the bias and slope of the integrals and perform baseline correction as described before. Display the **Process, Integration** panel (shown below), select **Partial** under **Integral Display Mode**, position `vnmrj`'s cursor on top of one of the integrals, select **Single Peak** under **Normalize Area To:**, type in a number in **Integral Area** and click on **[Set Integral Value]**. The list of integrals for the current spectrum only, normalized to the value entered will be shown on the right side of the panel. You can now generate a list of integrals for all the spectra with the macro `UMdli`. With this macro you can have the list printed or emailed to you in a format convenient to copy and paste to a line fitting program.



With the diffusion coefficient, the hydrodynamic radius of a compound in solution can be calculated from the Stokes-Einstein equation:

$$r_H = \frac{k_b T}{6 \pi \eta D}$$

where

$k_b$  = Boltzman constant,  $1.3806 \times 10^{-23} \text{ kg m}^2 \text{ s}^{-2} \text{ K}^{-1}$

T = temperature

$\eta$  = viscosity of the solution at temperature T

D = diffusion coefficient

## Calibration

The parameter `DAC_to_G` must be calibrated to do the calculations. This parameter is a conversion for the units of the gradient amplifier (DAC units) to gradient in gauss/cm. The parameter has already been calibrated in our spectrometers but you may wish to verify it or recalibrate it yourself. This is done with a sample of known diffusion coefficient like HDO in D<sub>2</sub>O (the residual solvent peak in D<sub>2</sub>O). Regulate the temperature and allow at least 10 minutes to equilibrate, run a diffusion experiment and after Fourier transformation and baseline correction select **Use Peak Heights**, expand the region around the HDO peak and type “`dosy_grad_calib`” to recalibrate. The macro will ask for the expected value of the peak in units of  $10^{-10} \text{ m}^2/\text{s}$  (e.g., for HDO at 25 °C enter 19.02). Known values<sup>4</sup> can be found in the table below. Use a diffusion gradient length of 2 ms, a diffusion delay of 100 ms and a long relaxation delay of at least 10 seconds for D<sub>2</sub>O at 25 °C (it is better to use D<sub>2</sub>O doped with 0.1 mg/mL GdCl<sub>3</sub> so that a shorter delay can be used). Type `DAC_to_G?` to print the new calculated value.

Sample	Diffusion coefficient <sup>4</sup>
10% D <sub>2</sub> O in 90% H <sub>2</sub> O at 25 °C	22.7 x10 <sup>-10</sup> m <sup>2</sup> /s
HDO in D <sub>2</sub> O at 5 °C	10.34 x10 <sup>-10</sup> m <sup>2</sup> /s
HDO in D <sub>2</sub> O at 25 °C	19.02 x10 <sup>-10</sup> m <sup>2</sup> /s
HDO in D <sub>2</sub> O at 45 °C	30.27 x10 <sup>-10</sup> m <sup>2</sup> /s
sucrose in D <sub>2</sub> O at 25 °C	4.4 x10 <sup>-10</sup> m <sup>2</sup> /s (this lab 01/18/10)

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## Appendix 1. Useful constants

Solvent	Temp, °C	Viscosity ( $\eta$ ), $\text{kg} \cdot \text{s}^{-1} \cdot \text{m}^{-1}$
D <sub>2</sub> O	20	1.2467 x 10 <sup>-3</sup>
	25	1.095 x 10 <sup>-3</sup>
H <sub>2</sub> O	20	1.0016 x 10 <sup>-3</sup>
	25	0.8909 x 10 <sup>-3</sup>
CDCl <sub>3</sub>	25?	0.55 x 10 <sup>-3</sup>
	20	0.57 x 10 <sup>-3</sup>
CD <sub>2</sub> Cl <sub>2</sub>	25	0.417 x 10 <sup>-3</sup>
	20	0.436 x 10 <sup>-3</sup>
CH <sub>3</sub> OH	20	0.59 x 10 <sup>-3</sup>
CD <sub>3</sub> OD	20	0.52 x 10 <sup>-3</sup>
Acetone-d <sub>6</sub>	20	0.34 x 10 <sup>-3</sup>
DMSO-d <sub>6</sub>	20	2.4 x 10 <sup>-3</sup>
Toluene-d <sub>8</sub>	20	0.58 x 10 <sup>-3</sup>
Benzene-d <sub>6</sub>	20	0.69 x 10 <sup>-3</sup>
Acetonitrile-d <sub>3</sub>	20	0.39 x 10 <sup>-3</sup>
Pyridine-d <sub>5</sub>	20	0.97 x 10 <sup>-3</sup>

From <http://www.chimorg.unifi.it/public/chimichi/nmrsolv.html> and other sources.

Some conversion constants:

$$1 \text{ Pa}\cdot\text{s (Pascal-second)} = 1 \text{ kg s}^{-1} \text{ m}^{-1}$$

$$1 \text{ P (1 poise)} = 1 \text{ g}\cdot\text{cm}^{-1}\cdot\text{s}^{-1}$$

The relation between poise and pascal-seconds is:

$$10 \text{ P} = 1 \text{ kg}\cdot\text{m}^{-1}\cdot\text{s}^{-1} = 1 \text{ Pa}\cdot\text{s},$$

$$1 \text{ cP} = 0.001 \text{ Pa}\cdot\text{s} = 1 \text{ mPa}\cdot\text{s}.$$

## Appendix 2. For versions previous to vnmrj 2.2C/chempack 4.1.

Earlier versions of vnmrj contain even more bugs than 2.2C; be careful. Essentially you can follow the same procedures described here but you will have to enter all parameters manually. The full list of parameters and other information about the experiment can be found in the manual page (go to the *Process/Text Output* panel and click on [Sequence Manual]). Run `DgcsteSL_cc` and verify or modify the parameters (suggested starting values are in parenthesis):

Parameter	Typical value	Comment
del	0.1-0.5 (0.2)	diffusion delay (in seconds)
gt1	0.001-0.005 (0.002)	Diffusion gradient length (in seconds)
gzlv11	10-2000	Diffusion gradient level, arrayed (in dac units)
d1	3-5 (5.0)	relaxation delay (seconds)
nt	8*n (8)	number of transients
spin	0	sample spinning must be off
ss	8	steady state transients
lb	0.5	line broadening

Set up a linear array of `gzlv11` values (Menu: *Acquisition>Parameter arrays*). Typically 20 values, from 10 to 2000 in increments of 100. In principle, the first value should be 0 but in practice this value gives unpredictable results; use 10 instead. Use the command `array('gzlv11', 20, 0, 100)`  
`gzlv11[1]=10` to setup the array or set it up from the menu *Acquisition > Parameter Arrays*.

When the experiment is finished, transform and phase the first or second spectrum. Integrate the spectrum and define all integration regions (even those not needed). Apply a baseline correction to all the spectra in the array manually or with `UMbc`. Normalize one region to 1 or to 100 and create a list of integrals for all the spectra in the array with the `UMdli` macro. Process the data manually.