DOSY and Diffusion

by NMR

A Tutorial for TopSpin 2.0

Version 2.0.0

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1. Introduction to Diffusion

Molecules in liquid or solution state move. This translational motion is, in contrast to rotational motion, known as Brownian molecular motion and is often simply called diffusion or self-diffusion. It depends on a lot of physical parameters like size and shape of the molecule, temperature, and viscosity. Assuming a spherical size of the molecule the diffusion coefficient **D** is described by the Stokes-Einstein equation

$$D = \frac{kT}{6\pi\eta r_s}$$

where k is the Boltzmann constant, T the temperature, η the viscosity of the liquid and r_s the (hydrodynamic) radius of the molecule.

Pulsed field gradient NMR spectroscopy can be used to measure translational diffusion of molecules and is sometimes referred to as q-space imaging. By use of a gradient molecules can be spatially labeled, i.e. marked depending on their position in the sample tube. If they move after this encoding during the following diffusion time Δ, their new position can be decoded by a second gradient. The measured signal is the integral over the whole sample volume and the NMR signal intensity is attenuated depending on the diffusion time Δ and the gradient parameters (**g**, δ). This intensity change is described by

$$I = I_0 \, e^{-D\gamma^2 g^2 \delta^2 (\Delta - \delta/3)}$$

where **I** is the observed intensity, **I₀** the reference intensity (unattenuated signal intensity), **D** the diffusion coefficient, γ the gyromagnetic ratio of the observed nucleus, **g** the gradient strength, δ the length of the gradient, and Δ the diffusion time. To simplify this equation some parameters are often combined

$$I = I_0 \, e^{-D q^2 (\Delta - \delta/3)} \qquad \text{or} \qquad I = I_0 \, e^{-DQ}$$

to emphasize the exponential decay behavior. If bipolar gradients are used for dephasing and rephasing a correction for the time τ between those bipolar gradients has to be applied:

$$I = I_0 \, e^{-D\,\gamma^2\,g^2\,\delta^2\,(\Delta - \delta/3 - \tau/2)}$$

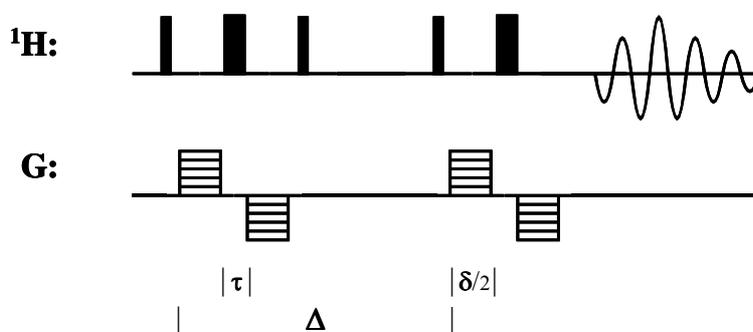The easiest pulse sequence to measure diffusion has already been described by Stejskal and Tanner[*] in 1965, the gradient spin echo sequence (SE):
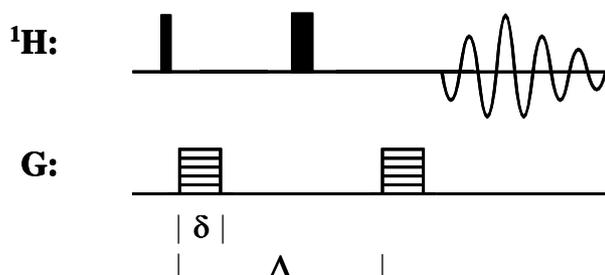
Here is an example for a stimulated echo sequence using bipolar gradients[†] (STEbp):

A good review about diffusion and DOSY can be found in [‡].

1.1. Gradient Linearity

For measuring accurate diffusion constants the quality of the gradient coil plays a very important role. The gradients used for diffusion measurements have to be linear across the sample volume, which actually means the field change has to be linear and therefore the field-gradient has to be constant. This, of course, is a question of gradient coil design. Typical high resolution solution NMR probes (HR probes) have not been designed with this particular feature in mind, because it is not really important for gradient selection experiments. However, for many years now, the quality of Bruker HR Z-gradient probes is good enough for diffusion

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[*] E. O. Stejskal and J. E. Tanner, *J. Chem. Phys.* **42**, 288 (1965).

[†] D. Wu, A. Chen, and C. S. Johnson, Jr., *J. Magn. Reson. A* **115**, 123 (1995).

[‡] C. S. Johnson, Jr., *Prog. NMR Spectrosc.* **34**, 203 (1999).

type experiments. In comparison, the XYZ-gradient system of HR probes shows some restrictions. The necessary linear range is smaller than the RF-coil height. This limitation can easily be overcome by using a restricted volume, for example with a Shigemi® tube. A volume of about 1 cm height placed in the center of the RF-coil leads to a NMR signal coming from the most linear area of this gradient system. The difference in quality between a Z-gradient and an XYZ-gradient probe can easily be understood by the limited space in the probe available for the gradient coil(s). This available space can either be used for a single gradient coil or compromises have to be accepted to fit 3 gradient coils into the same space.

1.2. Temperature Stability

One very important experimental parameter when you run a diffusion measurement is a good temperature stability. Having a bad temperature stability easily leads to a temperature gradient along the sample tube, i.e. you have different temperatures at the bottom and the top of the tube. Depending on the viscosity of the solvent and the actual temperature this may introduce convection in your tube: you generated a flow of the solvent. This has the same effect as diffusion, the molecules are moving, but most often much faster than they would do due to diffusion.

If you use low viscosity solvents (like $CDCl_3$) a similar effect occurs if the temperature is too close to the boiling point of the solvent. The solvent strongly evaporates, will condense at the top of the sample tube and either solvent drops will fall down or run down along the tube walls back into the solution. This reflux introduces a flow, which is very similar to convection and will disturb the diffusion measurement.

2. <u>Measuring Diffusion Data</u>

To run a diffusion measurement it is necessary to optimize the parameters that determine the decay function described in the previous chapter. To keep the timing constant throughout the whole experiment, we have chosen the gradient strength (**g**) to be the variable parameter, the diffusion time *big delta* Δ and the diffusion gradient length *little delta* δ are kept constant. All Bruker supplied pulse programs and AU programs are based on this choice. Nevertheless, the processing tools support the evaluation of data acquired by varying either one of the three possible parameters.

2.1. Optimizing Parameters

We need to optimize all 3 parameters to detect the whole decay function properly. Selecting the right values for Δ or δ is important to get good diffusion constants with as little error as possible (see figure 2.1).

A **B** **C**
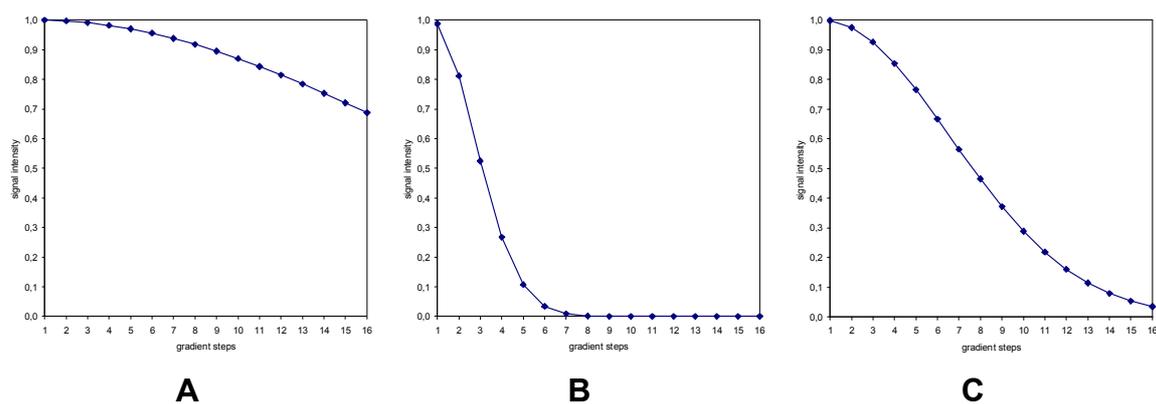
Figure 2.1: Simulated diffusion decay curves by varying the gradient strength from 2 to 95 % in 16 steps for the same diffusion constant, but with different selection for Δ and δ. They are chosen too small (A), too big (B), and properly (C) to sample data points along the whole decay curve.

The optimization is easily done by running a few 1D measurements. For that purpose we provide 1D versions of the diffusion pulse programs (see appendix). Execute the following steps:
- create a new dataset,
- select the "normal" spectral parameters (i.e. SWH etc.),
- select a diffusion sequence to use (i.e. STE, LED, with or without bipolar gradient pulse pairs, etc.),

- run a 1D spectrum with start values of 50 – 100 ms for Δ (*d20*) and 1 ms for δ (*p30*) with 2 % (*gpz6*) amplitude. This spectrum will be used as a reference for the optimization process, so you should store this, for example, in *procno* 2 (*wrp 2*). It is important to start with a gradient strength bigger than 0, because you may get unwanted echoes when you don't apply a gradient. We recommend a start value of 1 to 5 %.

- now increase the gradient strength (*gpz6*) up to 95 % (we recommend 95 % to make sure that there is no non-linear behavior of the gradient amplifier at the end of the amplification range, but you may go up to 100 %). Compare this spectrum with the reference spectrum and note the change in signal intensity.
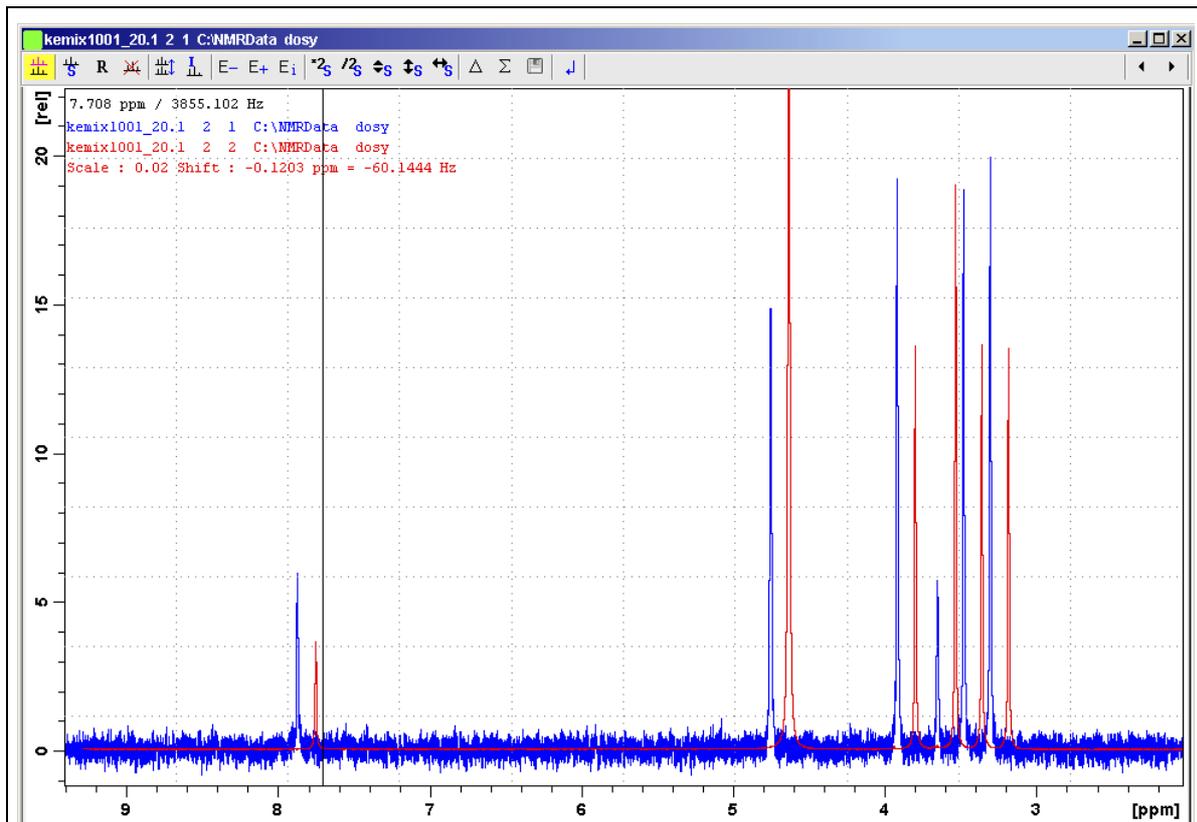
Figure 2.2: Dual display of reference spectrum (red) and well attenuated spectrum (blue). The blue spectrum has about 2% residual signal intensity compared to the red spectrum (Scale = 0.02).

The signal decay should go down to 5 % residual signal (i.e. 95 % signal attenuation due to diffusion). This comparison is conveniently possible in *dual display* (see figure 2.2). Depending on the accuracy with which the diffusion constant is to be determined and the resolution needed in the diffusion dimension in the case of signal overlap (fitting of more than 1

component), this value may have to be even smaller. The further you can attenuate the NMR signal, the better is the resolution you can achieve in the resulting DOSY spectrum.

The observable residual signal intensity is, of course, dependent on the signal-to-noise ratio (S/N). For bad S/N you may have to increase the number of scans (NS) or go with less signal attenuation. The smallest signal to be detected (i.e. at highest gradient strength) has to be above the noise. If the signal intensity is already totally gone, reduce the gradient strength (*gpz6*). If the signal is still to big, you have to increase either the diffusion time Δ (*d20*) or the gradient length δ (*p30*). Increasing δ is favorable, because it results in a bigger effect. δ^2 is determining the signal attenuation, while Δ is only affecting the exponential decay function linearly (see chapter 1). If you change Δ, you have to take the relaxation into account (T1 relaxation for all STE type sequences).

2.2. Running the Diffusion Measurement

After optimizing the parameters you can run the actual diffusion measurement, which is executed as a pseudo-2D acquisition. Instead of incrementing a delay you would do in a "normal" 2D spectrum, the gradient strength is incremented for the indirect dimension. You can easily generate this 2D dataset from the 1D dataset you used for the optimization procedure:

-   create a new *expno* with **new** or **edc**
-   switch to a 2D dataset by changing the dimension to 2D (in **eda** or AcquPars click on [123])
-   select a 2D pulse program instead of the 1D you used for the optimization

All other parameters are already set correctly, only the gradient ramp has to be generated. For this purpose we provide the AU program *dosy*.

2.2.1. The AU Program *dosy*

The AU program *dosy* is used to calculate the gradient ramp and needs some input parameters that define this ramp. These parameters are the start and the final value of the gradient ramp (0-100), the number of steps (i.e. number of increments = TD1), and the type of ramp (linear *l*, squared *q* or exponential *e*). The program will ask whether the acquisition should be started immediately or not. For automation purposes it is possible to call the AU program non-interactively by passing all parameters on the command line and giving a "*y*" to start the acquisition. In this case an additional 6th parameter is available, which enables to execute *rga* before the acquisition is started.

<u>example</u>: ***xau dosy 2 95 16 l y y***

        start value 2%, final value 95%, 16 steps, linear ramp, start acquisition,

        execute ***rga*** first.

dosy calculates the gradient ramp file ***Difframp*** and stores it in ***TOPSPINHOME/exp/stan/nmr/lists/gp***[†]. This file is used by the acquisition and contains the gradient strength values in percent gradient amplitude; 100% means maximum current, typically 10 A for the standard high resolution gradient amplifiers (GAB, GREAT 1/10, GREAT 3/10, ACUSTAR, BGPA). To have all the values used by ***dosy*** available as status parameters, the start and final value are stored as constants 20 and 21 (CNST20/21) and the ramp is converted into real gradient strength (in G/cm, see below) and stored in the *expno* of the dataset under the name ***difflist***. This file is necessary for the processing later.

Diffusion experiments don't have an imaginary part in the diffusion dimension, therefore the acquisition is setup like an QF experiment (***FnMODE*** = *QF*). ***dosy*** requires this parameter to be set and the Bruker pulse programs are written this way. ***dosy*** uses the parameter ***FnMODE*** in the case of a 3D experiment to try to detect the diffusion dimension. In general, 3D diffusion measurements (i.e. 2 spectroscopic and 1 diffusion dimension) are setup just like any other 3D acquisition. It is strongly recommended to determine the diffusion parameters using a 2D measurement. This is basically the same as running a 2D plane out of the 3D first.

The way to setup, optimize and run a diffusion measurement, as described above, is always the same and is independent from the way the data is processed afterwards using the different methods DOSY, fitting, DECRA or DOSY*m*. The only important difference: for DECRA it is necessary to select the square ramp type (option 'q'), otherwise you can't use DECRA as processing method.

2.3. Gradient Calibration

As mentioned above, the AU program ***dosy*** calculates the gradient ramp to be used during the diffusion measurement. For the acquisition we specify the gradient strength in percent of the maximum current provided by the gradient amplifier. For high resolution instruments this is typically 10 A, i.e. 100% means 10 A current through the gradient coil. In addition to this typical high resolution instrumentation, stronger amplifiers delivering higher currents and different probes with specialized gradient coils ("diffusion probe", etc.) are also available.

[†] ***TOPSPINHOME*** (or ***XWINNMRHOME***) is the directory where TopSpin is installed (typically ***C:\Bruker\TOPSPIN*** under Windows and ***/opt/topspin*** under Linux).

For further evaluation of the diffusion data we need the actual gradient strength in absolute values, i.e. in G/cm (or T/m). This value obviously depends on the design of the gradient coil. A high resolution Z-gradient probe delivers 5.35 G/cmA, the Z-gradient coil of an XYZ-gradient probe about 6.5 G/cmA and the X and Y coils of an XYZ-gradient probe about 5.0 G/cmA. To convert the percent values into absolute values we need a gradient calibration constant, which is used by the AU program *dosy* to calculate and store the above mentioned list (*difflist*) containing absolute gradient strength values. This list is used by the processing tools to calculate the correct diffusion constants.

In addition *dosy* takes the shape of the gradient used for dephasing/rephasing into account (*GPNAM6*). In general we recommend sine shaped gradients for all gradient experiments. Those may be used for diffusion experiments as well. As long as δ is small compared to Δ, a shape different to a rectangle has no negative effect. The effective gradient strength, of course, is reduced and the shape factor (i.e. the integral of the shape compared to a rectangle) is used when calculating the absolute gradient strength values for *difflist*. This factor is read from the shape file automatically. If you prefer a rectangular gradient, we recommend the use of trapezoidal shapes. The slope should match the rise/fall times of the gradient amplifier (< 50 μs). We provide an AU program *difftrap* to create the necessary trapezoidal shape. If you set *GPNAM6* to *difftrap*, *dosy* will automatically calculate a new trapezoidal shape *difftrap* matching the rise/fall times and the defined length of *p30*.
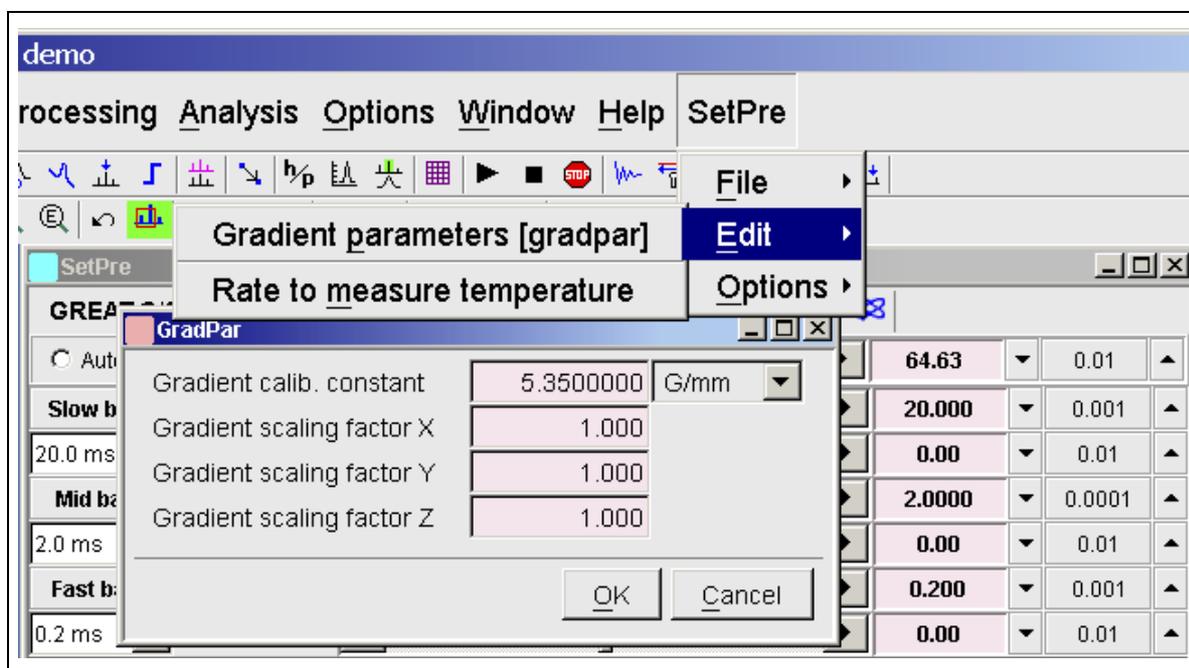

Figure 2.3: use "setpre" or "gradpar" to define the gradient calibration constant

The calibration constant is set and stored using **setpre** (*SetPre | Edit | Gradient parameters*) or **gradpar** directly. With the above mentioned 5.35 G/cmA for the gradient system of a standard HR-probe the calibration constant is typically 53.5 G/cm or 5.35 G/mm at 10 A maximum output of the gradient amplifier (100 % gradient amplitude). This value is directly written into the file **TOPSPINHOME/conf/instr/gradient_calib**, which contains the calibration value for the current probe. In addition **setpre** can be used to store this calibration constant for each gradient probe together with the preemphasis values. Even if the adjustment of preemphasis is not necessary or not possible (depending on the probe and gradient amplifier), you should use **setpre** to store the probe calibration (*SetPre | File | Write ...*). The values are written to disk into a probe dependent directory (**TOPSPINHOME/exp/stan/nmr/parx/preemp/<probe id>**). The probe id is determined by the probehead list available via the **edhead** command. After a probe change, **setpre** should be used to recall these values.
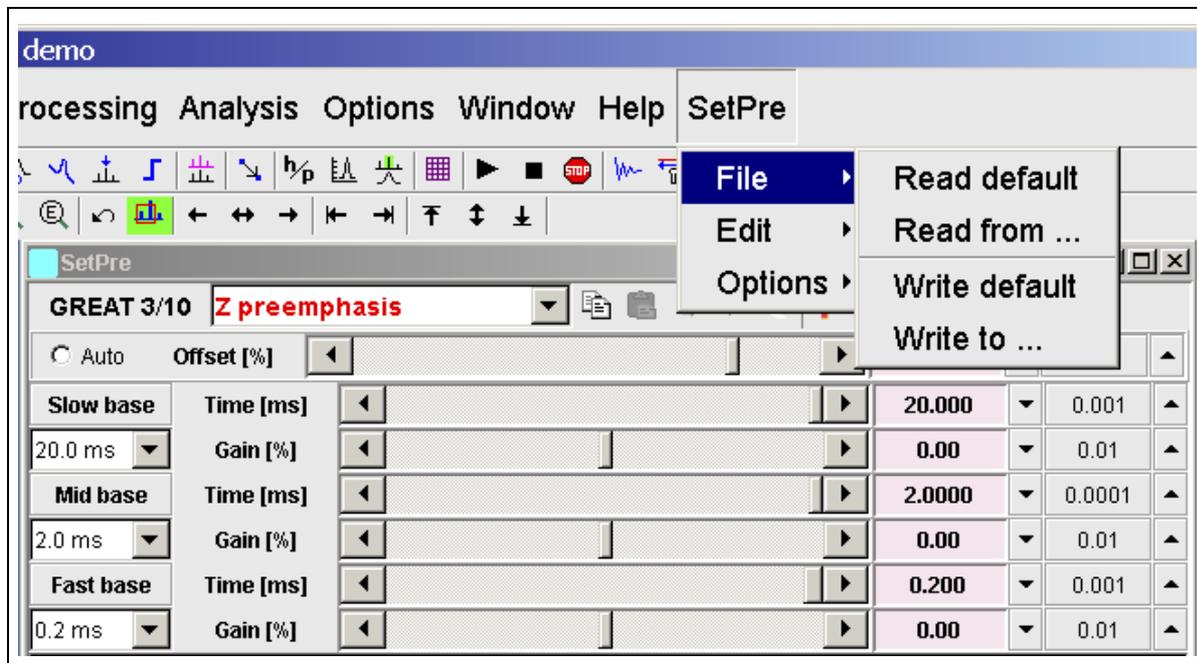
Figure 2.4: saving and restoring preemphasis and gradient calibration values with "setpre"

If the gradient calibration value was incorrect at the time the AU program **dosy** was executed, the file **difflist** obviously contains wrong absolute gradient strength values and this will lead to wrongly calculated diffusion constants and a wrong diffusion axis after DOSY processing. To correct this, first enter the right gradient calibration constant (see above) and then recalculate the **difflist** using the AU program **dosy**: **xau dosy restore.**

3. Processing Diffusion Data

As previously described, measuring diffusion is carried out by observing the attenuation of the amplitudes of NMR signals. The processing software has to evaluate this decay behavior and extract the diffusion coefficient (and maybe an error value) out of the signal decay curve. In the literature several different approaches for this data fitting process have been described. Currently we offer a fitting approach similar to standard exponential fitting algorithms.

Even though diffusion measurements are recorded as 2D data, they are basically a set of 1D spectra. The processing therefore starts with processing these 1D spectra (i.e. the F2 dimension of the 2D dataset). You have to select a size (SI[F2]) and an apodization function (window function) and determine the phase correction, then process with *xf2*. If you plan to use the DOSY processing tools you should use zero filling in F1 (SI[F1]). This has to be defined prior to the F2 processing (*xf2*), because *xf2* already creates the files on disk with the necessary size given by SI[F2] * SI[F1].

The 2D file now contains a set of 1D spectra with decreasing signal intensities: the first row with strong intensity and the last one with very weak intensity. This often leads to a distortion of the baseline level, i.e. the row with strong intensity has a baseline which is often shifted into the negative. Nevertheless, the intensity from the baseline up to the top of the signal is correct. The processing software uses the absolute peak height, we therefore have to correct the spectra by shifting the baseline back to 0 absolute level. This is conveniently done with the automatic baseline correction (*abs2*): set the limits ABSF1 and ABSF2 in F2 to 1000.0 and -1000.0 (outside the limits of the actual dataset), set the degree of the polynomial fitting function to 0 (ABSG[F2]) and execute *abs2*. This will lead to an individual DC adjustment of the baseline for each row.

Before you process with one of the diffusion processing tools, you should once call the AU program *setdiffparm*. This will transfer some important parameters like Δ (D20) and δ (P30 or P30*2, depending on the pulse program) into the appropriate parameters for the next processing step (DOSY, SimFit, CONTIN, DECRA, or DOSY*m*). A wrong setting will obviously lead to wrong diffusion constants.

3.1. Processing with the DOSY Software

To generate a 2D spectrum with chemical shifts on the F2 axis and diffusion constants on the F1 axis, each column has to be fitted first. Out of the fitting results, 2D cross peaks are created where the centers of the cross peaks correspond to the calculated diffusion constant. The width

of the peaks correlates with the fitting error. Only this way of presenting the diffusion data is called DOSY. All columns of the F2 transformed spectrum are fitted independently using a Levenberg-Marquardt algorithm. Columns containing only noise are left out and zeroed. Fitting a decaying curve is much more difficult than a straightforward Fourier transform and therefore needs much more parameters describing the fitting procedure. After execution of the AU program *setdiffparm* and calling the editor *eddosy* for the diffusion processing parameters, all parameters are preset with meaningful defaults. *setdiffparm* may be called from within the *eddosy* editor window (🔳). The icon P allows to switch back to the normal processing parameters *edp*, the icon G allows to setup parameters for DOSY*m* processing.
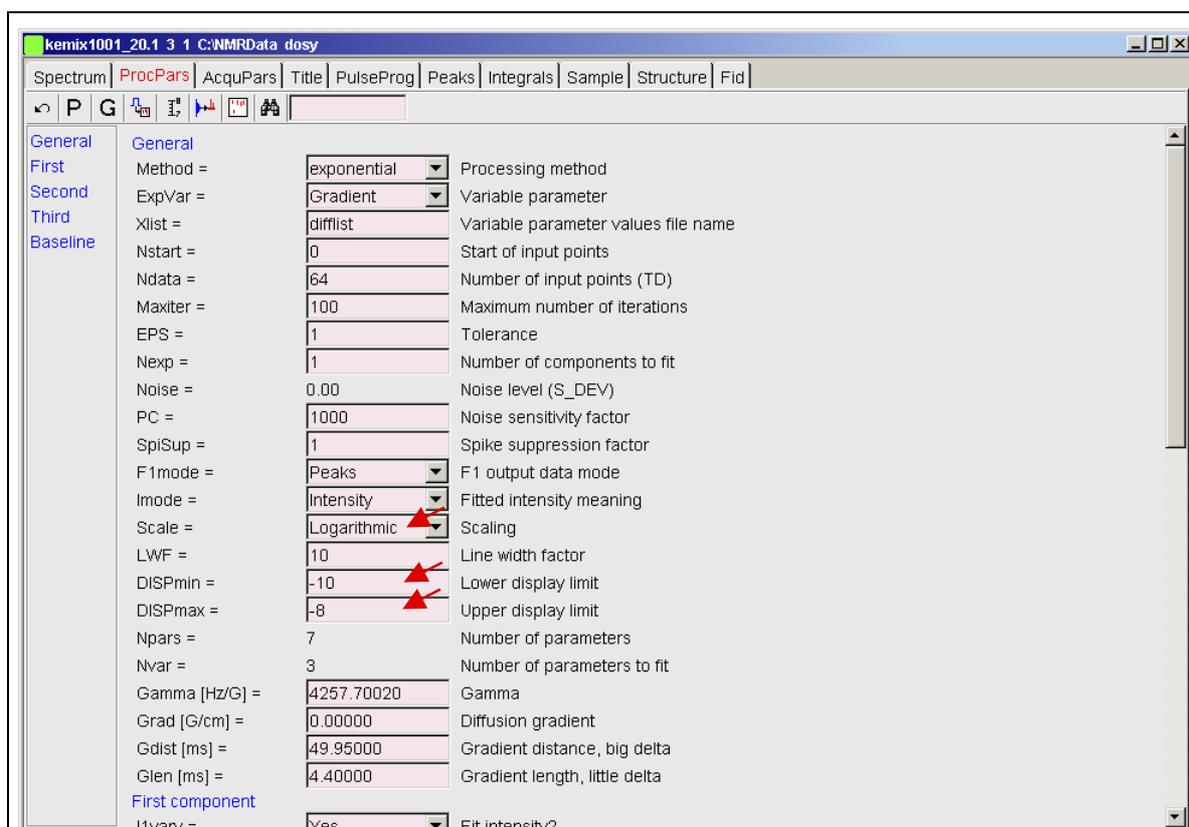
*Figure 3.1: Part of the parameters in the **eddosy** editor. The AU program **setdiffparm** sets the values determined by the acquisition (**Gdist**, **Glen**, etc.) and presets some other parameters with useful defaults. (red arrows see text)*

The only parameters we have to think about for a first trial processing step are the display limits and the type of scaling in F1 (linear or logarithmic diffusion axis). These are indicated by the red arrows in *Figure 3.1.* In contrast to a Fourier transform that delivers well determined frequency limits (left and right edge of spectrum), the diffusion fitting offers a possible area from 1 to 0, which is infinitely long on a logarithmic scale and is huge with respect to the very

small area the compounds in your sample may show up with. As a consequence we have to specify display limits. Assuming the gradient calibration constant is correct, small molecules will show up between –8 and –10 ($1 \cdot 10^{-8}$ and $1 \cdot 10^{-10}$ m^2/s). If you are not sure about the correct display limits you may call the command ***dosy2d setup*** (📋) first. This scans the data for possible solutions and uses these values to set the display limits. No data is written back to disk. The actual processing is executed by the command ***dosy2d*** (📋).

As a result you should obtain a 2D DOSY spectrum with chemical shifts along the F2 axis and diffusion coefficients along the F1 axis. The F1 axis after processing will show either [m^2/s] (logarithmic scale) or [m^2/s *1e-9] (linear scale). If you have chosen a logarithmic scale just read the values as **log D** values in m^2/s, i.e. -8.6 means $10^{-8.6} = 2.5 \cdot 10^{-9}$ [m^2/s]. In the case of a linear scale you will directly see the diffusion coefficient.
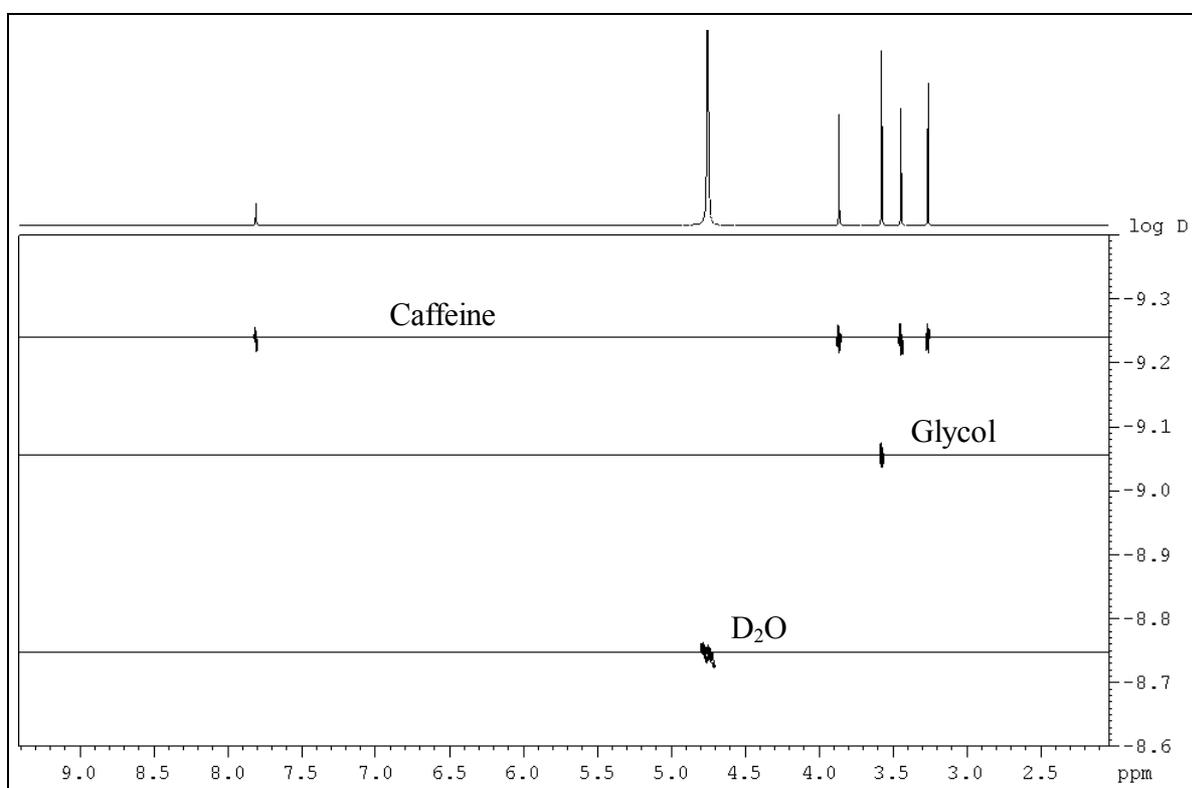
Figure 3.2: Example of a DOSY spectrum showing the different diffusion behavior of Ethylenglycol and Caffeine dissolved in D$_2$O. Parameters used for acquisition are given in the Appendix. On top is the 1D spectrum of the mixture.

For the processing of 3D datasets the parameter setup is exactly the same and the processing parameters should be optimized on a 2D measurement. The raw data has to be processed in both spectroscopic dimensions first, i.e. ***tf3*** and ***tf2*** or ***tf3*** and ***tf1***, depending on which

direction is the diffusion dimension. The processing for a pseudo-3D DOSY is executed with *dosy3d*.

3.1.1. Parameter description

Method – choice of processing method options.

ExpVar - variable parameter:

This parameter defines the experimental variable. It can take values of "*Gradient*", "*Grad_distance*", or "*Grad_length*", corresponding to the variable gradient strength **g** (= gradient amplitude), diffusion time *big delta* Δ (= gradient distance) and diffusion gradient length *little delta* δ. It is used to select the proper fitting formula.

Xlist - variable parameter values file name:

The values of the experimental variable *ExpVar* are kept in the specified file <disk>/data/<user>/nmr/<name>/<expno>/<Xlist>. The default value for *Xlist* is "*difflist*" and contains the gradient strength values used.

Nstart - start of input points:

In some cases, a few data points at the beginning of the curve to be fitted may have wrong values. *Nstart* allows to exclude them to produce better results.

Ndata - number of input points:

Number of points actually used for the fitting. *Ndata* must be bigger than the number of the fittable parameters *Nvar*. *Nstart*+*Ndata* must not be bigger than *TDeff*.

Maxiter - maximum number of iterations:

Defines the stop condition for the Levenberg-Marquardt iteration loop. Default value is 100.

EPS – tolerance:

Another stop condition for the iteration loop. The loop breaks when the RMS deviation of the fit decreases by a value less then *EPS*. One may understand *EPS* as "minimal average improvement of fitted point" to be accepted. As we deal with integer data, *EPS* = 1 is a good candidate for the default value. *EPS* and *Maxiter* work together to limit the total processing time. Typically, it takes 20-50 iterations to achieve acceptable convergence and the loop breaks by the *EPS* condition. But for slowly converging cases, iteration stops after *Maxiter* cycles.

Nexp - number of exponents to fit:

DOSY fitting is able to discover up to three simultaneous decays in the experimental data. This allows overlapping peaks in the spectrum to be resolved in the DOSY dimension. The *Nexp* parameter defines how many of *In* and *Dn* (see below) are taken into account by the fitting.

Noise - noise level calculated by *xf2*. This parameter cannot be changed by the user and is displayed for convenience only.

PC - noise sensitivity factor:

If all intensity values of a data column are smaller than *PC* * *Noise*, the column is qualified as pure noise and zeroed on output. Default value is 4.

SpiSup - spike suppression factor:

Occasionally, the fit produces unlikely good convergence for some columns resulting in sharp spikes in the DOSY spectrum. The parameter *SpiSup* allows to set the minimal allowed peak width to a reasonable value = *SpiSup* * *Noise*.

F1mode - F1 output data mode:

The default value "Peaks" means that the F1 data will be generated as gaussian shaped peaks with the intensities, positions and linewidths corresponding to the fitted intensities, diffusion values and their standard deviations using the *Scale* as defined by the user. If *F1mode* has the value "Decays", then the Stejskal-Tanner decays along F1 will be generated corresponding to the fitted parameters. These results may be further fitted by *dosy* again. The main feature of this mode is the ability to generate simulated diffusion decays with known properties to test the quality of the data.

Imode - fitted intensity meaning:

This switch determines whether the calculated peaks in the 2D spectrum will have correct integrals (*Imode* = Integral, by default) or correct intensities (i.e. peak heights) corresponding to the fitted intensities *In* (*Imode* = Intensities).

Scale - scaling in DOSY direction:

Sets the axis to linear or logarithmic scale in the DOSY direction.

LWF - line width factor:

Allows to adjust the peak width in the DOSY direction by multiplying the calculated width with *LWF*. This is similar to a line broadening window function (exponential multiplication). A special case is setting *LWF* = 0. This produces peaks with one pixel width, i.e. the fitting error will not correspond to the width of a cross peak. The default value is 1.

DISPmin, ***DISPmax*** - lower and upper display limits for F1:

Reasonable values depend on the chosen scale. Can be preset automatically by *dosy2d setup*.

Npars - number of parameters:

Shows the number of parameters used by the fitting, equal to five (***Gamma***, ***Grad***, ***Gdist***, ***Glen***, ***Bline***) plus two for each fitted decay (***In***, ***Dn***).

Nvar - number of parameters to fit:

Number of fittable parameters with the "vary" tag set to "Yes".

Gamma - gyromagnetic ratio of current nucleus:

4257.64 Hz/G for protons, is preset by the AU program ***setdiffparm***.

Grad - diffusion gradient.

Gdist - gradient distance (big delta Δ).

Glen - gradient length (little delta δ).

These three values are experimental variables, one of them, defined by ***ExpVar***, had been varied in the experiment. This value is ignored, instead we use ***Xlist*** as the source. The other two values were experimental constants.

I1vary　　 - fit intensity: Yes/No

I1　　　　 - intensity of component 1.

I1min　　 - minimum intensity of component 1.

I1max　　 - maximum intensity of component 1.

This block of four parameters defines the properties of the first component intensity. ***I1vary*** = Yes means this parameter should be fitted, otherwise it is fixed. ***I1*** defines the start value for the fitting. ***I1min*** and ***I1max*** define limits for the search.

D1vary　　 - fit diffusion coefficient: Yes/No.

D1　　　　 - diffusion coefficient.

D1min　　 - minimum diffusion coefficient.

D1max　　 - maximum diffusion coefficient.

Like ***I1****, define the properties of the first component diffusion coefficient.

I2*,I3*,D2*,D3* parameters have the same meaning as ***I1**** and ***D1**** for the second and the third component, respectively.

Bvary,Bline,Bmin,Bmax deal with baseline fitting in the same way as the ***In**** and ***Dn**** parameters. Generally, we recommend to adjust the baseline with TopSpin tools (such as

abs2) before the DOSY fitting and set *Bvary = No* and *Bline = 0*. The free baseline fitting during the DOSY processing is much less stable.

Nonneg – Non-negative data: only used by CONTIN, see chapter 3.4. for details.

Dfmin – Minumum degrees of freedom: only used by CONTIN, see chapter 3.4. for details.

Qmode – quadrature mode: only used by CONTIN, see chapter 3.4. for details.

RegMult – Regularization parameter multiplier: only used by CONTIN, see chapter 3.4. for details.

!

Note: The user should not overestimate the ability of DOSY to resolve decays with similar constants, especially in presence of noise. Simulations show that the algorithm fails to resolve exponents of the form $\exp(-x)+2 * \exp(-2x)$ when there is more than 5% noise in the data.

The parameters *ExpVar*, *Xlist*, *Scale*, *Gamma*, *Grad*, *Gdist*, *Glen* must be set by the user in order to obtain proper results. Other parameters may be automatically preset by the command *dosy2d setup*. *dosy2d setup* changes the parameters only, it does not alter the data.

3.2. Processing with the T1/T2 Software

The data obtained with the acquisition procedure described above can be evaluated with the TopSpin T1/T2 package as well (only for 2D measurements). Fitting diffusion decays is one of the available fitting functions for the **SimFit** algorithm. The AU program *setdiffparm* presets some default values for this fitting procedure (check in editor, see Figure 3.3). The first part of the processing is the same as described above for the DOSY processing (*xf2* and *abs2*, Chapter **3.1**), with only one difference: you don't need zerofilling for this type of processing.

The procedure to use **SimFit** is described in detail in the processing manual. The basic procedure can be outlined as follows:

- enter NMR Relaxation Guide: *Analysis | T1/T2 Relaxation*,
- *extract a slice* ( ), select spectrum slice 1 (i.e. the row with the highest intensity), a new window with the selected 1D will appear,
- select *Define Ranges* ( ), define the peaks to be fitted as integral regions ( ) and export them to the relaxation module ( ),
- you enter the *Relaxation Window* ( ) and can check the fitting parameters (or), **Function Type** *vargrad* and **List file name** *difflist* have been set by *setdiffparm* (see *Figure 3.3*),
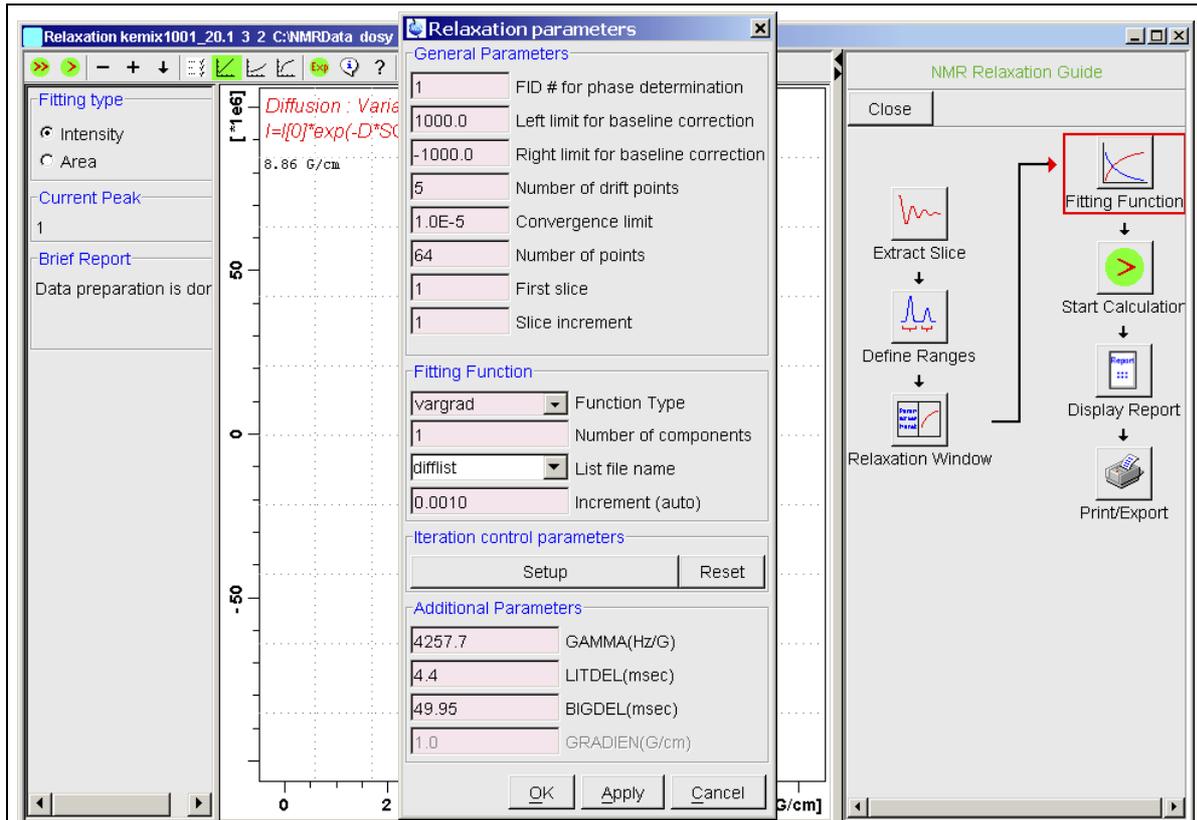- execute the fitting ( ).

*Figure 3.3: Part of the parameters in the relaxation parameters editor. The AU program **setdiffparm** sets the values determined by the acquisition (LITDEL, BIGDEL, etc.) and presets some other parameters with useful defaults.*
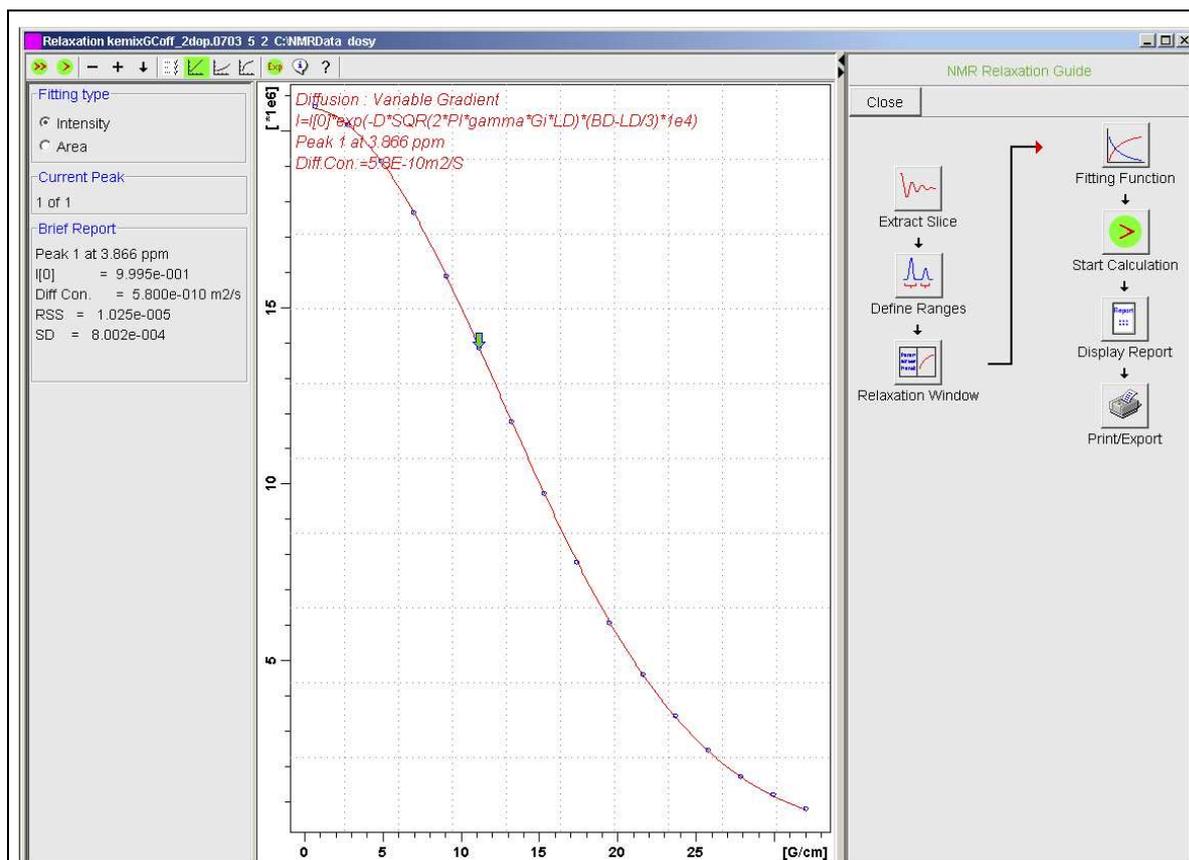
Figure 3.4: TopSpin display after fitting with SimFit (T1/T2 Analysis). The fitting curve for the peak at 3.87 ppm (Caffeine) is shown.

Figure 3.4 shows a typical result after processing with **SimFit**. The calculated diffusion constant (5.8e-10 m^2/s) is the same a shown in the DOSY spectrum (see *Figure 3.2*): -9.24 [*(log)*m2/s] $\Rightarrow$ $10^{-9.24}$ = $5.75 \cdot 10^{-10}$ [m^2/s].

3.3. Processing with DECRA

DECRA or *D*irect *E*xponential *C*urve *R*esolution *A*lgorithm is implemented as an AU program named *decra*. DECRA is based on algorithms described in the original papers by **B. Antalek** and **W. Windig**[#].

The algorithm provides another means to the analysis of diffusion spectra: instead of nonlinear fitting each 2D column separately, DECRA processes the 2D spectrum as a whole. The result

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[#] W. Windig, B. Antalek, Chemometrics and Intelligent Laboratory Systems, **37**, 241-254 (1997),

B. Antalek, Concepts in Magnetic Resonance, **14** (4), 225-258 (2002).

are the 1D subspectra corresponding to the components of the mixture separated from each other (almost as LC-NMR, but without LC). In addition to the spectra of the separated components the diffusion constants and the relative amounts of the components are calculated. The present implementation of DECRA as an AU program and the part of the algorithm concerning autodetection of the number of components has been developed by Georgy Salnikov[+]. Many Thanks to him for this nice work.

The program is easy to use. It requires less parameters than *dosy2d* (see below), no special setup or prior knowledge is necessary (unlike "*dosy2d setup*"), and therefore less user experience. Although the resulting 1D spectra might be not as visual as a DOSY spectrum, DECRA produces rather fool-proof results. The short usage instructions one can find, as usual, in the comments at the beginning of the AU program source. Typically you just call *decra*. You will be asked in which *procno* the first component spectrum should be saved (it will use the following numbers to save further component spectra). The second question is how many components you expect, the default answer is *auto*.

You can call decra with the following command line options:
decra [p = <procno>] [o = <offset>] [n = <ncom>] [i = y]

<procno> - starting procno (within current expno):
   Into this procno and the following the resulting 1D spectra of the resolved components will be written. The default is the current procno + 1.

<offset> - DECRA offset between subdatasets A and B (see the papers):
   Will not be requested if not given on the command line. By default 1 row.

<ncom> - number of components to resolve:
   'n = a' will do autodetection. Will be requested if not given on the command line. By default the autodetection of components is proposed.

i = y - if specified on the command line, the imaginary data will be analyzed as well. Otherwise only the real data will be analyzed.

For the DECRA analysis to work the data must have been acquired using variable gradient strength and with the square ramp option (*dosy* AU program), i.e. the squares of the gradient values must be distributed linearly along the ramp.

The program creates a text file with the name *DECRA.info* in each of the generated procnos. It contains some relevant information about the DECRA flow and the component resolved in that procno, such as relative amounts and diffusion constant of the component.

Although the program usually can autodetect the real number of resolvable components, you should not blindly rely on this capability. You should always check the resolved subspectra visually and the contents of the *DECRA.info* files for reasonable diffusion values.

From the theory DECRA outperforms *dosy2d* in the following aspects:
- It evaluates the 2D data as a whole thus reducing the probability of artifacts due to bad data in some particular columns. Particularly, overestimating the number of components in DECRA should be impossible, and the reliability of the results for overlapped peaks is much higher compared to the multiexponential *dosy2d* fitting. The DECRA algorithm is also more stable than fitting in the presence of big noise.
- In principle, it can achieve better separation of components than DOSY, in particular if the components have similar diffusion coefficients and/or massive regions of overlapped peaks.
- The 1D spectra of separated components produced by DECRA can be integrated and/or phase corrected (if imaginary data were available) after the DECRA processing. The intensities of the distinct subspectra are even comparable with each other so that the concentrations of the components may be estimated.
- The quality of high resolution multiplets in the subspectra separated by DECRA is much better compared to *dosy2d*, as the DECRA multiplets have undistorted relative intensities.
- In the present implementation, DECRA can in most cases automatically detect the actual number of resolvable components and generate all the corresponding subspectra.
- DECRA is fast: a typical 2D spectrum (32 rows, 16K words each) is processed by DECRA in several seconds. This is independent from the number of components to separate, a monoexponential fitting by *dosy2d* may take about 10 seconds, a multiexponential fitting even much longer.
- DECRA requires no preset of diffusion limits and therefore no setup phase.

However, DECRA has also a few disadvantages in comparison with *dosy2d*:
- The main drawback is the requirement that data must be acquired using a squared gradient ramp. Therefore, if the diffusion coefficients of the components differ a lot, several measurements may be necessary to get the best separation of subspectra of all components.

- As DECRA has to load the whole data matrix into memory, it is restricted to 2D processing only (very big spectra can overload the computer resources). Memory requirements are therefore much bigger than for DOSY.
- The sensitivity to systematic errors (incomplete relaxation, inaccurate gradients, etc.) resulting in the unability to resolve some of the components may be higher. But how sensitive it really is in comparison with dosy2d, is not yet tested.

In practice, in my personal experience, the reality does not show what the theory promised (as more often observed). You will not always get subspectra with clear separation of different components of a mixture.

3.3.1. Parameter description

The DECRA processing uses the following *eddosy* parameters. Their meanings are the same as described above (Chapter 3.1.1.):

Xlist - variable parameter values file name.
Nstart - start of input points.
Ndata - number of input points.
Noise - noise level calculated by *xf2*.
PC - noise sensitivity factor.
Gamma - gyromagnetic ratio of current nucleus.
Gdist - gradient distance (big delta Δ).
Glen - gradient length (little delta δ).

3.4. Processing with CONTIN

Before using the CONTIN mode for DOSY processing you should have read the given references. TopSpin's CONTIN implementation is in fact directly derived from Provencher's algorithm[‡].

[‡] S. W. Provencher, A constrained regularization method for inverting data represented by linear algebraic or integral equations, Computer Physics Communications, **27**, 213-227 (1982).

S. W. Provencher, CONTIN: A general purpose constrained regularization program for inverting noisy linear algebraic and integral equations, Computer Physics Communications, **27**, 229-242 (1982).

S. W. Provencher, CONTIN (Version 2) Users Manual, Technical Report EMBL-DA07 (March 1984).

These three are available through http://s-provencher.com/pages/contin.shtml.

First of all, it must be noted that the mathematical problem considered here is called *Inversion of Laplace Transform*, or *Inverting Laplace Transform*. The wrong name *Inverse Laplace Transform* does exist in mathematics as well, but has definitively another meaning. To avoid ambiguity, please use only the right term *Inversion of Laplace Transform*!

Secondly, it is well known that inverting Laplace transform is a typically degenerate problem. An infinite number of solutions (particularly if we want more data points on output than there were in input) can be found, but the worst fact is that these various possible solutions can arbitrarily differ from each other (and from the expected true solution) at the same time still yielding approximately the same accordance with the input data (inside the range of the experimental noise). Therefore, in contrast to the previously existing DOSY fitting (***dosy2d***/***dosy3d***), which finds the best solution (with a restricted number of discrete exponents), CONTIN is searching for a reasonable solution (with a continuous distribution of exponents) among many various solutions which are approximately equally 'best'. The CONTIN program equilibrates between very noisy solution with many very sharp spikes (but which approximates the data rather good) and very smooth one having very broad lines (but which still approximates the data sufficiently good). At the same time, taking into account existing information (for example, knowing that our data must be non-negative) can help in the trade-off to choose the right one. This technique is called 'constrained regularization', and choosing the proper regularization parameters can in principle help to shift the choice of solution in favor of either better agreement (but with higher probability of spikes) or better smoothness (but with broader peaks). However, giving the continuous distribution of exponents, CONTIN yields generally more broad peaks than the discrete DOSY fitting. This phenomenon should not be considered as a mistake, this is a normal behavior of the mathematical nature.

CONTIN is implemented as a part of the TopSpin processing modules and is invoked with the same commands as the DOSY fitting: ***dosy2d*** for 2D or ***dosy3d*** for 3D data correspondingly. The algorithm used for DOSY processing (fitting or CONTIN), depends on the value of the new DOSY parameter ***Method*** which can have values *exponential* (for non linear least squares fitting), *contin* (which we are just describing), *decra* and *Gifa-Maxent*. All algorithms use the same parameterfile *dosy*, but possibly only a subset of the parameters. Each method has a few specific parameters not used by the other methods. The identity of the meanings of parameters

W. H. Press, S. A. Teukolsky, W. T. Vetterling, B. P. Flannery, Numerical Recipes in C: The Art of Scientific Computing, Chapter 18: Integral Equations and Inverse Theory (available through http://www.nr.com/).

allows to process datasets with fitting and CONTIN in different runs without changing any parameter except *Method*.

As CONTIN in principle can produce any distribution of exponents in any given exponent range, it cannot do any setup searching a reasonable display range similar to *dosy2d setup* available for fitting. Therefore, in principle, such a setup mode should be prohibited for CONTIN, but it would not be convenient. Instead, the setup mode is allowed for *Method* = *contin* as well, but *dosy2d setup* is always implicitly executed in the fitting mode. This feature does not lead to ambiguity due to the identity of all parameter meanings for both methods. Therefore, it is possible to set *Method* = *contin*, then execute *dosy2d setup* and immediately, without calling *eddosy* in between, start *dosy2d* in the CONTIN mode.

3.4.1. More details about CONTIN

At the very beginning of CONTIN processing the program must translate the Fredholm's integral equation into the corresponding system of linear algebraic equations. To do it, the program firstly divides the region between *DISPmin* and *DISPmax* on a grid consisting of *SI1* data points intended for the final output. How to calculate the grid, is defined by the parameter *Scale*, which may be *Logarithmic* or *Linear*, same as for DOSY fitting. Then the program integrates the Fredholm's equation along the grid. This procedure is called *quadrature* and the corresponding DOSY parameter is called *Qmode*. This quadrature may be done in either *Trapezoidal* or *Simpson* mode, the default is *Trapezoidal*.

The CONTIN program can take into account the presence of an arbitrary constant baseline in the data. Whether the baseline should be corrected, is controlled by the parameter *Bvary* which defaults to *No*. The data transferred on output will have the baseline, if varied, added to them, as was done for DOSY fitting.

If it is a priory known that the data must be non-negative – the typical situation for NMR data - CONTIN will more easily find the right solution. Therefore the parameter *Nonneg* should be set to *Yes*, which is also the default value (the baseline, if *Bvary* = *Yes*, may still become negative, *Nonneg* controls the exponents only). However, if the data may be positive as well as negative, it is possible to set *Nonneg* to *No*. Usually in the absence of other constraints the solution becomes unacceptably wildly oscillating. To (partly) overcome this problem, if *Nonneg* = *No*, the program automatically assigns the ordinates on both endpoints of the solution to zero. However, if this technique helps sufficiently, is a question of a chance...

Then the CONTIN program tries to find an appropriate constrained regularized solution. As already said, as constraints either nonnegativity of all solution points, or equality to zero of

both endpoints can be set. Choosing the proper regularizer is the most arcane part of the analysis. The algorithm programmed in the CONTIN subroutine itself firstly scans six solutions with widely deviating regularizer parameter (the regularizer parameter is called *alpha* in the CONTIN manual by Provencher) and computes a special probability criterion (which is called 'PROB1 TO REJECT') for them. From these six (or less if the best are found earlier) CONTIN chooses the two, whose 'PROB1 TO REJECT' is nearest to 0.5. Then CONTIN generates other six intermediate alpha values which lie in the middle between the two found coarse solutions, and refines them in the same manner. The refined solution which has 'PROB1 TO REJECT' closest to 0.5 becomes the so called *chosen solution*. The 'PROB1 TO REJECT' criterion is then analyzed by the calling dosy2d function. If it lies between 0.1 and 0.9 (which is usually so for sufficiently good data), the chosen solution is accepted immediately. If 'PROB1 TO REJECT' is outside the acceptance region, then dosy2d divides the starting alpha by 10 and calls CONTIN for the same column again. In such manner up to six trials (each with alpha divided by 10 in comparison with the preceding alpha) may be done. If some trial yields a solution with accepted 'PROB1 TO REJECT', it is placed into the output dataset. If after the six trials no 'PROB1 TO REJECT' value comes into the region between 0.1 and 0.9, the latest solution (with the smallest alpha) is placed on output. More than six trials will not be done because most probably they would not succeed too. If the user has some idea which regularizer parameter alpha might be most suitable, he can enter the value in the DOSY parameter *RegMult*. By default *RegMult = 1.0* (which means: don't modify the values suggested by CONTIN) and it may be either bigger or less than unity. Under the same other circumstances smaller values of *RegMult* may produce narrower CONTIN peaks with slightly better agreement with the data (and slightly higher probability of spikes), although the technique of the solution scanning in CONTIN and automatic reduction of *RegMult* on unacceptable 'PROB1 TO REJECT' may compensate *RegMult* deviations and still yield approximately the same solution.

Another (less important) parameter for regularizer control is *DFmin*. While computing solutions, CONTIN checks that the estimated number of degrees of freedom in the solution is not smaller than *DFmin*. If CONTIN finds that it is too small, the regularizer is automatically adjusted to enlarge it which usually may lead either to slightly broader peak or to appearance of one more additional small peak. Therefore, *DFmin* could be very approximately understood as a minimum number of distinct exponents in the solution (not counting the baseline if *Bvary = Yes*) which must have been found. Setting *DFmin* to *-1.0* totally disables the *DFmin* control, so that any number of degrees of freedom will be accepted. By default *DFmin = 2.0*, as recommended in the CONTIN manual by Provencher.

Perhaps, for the most flexibility of the regularizer, one could set **DFmin** to *-1* (switch degrees of freedom control off, as there may be columns with a peak from the only component), leave **RegMult** at *1* (or reduce it, let's say, to *0.001*), and set **Bvary** to *Yes*. Analyzing debug output of CONTIN I have really seen improvement of 'PROB1 TO REJECT' for some data columns after setting **DFmin** to *-1* and/or reducing **RegMult**, although some other columns did not show any tolerance to these parameters. Thus, whether manual adjustment of **DFmin** and **RegMult** leads to an improvement, highly depends on the data. Setting **Bvary** to *Yes* sometimes improves the accordance of the solution with the data and its quality (narrower peaks, better 'PROB1 TO REJECT').

While processing, the program checks if some points of the processed column lie above the **Noise*PC** bound, if no point does, the whole column is believed to contain pure noise and is completely zeroed on output. Exactly the same technique was used in DOSY fitting too.

Last, but not least the **F1mode** Parameter controls, what information to send on output at all (*Peaks* or *Decays*), the meaning being identical to that used in DOSY fitting.

All the other parameters: **Maxiter**, **EPS**, **Nexp**, **SpiSup**, **Imode**, **LWF**, **Npars**, **Nvar**, and all the component- and baseline-specific parameters (except **Bvary** which was described earlier) are meaningless for CONTIN and will be silently ignored. Analogously, the four CONTIN specific parameters: **Nonneg**, **Qmode**, **DFmin**, and **RegMult** will be silently ignored by DOSY fitting.

As CONTIN processing usually takes significantly more CPU time than fitting, the program shows some progress feedback in the TopSpin status line. In about 15 seconds after the message „F1: processing column xxx to xxx [xxx]" CONTIN writes in intervals of 5 seconds messages like „CONTIN: analyzing column xxx of xxx..." so, that one can be sure that the program is not hanging or cycling and approximate somehow the time to wait for finish.

If some errors or warnings come from the CONTIN algorithm, the program accumulates them and shows the latest message after the very end of processing. Several special kinds of CONTIN warnings (two up to now) are not shown because they come rather often while being not fatal and still leading usually to quite acceptable solutions.

3.5. Processing with DOSY*m*™

DOSY*m* **(D**iffusion **O**rdered **S**pectroscop**Y** *m*odule**)** is an efficient, easy to use, processing module especially designed for Bruker TopSpin NMR Software. Similar to the above described DOSY processing (chapter 3.1.), it delivers DOSY spectra. Instead of a plain fitting method, it uses a maximum entropy algorithm to obtain the result. For a complex mixture of small molecules with highly overlapping signals, maximum entropy processing typically is able to distinguish much better between the different compounds. Further information is available in the **DOSY*m*** manual. TopSpin provides the interface to use this module, but the module is not part of the TopSpin program suite, you have to buy a license separately. Bruker is able to deliver an evaluation version to you.

[TM] DOSY*m* is a trademark of NMR*tec* S.A.S., France: http://www.nmrtec.com

4. Appendix

4.1. Bruker Release Pulse Programs for Diffusion

1D pulse programs for setup or as diffusion filtered sequence:

|	
stegp1s1d	STE with 1 spoil gradient
stebpgp1s1d	STE with bipolar gradient pulse pair, 1 spoil gradient
stebpgpin1s1d	STE with bipolar gradient pulse pair, 1 spoil gradient, and INEPT transfer to observe an X nucleus
stebpgp1s191d	STE with bipolar gradient pulse pair, 1 spoil gradient, 3-9-19 WATERGATE solvent suppression
ledgp2s1d	LED with 2 spoil gradients
ledbpgp2s1d	LED with bipolar gradient pulse pair, 2 spoil gradients
dstegp3s1d	double STE with 3 spoil gradients, for convection compensation

2D pulse programs:

stegp1s	STE with 1 spoil gradient
stebpgp1s	STE with bipolar gradient pulse pair, 1 spoil gradient
stebpgpin1s	STE with bipolar gradient pulse pair, 1 spoil gradient, and INEPT transfer to observe an X nucleus
stebpgp1s19	STE with bipolar gradient pulse pair, 1 spoil gradient, 3-9-19 WATERGATE solvent suppression
ledgp2s	LED with 2 spoil gradients
ledbpgp2s	LED with bipolar gradient pulse pair, 2 spoil gradients
ledbpgpml2s2d	LED with bipolar gradient pulse pair, 2 spoil gradients, MLEV17 TOCSY transfer, "diffusion filtered TOCSY"
ledbpgpml2s192d	LED with bipolar gradient pulse pair, 2 spoil gradients, MLEV17 TOCSY transfer, 3-9-19 WATERGATE solvent suppression, "diffusion filtered TOCSY"
dstegp3s	double STE with 3 spoil gradients, for convection compensation

3D pulse programs:

ledbpgpco2s3d	DOSY-COSY, LED with bipolar gradient pulse pair, 2 spoil gradients, COSY transfer, magnitude mode
ledbpgpml2s3d	DOSY-TOCSY, LED with bipolar gradient pulse pair, 2 spoil gradients, MLEV17 TOCSY transfer

ledbpgpno3s3d   DOSY-NOESY, LED with bipolar gradient pulse pair, 2 spoil gradients, NOESY transfer

4.1.1. Example pulse sequence: ledbpgp2s (LEDbp)
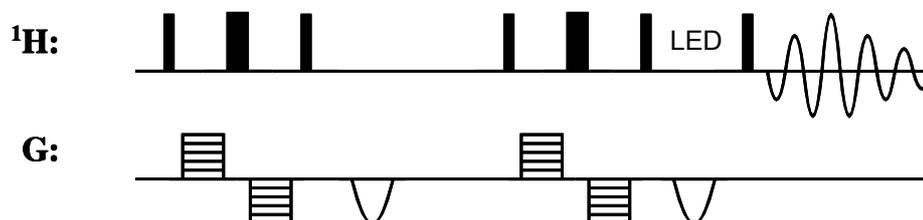
4.2. Important Parameters used in Diffusion Pulse Programs

P30:   is the length of the diffusion gradient *little delta* δ; for a pulse sequence using bipolar gradients it is $\delta/2$. The strength is defined by **gpz6**.

D20:   is the diffusion time *big delta* Δ.

D21:   is the additional delay in LED type pulse sequences (LED = longitudinal eddy current delay); it acts like a Z-filter; typically 5 ms.

P19:   additional spoiling gradients placed in the LED delay or the Z period of the diffusion time in STE type pulse sequences.

D16:   is the gradient recovery delay; a probe dependent parameter; for HR probes with Z gradient coil typically $50 - 100$ μs, for probes with XYZ gradient it may be up to 500 μs.

4.3. Experimental parameters for the example DOSY spectrum

Figure 3.2 shows a DOSY spectrum as an example. Here are the parameters used.

<u>Acquisition parameters:</u>

PULPROG:	ledbpgp2s	TD[F2]:	8K	TD[F1]:	16
NS:	32	DS:	8	D20:	50 ms
D21:	5 ms	P30:	2.2 ms	P19:	1.1 ms
GPZ6:	100 %	GPZ7:	-17.13 %	GPZ8:	-13.17 %

gradient ramp from 2 % up to 95 % in 16 linear steps (TD1) calculated with AU program *dosy*. The gradient calibration value was 5.35 G/mm.

<u>F2 processing parameters:</u>

SI[F2]:	4K	SI[F1]:	256	WDW[F2]:	EM
LB[F2]:	2	ABSF1[F2]:	100	ABSF2[F2]:	-100

ABSG[F2]: 0

execute *xf2* followed by *abs2*.

DOSY processing parameters (only non-default values given):

PC:	10	Scale:	Logarithmic	LWF:	5
DISPmin:	-10	DISPmax:	-8	Gdist:	49.95 ms
Glen:	4.4 ms				

execute *dosy2d*.

In general, the DOSY parameters for all processing methods are accessed via the *eddosy* editor or the FETCHDOSYPAR and STOREDOSYPAR macros in AU programs.