

Diffusion Ordered Spectroscopy (DOSY)

1. **"Basic" Stimulated Echo DOSY:** the stimulated echo experiment (Fig 3) is (see papers by Stejskal & Tanner) is the standard backbone of many diffusion experiments.
2. **Convection-Compensated (CC) DOSY:** in a variable temperature experiment or when utilizing a cryoprobe, convection currents will exist in your sample. These can affect your NMR data, so you should utilize a cc-DOSY experiment in these cases. (Fig. 4)

For the lab, the sample is an "energy drink" mixture comprised of caffeine, taurine, and glucose 6-phosphate, combined with the aqueous standard DSS in D₂O.

1. DOSY

DOSY is a method of measuring translational self-diffusion of molecules using pulsed field gradient NMR spectroscopy. Self-diffusion depends on physical parameters such as size and shape of the molecule, temperature and viscosity. Assuming a spherical-sized molecule, the diffusion coefficient D [m²/s] is described by Stokes-Einstein equation:

In NMR, we don't measure the diffusion coefficient directly; instead we measure NMR

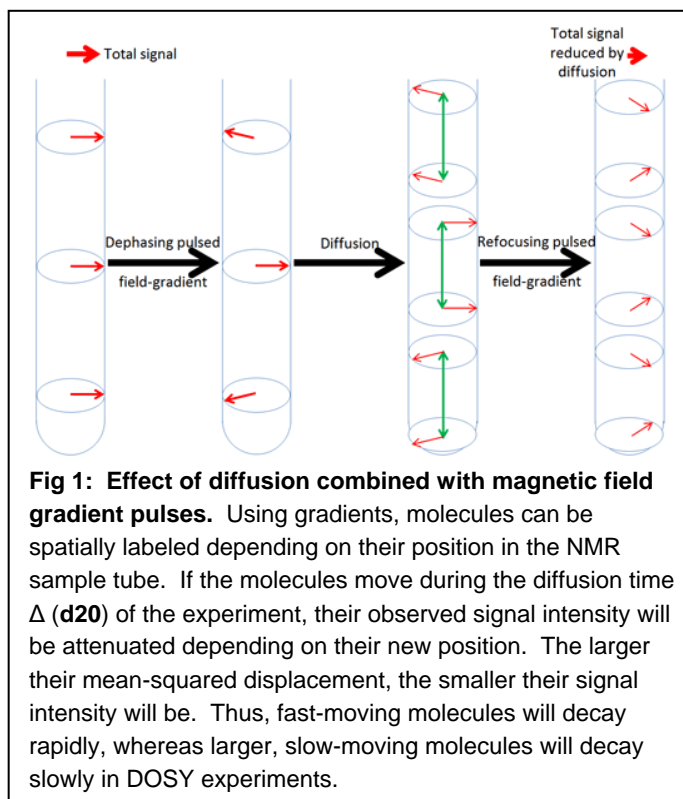
$$D = \frac{k_B T}{6\pi\eta r} \quad \text{in which}$$

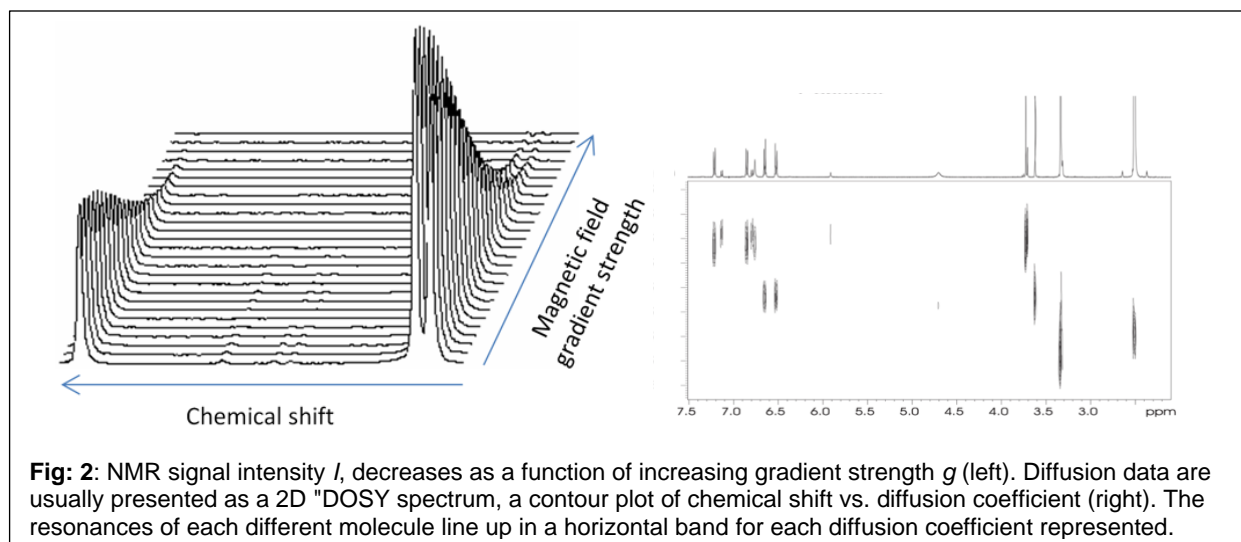
$k_B \equiv$ the Boltzmann constant [J/K]
 $T \equiv$ temperature [K]
 $\eta \equiv$ the viscosity of the liquid [Pa · s]
 $r \equiv$ the hydrodynamic radius of the molecule [m]

signal intensity as a function of diffusive behavior and fit the data to an equation. Observed signal intensity I is attenuated depending on the value of the diffusion time Δ (d20) and the gradient parameters: the gradient strength g (depends on the probe) [T/m], and the length of the gradient pulse δ (p30) [s]. See Figures 1 & 2 for more information.

$$I = I_0 \exp \left[-D \gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3} \right) \right] \quad \text{in which}$$

$I \equiv$ NMR signal intensity for a given Δ , g and δ
 $D \equiv$ diffusion coefficient [m²/s, sometimes cm²/s]
 $\gamma \equiv$ gyromagnetic ratio of the observed nucleus [MHz/T]
 $g \equiv$ applied gradient strength [T/m, sometimes G/cm]
 $\delta \equiv$ the length of the gradient pulse [s]





A successful DOSY experiment requires careful optimization/set-up of the following:

- **Excellent Temperature Uniformity:** thermal gradients/convection have an effect.
- **Sample Preparation:** if you are using the convection-compensated sequence and believe convection could be still causing a problem (e.g., your D is off by an order of magnitude or you only see one diffusion constant when you expect 2 or more) there are a few things you can do:
 - Only add enough (40 mm) of sample to the NMR tube
 - Use a 3 mm NMR tube which reduces convection
 - Use a Shigemi tube which reduces temperature gradients across a smaller sample volume.
- **Shimming, Lineshape & Resolution:** If your NMR signals blend into each other, it will be hard to resolve the DOSY spectrum. 1D spectra must have good SNR, be well-phased, and have a flat baseline.
- **Experimental Parameters** such as pulse sequence, gradient strength, duration, range, etc. will need to be carefully chosen based on the size and diffusive behavior of the molecules being studied. Larger/slower molecules need stronger/longer gradients to generate usable data, but pushing these parameters to their limits may lead to non-linear responses, eddy current effects, etc. that can compromise the results. Some pulse sequences work well for smaller/faster vs. larger/slower and long spin-spin relaxation time T_2 vs. short T_2 samples.

The basic steps of any DOSY experiment are:

- 1) Obtain a 1D spectrum
- 2) Load the DOSY pulse sequence/parameter set
- 3) Measure a pair of 1D NMR spectra: one with small g and one with large g , Compare the signal intensities.
- 4) Run a 2D series with data points in between the weak and strong gradients.
- 5) Extract the integrals for each signal of interest. Process the data and fit the attenuation curves to: $I = I_0 \exp \left[-D \gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3} \right) \right]$. You can either use the exponential form of the equation or the linearized form: $\ln \left(\frac{I}{I_0} \right) = -D \gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3} \right)$.

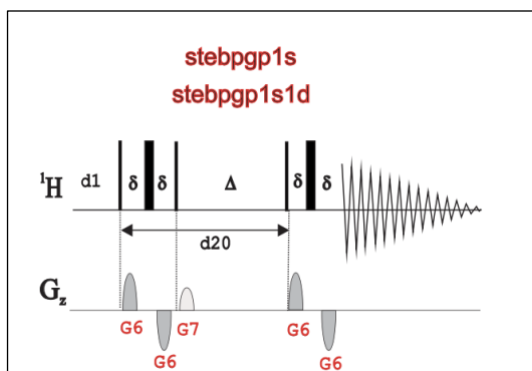


Fig. 3: The "basic" DOSY experiment varies the gradient strength while holding the diffusion time $d20$ (Δ) constant. A stimulated echo allows you to use a Δ that depends on T_1 relaxation rather than T_2 . `stebpgp1s(1d)` pulse sequence stands for STimulated Echo with BiPolar Gradient Pulses and 1 Spoiler gradient. This sequence is the backbone sequence of many diffusion pulse sequences.

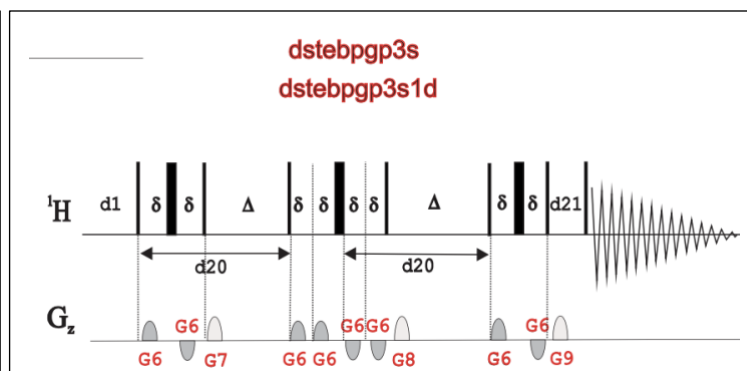


Fig. 4: The convection-compensated DOSY experiment `dstebpgp3s(1d)` is a Double STimulated Echo with BiPolar Gradient Pulses and 3 Spoiler gradients. contains a stimulated echo as its backbone. You vary the gradient strength (holding the diffusion time $d20$ (Δ) constant. The double stimulated echo cancels constant velocity effects, which minimizes the effect of translational motion due to convection currents. However, this sequence results in $\frac{1}{2}$ of the NMR signal compared to non-cc sequences.

DOSY Data Acquisition

Set the VT:

1) For an "ambient" room temperature measurement, set the VT to 25°C (298 K) and allow the sample to equilibrate for 5-10 minutes before you start acquiring data. To this, type `edte` and set the temperature. While you are waiting, you can start to set-up the different experiments-- but don't actually run anything yet! If there has been a group before you in lab, the sample will already be equilibrated and you can move forward.

Get a "routine" 1D ^1H spectrum:

2) For this, you can create a **new** experiment and read in the standard proton parameter set i.e., `rpar 1h.av500 all`. Lock to `D2O`, `getprosol`, and `atma`, but *wait until the sample is equilibrated to do any acquisition* (i.e., wait to `topshim convcomp;rga;zg`). You can feel free to change any parameters (`sw`, `o1p`, `ns`, `d1`, etc.) in `ased`, or set up the experiments you need for step 3 while you're waiting for the sample to equilibrate.

Once the sample has reached temperature equilibrium, you can tune the probe, shim, and acquire data. Before you start the next step, make sure you calibrate your 90° pulse length with `pulsecal`. A carefully calibrated pulse is very important for accurate diffusion measurements!

1D Optimization of Δ (`d20`) and δ (`p30`)

3) Create another experiment (you can use `iexpno`). Use `pulprog` to change the pulse sequence to `stebpgp1s1d`. For other acquisition parameters ("AcquPars"/`eda/ased`), use the following values:

recycle delay [s]	<code>d1</code>	2
number of scans [#]	<code>ns</code>	8
dummy scans [#]	<code>ds</code>	4

shaped gradient for diffusion-encoding gradient (gpnam6) and spoiler gradient (gpnam7)	gpnam6, gpnam7	SMSQ10.100
gradient 7 strength [%]	gpz7	-17.13
90° pulse length [us]	p1	Your calibrated 90° pulse length

4) All diffusion experiments allow you to optimize your diffusion experiment parameters. There are three main parameters in the diffusion experiment:

- Gradient strength g : this parameter in TopSpin is **gpz6** [1-98%]
- Gradient pulse length δ : this parameter in TopSpin is **p30** [0.5-3 ms]
- Diffusion time Δ : this parameter in TopSpin is **d20** [~50 ms to T_1 of the slowest relaxing compound]

5) To start with, set **gpz6** = 2 %, **p30** = 1000 us (1 ms), and **d20** = 0.1 s. For any remaining acquisition parameters in **ased**, use the suggested value in brackets shown to the right of the field.

6) Set **lb** = 3 (under **edp**/"ProcPars") and then set the receiver gain with **rga** and collect/process a 1D spectrum with **zg;ef;apk;abs**. If the spectrometer complains about acqt0 after you type zg, you can ignore the error and continue. ☺ (Aside: my favorite TopSpin error message is: "Error: no error."). You may need to phase the data manually; see the handout on Canvas if you need assistance.

7) Create another experiment with **iexpno**. Change the value of **gpz6** = 75. Then acquire and process a new 1D spectrum with **zg;ef;apk;abs**. You may need to phase the data manually.

- This spectrum should be much less intense. Overlap this spectrum using the **Multiple Display Tool [.md]** with the spectrum that has **gpz6** = 2. Use the controls at the top of the Multiple Display subroutine to adjust the two spectra until they both have the same intensity.
- The screen will display the attenuation factor of the spectra. Ideally the data with 75% gradient should have an intensity ~5-10% of the spectrum with 2% gradient.

8) If the spectrum with 75% gradient < 5%, decrease **d20**. If it is > 10%, decrease **d20** and/or increase **p30**.

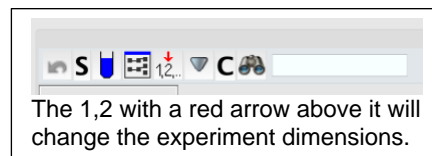
- Do NOT make **p30** > 2000 us (2 ms) because you could damage the probe.
- Make a note of your **d20** and **p30** values.

2D Set-Up and DOSY Acquisition: After optimizing the **p30** and **d20** values, you can now run the diffusion experiment.

9) Create another experiment (you can use **iexpno**). Use **pulprog** to change the pulse sequence to **stebppg1s**. This is a pseudo-2D version of the previous pulse program; it is actually a series of 1D experiments with the gradient strength incremented for the second dimension.

10) In the **AcquPars (eda)** tab:

- Change the experiment dimension from 1D to 2D.
- Set **td(F1)** = 16.
- Set **FnMODE(F1)** = QF.
- Set **p30** and **d20** to the values from step 8.



11) Type **ased** and make sure all of the parameters such as **gpnam6**, etc are set correctly.

12) Use **expt** to check how long the experiment will take.

13) Run the command **dosy** to start the acquisition. Do not use zg to start the experiment!

- A number of windows will appear.
- Set the first gradient amplitude = 2%, final gradient amplitude = 75%, number of points = 16, and ramp type = linear.
- The command dosy calculates the gradient ramp strengths and converts them to G/cm units which is stored in the experiment number of the dataset (/filename/expno) under the name **difflist**. This file is needed for data processing (both automated and manual processing).

14) You will need to do the same thing for the convection-compensated sequence. For the CC-DOSY experiment, create a new experiment with **iexpno** and run through steps 3-13, after you change the **pulprog** to **dstebpgp3s1d** (steps 3-8) and **dstebpgp3s** (steps 9-13). There are a couple of additional parameters for the double stimulated echo sequence that you need to change:

recycle delay [s]	d1	2
long eddy current delay [s]	d21	0.005 (5 ms)
number of scans [#]	ns	16
dummy scans [#]	ds	4
shaped gradient for diffusion-encoding gradient (gpnam6) and spoiler gradients (gpnam7, gpnam8, gpnam9)	gpnam6, gpnam7, gpnam8, gpnam9	SMSQ10.100
length of diffusion gradient pulse [us]	p30	1500
gradient 7 strength [%]	gpz7	-13.17
gradient 8 strength [%]	gpz8	-17.13
gradient 9 strength [%]	gpz9	-15.37
90° pulse length [us]	p1	Your calibrated 90° pulse length

For the CC-DOSY experiment, we suggest using a range of 2 → 50% for gradient strength, rather than 2 → 75% in the first part of the lab, i.e., for step 7 use **gpnam6** = 50 and for step 13 use final gradient amplitude = 50. Use the same **d20** value of 0.1 s.

Data Processing

Note 1: absolute gradient strength values are saved in a file named *difflist* in the expno of the DOSY data set after executing the **dosy** macro to run the experiments. The gradient strength values are stored in G/cm.

Note 2: the peaks need to be aligned in the stack of DOSY spectra for optimal processing. Set the chemical shift of one particular peak to the same value in each increment of DOSY.

Data Processing in TopSpin

1) In the "ProcPars" tab (**edp**), set the following parameters:

zero-fill F2	si(F2)	$\geq \text{td(F2)}$
zero-fill F1	si(F1)	16 (same as td(F1))
phasing-mode for F2	ph_mod(F2)	pk
zeroth order phase correction constant F2	phc0(F2)	Value of phc0 from your $\text{gpz6} = 2\%$ acquisition
first order phase correction constant F2	phc1(F2)	Value of phc1 from your $\text{gpz6} = 2\%$ acquisition

2) Run the command **xf2**. The pseudo-2D file now contains a set of 1D spectra with decreasing signal intensities. The first row will have a strong intensity and the last row is the most attenuated signal.

3) Run the command **abs2** for an automatic baseline correction.

4) Run the AU program **setdiffparm** to transfer important parameters such as diffusion time and gradient pulse time into the processing software.

5) Run **eddosy**. A new processing window will appear. Clicking on the "P" icon will switch back to the normal processing window.

6) The only parameters we have to think about for a first trial processing step as the display limits and type of scaling in F1 (linear or logarithmic). Typically small molecules show up between -8 and -10 (1×10^{-8} and 1×10^{-10} m²/s).

7) The actual processing is executed by running the command **dosy2d**. This provides a 2D DOSY spectrum with chemical shifts on the F2 axis and diffusion coefficients along the F1 axis. The F1 axis will show either [m²/s] (logarithmic scale) or [m²/s $\times 1\text{e-}9$] (linear scale).

Data Processing with MestReNova from MestreLab:

Mnova can process your DOSY spectra automatically. Follow the guides provided by MestreLab. However, a word of caution: MNova uses a Bayesian algorithm for the DOSY transform. The accuracy of the Bayesian transform will depend on the quality of your data, i.e. SNR, and the calculation parameters you use. If you put garbage in, it will spit garbage out. Good values are easily found for major components, but the Bayesian transform can be misleading if you are interested in minor components. It is always good practice to compare the Mnova results with those from Topspin or manual processing. Also, the peaks need to be aligned in the stack of DOSY spectra for optimal processing. Set the chemical shift of one particular peak to the same value in each increment of DOSY.

Manual Data Processing:

You can extract the intensity or integrals of the desired peaks at different gradient amplitudes and use a program of choice (e.g., Matlab, Excel, Mathematica, etc.) to fit the peak intensities or integrals to equation 2. Always do the dimensional analysis and make

sure your units match (e.g., Tesla \rightarrow Gauss, cm \rightarrow m). Again, compare your results with those from TopSpin or Mnova.

When you are done in the lab, make sure to log out. Type **CTRL + ALT + BACKSPACE** to log out of the computer.

What to turn in:

- A high-resolution 1D proton NMR spectrum ("routine" is ok), assigning the various resonances to the different components.
- The two DOSY spectra (the "standard" STE and convection-compensated). Assign each "horizontal band" to a component.

Questions

1. List the components of the mixture and their diffusion coefficient you collected for Parts 1 and 2 of the lab. Are these apparent diffusion coefficients consistent with the values that you would expect for the molecular size of each compound? (See the table of published values below).

Component	D [m ² /s] from "basic" DOSY	D [m ² /s] from CC-DOSY
Caffeine		
Taurine		
Glucose		
DSS		
Residual H ₂ O		

Published Values for Select Diffusion Coefficients (1) Brian Antalek, "Using Pulse Gradient Spin Echo NMR for Chemical Mixture Analysis: How to Obtain Optimum Results," *Concepts Magn. Reson.*, **14**, 225-258 (2002); (2) University of Michigan NMR Lab

SAMPLE	DIFFUSION COEFFICIENT (1,2)
10% D ₂ O in 90% H ₂ O at 25 °C	22.7 x10 ⁻¹⁰ m ² /s
HDO in D ₂ O at 25 °C	19.02 x10 ⁻¹⁰ m ² /s
n-dodecane at 25 °C	8.11 x10 ⁻¹⁰ m ² /s
sucrose in D ₂ O at 25 °C	4.4 x10 ⁻¹⁰ m ² /s

2. Use the results from the CC-DOSY experiment to calculate the hydrodynamic radius of each compound in solution using the Stokes-Einstein equation: $r = \frac{k_B T}{6\pi\eta D}$. Do they agree for what you might expect for each molecule? The viscosity η of a 0.1 M solution in CDCl₃ is 0.54 Cp. (For comparison, you could calculate a molecular radius from the density and molecular weight of each component.)

Component	Hydrodynamic Radius r
Caffeine	

Taurine	
Glucose	
DSS	
Residual H ₂ O	

3. In the case of severe thermal gradients, artifacts from convection can present in your data as a cosine modulation to the usual signal amplitude decay profile. In the case where convection dominates over diffusion, the signal amplitude may become negative before again showing positive. In the case of convection, the NMR signal intensity can be approximated as:

$$I \approx I_0 \cos(\gamma \delta g \Delta v) \exp \left[-D \gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3} \right) \right] \quad \text{in which}$$

$I \equiv$ NMR signal intensity for a given Δ , g and δ
 $D \equiv$ diffusion coefficient [m²/s, sometimes cm²/s]
 $\gamma \equiv$ gyromagnetic ratio of the observed nucleus [MHz/T]
 $g \equiv$ applied gradient strength [T/m, sometimes G/cm]
 $\delta \equiv$ the length of the gradient pulse [s]
 $\Delta \equiv$ the diffusion time [s]
 $v \equiv$ convective flow velocity [m/s]

Convection-modulated data sometimes can appear to fit the usual exponential decay function, but the apparent diffusion coefficient D_{app} will be larger than the true D .

$$D_{app} \approx D + \frac{1}{2} \Delta v^2 \quad \text{in which}$$

$D_{app} \equiv$ apparent diffusion coefficient [m²/s, sometimes cm²/s]
 $D \equiv$ diffusion coefficient [m²/s, sometimes cm²/s]
 $\Delta \equiv$ the diffusion time [s]
 $v \equiv$ convective flow velocity [m/s]

Calculate the effect of convection on the measurements you made: what is the flow velocity of water in your sample? (You can use the literature value $D = 22.7 \times 10^{-10}$ m²/s for H₂O/D₂O.) Does the "convection-compensated" sequence work well for our data? What about the "basic" STE sequence? (Aside: flow velocities as small as 0.05 mm/s can be sufficient to give an erroneous value for a species whose actual $D = 1 \times 10^{-10}$ m²/s!)

4. Convection is one factor that can lead to an erroneous value of the apparent diffusion coefficient. What are other factors that could contribute to an inaccurate measured diffusion coefficient? How could you control or mitigate these issues?

5. Compared to the standard STE-DOSY experiment, the CC-DOSY experiment utilized 2 as many scans and a smaller final gradient amplitude. ($n_s = 16$, vs 8; maximum $g_{p26} = 50\%$ vs 95%). Yet if you overlay the 1D spectra from these experiments, the CC-DOSY has a smaller NMR signal amplitude. What gives rise to lower SNR in the CC-DOSY compared to the STE-DOSY?

6. How you modify the NMR experiment (what parameters would you change and how?) to measure a smaller diffusion coefficient? What about a larger one? Recall that the equation for the DOSY NMR signal is $I = I_0 \exp \left[-D \gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3} \right) \right]$.