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The Diffusion Ordered Spectroscopy (DOSY) Pulse Sequence and Defence Applications

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Human Protection and Performance Division
Defence Science and Technology Organisation

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ABSTRACT

This technical report will discuss all aspects of the DOSY two dimensional NMR experiment, including setting up the experiment, the collection of raw data, and the data processing required. Details of the DOSY experiment are explained through data collected on a variety of samples involving several chemical classes. At the end of the technical report is a discussion on the application of the DOSY pulse sequence to aspects of defence science related mixture analysis.

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Executive Summary

Mixture analysis of organic chemicals has historically been performed using either high performance liquid chromatography or gas chromatography coupled to mass spectrometry. While historically significant and a proven and reliable analysis technique, they are destructive techniques and do not contribute definitive information regarding the structure of the chemical being analysed.

Without question Nuclear Magnetic Resonance (NMR) Spectroscopy is the most powerful analytical technique available to organic chemists in terms of determining the structure of organic chemicals as it yields in most cases unambiguous and definitive structural information. Information about the structure of the organic chemical in question can be derived through the running of many different homonuclear and heteronuclear one- and two-dimensional experiments.

Recently, a technique has become available for the NMR analysis of mixtures of organic chemicals which utilises a technique called Pulsed Gradient Spin Echo (PGSE) NMR, or so-called Diffusion Ordered Spectroscopy (DOSY). This method relies on the different rates of diffusion of chemicals through a solution, are directly related to the physical properties of the chemical components making up the mixture.

This technical report will address critical technical issues related to DOSY data, including the set up and collection of experimental data, the processing of these collected data, as well as the sensitivity of the experiment.

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Simon Ovenden graduated with a BEd-Sci in 1994 and with a BSc(Hons) in 1995 from The University of Melbourne. In 1999 he completed a PhD in marine natural products chemistry from the same institution. He then completed two years post doctoral research in Singapore at the Centre for Natural Product Research isolating and elucidating novel natural products as potential drug leads. Following this Simon spent three years at Cerylid Bioscience in Melbourne, then approximately one year at the Australian Institute of Marine Science, in both cases as a Senior Research Scientist researching novel natural products as potential drug leads from Australian biota. He joined DSTO in 2006 as a Defence Scientist in Analysis and Verification, where he is applying his background in Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry.

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1. Introduction

The chemical analysis of complex mixtures of chemicals continues to be a challenging area of work. Traditionally, mixture analysis has been performed using either high performance liquid chromatography (HPLC) or gas chromatography (GC). However, this technique alone does not elucidate definitive structural information. Additional information obtained through mass spectrometry coupled to these separation techniques (i.e. LC-MS and GC-MS) allows for more structural information to be obtained, but there are still issues that can impede unequivocal structural determination.

Unquestionably, the most unambiguous technique available to the analytical chemist for structure determination is Nuclear Magnetic Resonance (NMR). Definitive structure determination is able to be obtained through the running of many different experiments, in particular ^1H and ^{13}C one-dimensional experiments, as well as homonuclear and heteronuclear two-dimensional experiments. Despite this, there are limitations to NMR analysis of mixtures that are not trivial to overcome. In particular, for successful NMR analysis of potential chemical warfare agents, the analyte needs to be of reasonable purity, and the quantity of sample provided for analysis needs to be at least 0.2 mg.

A technique available to researches for the NMR analysis of mixtures utilises Pulsed Gradient Spin Echo (PGSE) NMR, called Diffusion Ordered Spectroscopy (DOSY).¹⁻⁴ This method relies on the different rates of diffusion of molecules through a solution, due to the inherent difference in the physical properties, to separate the components making up the mixture. DOSY spectroscopy has previously been utilised with some success in the analysis of ligand binding to both immobilised and membrane bound proteins.⁵ However, it has found application in other fields, such as the metabolic profiling of secondary metabolites produced in lettuce (Metabolomics).⁶

This technical report will address critical technical issues related to DOSY data, including the set up and collection of experimental data, the processing of these collected data, as well as the sensitivity of the experiment. Several mixtures of compounds have been analysed, ranging from small molecules (nerve agent simulants dimethyl methylphosphonate, or DMMP and diisopropyl methylphosphonate, or DIMP, see Figure 1) to larger molecules (bradykinin, Figure 1), as well as a synthetic reaction mixture containing several different compounds.

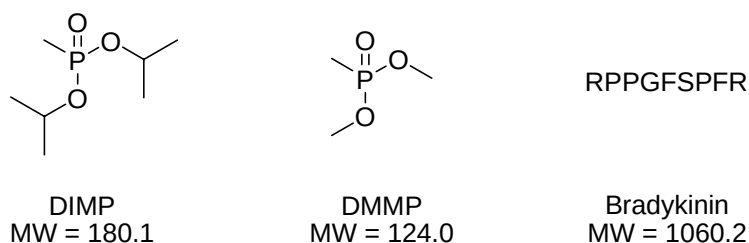


Figure 1. Structures of DIMP, DMMP and the amino acid sequence of bradykinin.

2. Results and Discussion

There are several pulse sequences available for the collection of DOSY data. The main variations are centred around an additional delay time for Longitudinal Eddy-current Delay (led) versus Stimulated spin Echo (ste) based pulse sequences. Also added to the pulse sequences are additional spoil gradients (identified as G7 in Figure 2 below), water suppression pulses (so called WATERGATE based sequences) to remove the water signal from the generated spectra, and additional dimensions (i.e. three dimensional NMR) for increased structure information. For the purposes of this study, only the stebpgp1s pulse sequence was investigated.

2.1 The stebpgp1s (DOSY) pulse sequence

Shown below in Figure 2 is the pulse sequences stebpgp1s that was used for these studies.

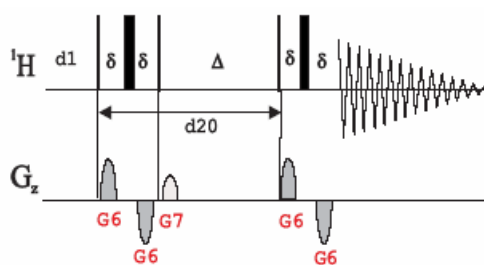


Figure 2. A schematic diagram of the stebpgp1s (DOSY) pulse sequences.

Of particular importance are the parameters Δ (d20), δ (p30), gpz6, as well as optimisation of the 90° pulse length (p1) and pulse power (p11).

Diffusion Time (Δ , d20)

This is the delay time between gradient pulses, that is, the time between the bipolar pulses pairs G6 shown above in Figure 2. Hence, it is the time that components within the mixture are allowed to diffuse through the mixture that is being analysed. Typical times for d20 are 50-100 msec.

Gradient length (δ , p30)

This is the length of time that the gradient is applied for (shown as δ in Figure 2). Typical times for p30 range from 1-5 msec. For pulse sequences on Bruker NMR spectrometers, the value added is equal to $\delta/2$.

Gradient power (gpz6)

The value of gpz6 is the amount of power that is applied to attenuate the gradient pulse (p30). This value is generally run as a linear gradient from 2% through to 95% in 16 equivalent steps.

When optimising diffusion parameters for the DOSY experiment, the one-dimensional form of the experiment is run (in this case stebpgp1s1d). Values for d20 and p30 are selected with gpz6 set to 2%, and the experiment run. The data is processed using the standard processing procedure for one-dimensional NMR (fp or efp, and apks if phasing is

needed) and the processed data stored in another procedure (wrp 2). The value of gpz6 is then reset to 95% and the experiment re-run and re-processed. When the two spectra are compared there should be a 95% reduction in signal. If the attenuation is not set correctly, then adjustments in the value of d20 and p30 need to be made. It is worth noting that adjustments to the value of p30 (δ) has a larger effect as it directly affects the signal attenuation, while d20 (Δ) has a linear effect on the exponential decay function, and as a consequence of this, a reduced effect on decreasing signal attenuation.

2.2 Non-sequence based NMR parameters

In addition to the above parameters, there are several non-sequence dependant parameters crucial to successful collection of DOSY data. These include sample depth, NMR probe temperature stability and solvent viscosity, and spinning versus non-spinning of sample.

Sample depth

The synthetic reaction mixture MLA-1-037/B is the product of a reaction containing two major and several minor components. The mixture was dissolved in 600 μL of D_2O and the diffusion parameters were optimised (d20 = 60 msec; p30 = 2.6 msec). When the one-dimensional data were collected, non-uniformity was observed in the baseline of the NMR spectrum around the methyl triplet signals at δ 0.97 and δ 1.08 (see Figure 3). This is despite the fact that excellent ^1H NMR spectra were previously recorded. A reduction of the sample volume for analysis to 400 μL significantly reduced the observed non-uniformity in the baseline of the NMR. The most likely cause of this problem is the non-linearity of the gradient across the probe, and hence the sample. As the sample volume is reduced, there is a reduction in this problem.

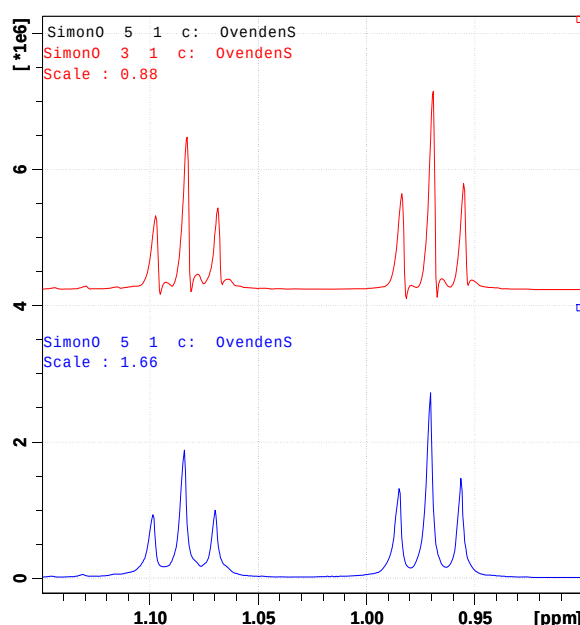


Figure 3. One dimensional NMR data for the reaction mixture *stebpgp1s1d*, with an expansion between δ 0.90 and δ 1.15. Top spectrum: mixture dissolved in 600 μL of D_2O ; Bottom spectrum: mixture dissolved in 400 μL of D_2O

Probe temperature stability and solvent viscosity

The temperature stability of the NMR probe is important for DOSY NMR. Temperature gradients across the sample being analysed will inevitably cause differences in the rate of diffusion across the sample, leading to generation of broad distorted cross-peaks, and hence poor quality data. Also related to this issue is the viscosity of the NMR solvent. If a temperature gradient occurs across the NMR sample, a flow of solvent is generated through the sample caused by convection currents, and hence different rates of diffusion of the analyte will be observed, leading again to poor quality data. Reduction in sample volume helps suppress this issue, as does appropriate solvent selection (i.e. d_6 -DMSO and D_2O as opposed to $CDCl_3$). Temperature stability across the probe can also be improved through an increase in the gas flow rate into the probe. To try and achieve the most thermally stable set of conditions, for the experiments conducted in this report the gas flow across the probe was set at 800 L/hr.

Spinning versus non-spinning of the sample

Spinning the sample to be analysed has the effect of a reduction of the convection current issue that was outlined above. However, this is offset to a certain degree by the introduction of other interference factors such as vibration.

In general, when performing DOSY analysis, it has been shown¹ from previous studies that for best data, reducing the sample height to as low as possible without loss of peak shape as well as using reduced volume NMR tubes such as 3 mm, are the best for successful analysis. The reasons for this are decreases in errors due to gradient non-linearity across the sample, and reduction in convection currents introduced through temperature gradients across the sample. This allows for more consistent diffusion to be attained. As only 5 mm NMR tubes were available for this study, it was decided to spin the sample, increase gas flow across the probe to 800 mL/hr, and have reduce sample volume to 400 μ L.

2.3 Data Processing

As the DOSY NMR experiment is essentially a series of one-dimensional experiments where the signal is slowly attenuated, the collected one-dimensional data needs to be processed and collated into a two-dimensional spectrum. The processing parameters important to the F2 parameters (in other words, the individually collected one-dimensional NMR spectra that make up the DOSY sequence), and the parameters associated with DOSY diffusion processing their corresponding definitions and suggested default values are discussed below in Table 1.

Table 1. Definition of the processing parameters for DOSY, and suggested values

F2 Processing parameters	Definition	Suggested values
SI[F1]	Zero filling in F1 (diffusion dimension)	1k
SI[F2]	Zero filling in F2 (¹ H NMR dimension)	32k
ABSF1[F2]	Sets limits for automatic baseline correction.	100
ABSF2[F2]	Sets limits for automatic baseline correction.	-100
ABSG[F2]	Sets the type of polynomial that is applied to the dataset for baseline correction.	0
WDW[F2]	Window function that is applied to the data set. Allows for strongest portion of the FID to be applied to the whole data set.	EM
LB[F2]	Line broadening. Allows for increase of signal to noise, at the expense of resolution.	2 Hz
DOSY Processing parameters	Definition	Suggested values
PC	Noise sensitivity	10
LWF	Line width factor	5
Scale	Sets the scale for the diffusion parameters.	Can be Logarithmic or linear
DISPmin and DISPmax	Sets scale limits for F1 (diffusion)	-10 and -8
Gdist	Gradient distance	Determined by value of Δ
Glen	Gradient length	Determined by value of δ

2.4 DOSY NMR data

2.4.1 DIMP and DMMP

A mixture of DIMP (0.9 mg) and DMMP (1.2 mg) in 400 μ L of *d*₆-DMSO was used to determine the optimum temperature at which to perform the analysis. Additionally, this mixture was subjected to a series of 1:2 dilutions to analyse the sensitivity of the experiment.

Temperature

This analysis was performed with td[F1] and ns set at 32. A DOSY experiment was run at four different temperatures (298 K, 303 K, 308 K and 313 K) to try and establish an optimum temperature at which to run the experiment, the results of which are shown in Table 2 below, and also in Figure 4 on page 6. This analysis showed that while the diffusion coefficient for DMMP and DIMP increased as temperature increased, the difference between the diffusion coefficients for these compounds remained essential the same. Therefore from this analysis it appears that sample temperature has an impact on absolute diffusion, but relative diffusion remained consistent, and hence no discernable difference in DOSY spectra could be determined.

Table 2. Diffusion coefficient values at different temperatures for DMMP and DIMP

Temp (K)	Diffusion Coefficient (m ² /s)		
	DMMP	DIMP	Δ
298	-9.038	-9.117	-0.079
303	-9.003	-9.074	-0.071
308	-8.968	-9.042	-0.074
313	-8.944	-9.024	-0.080

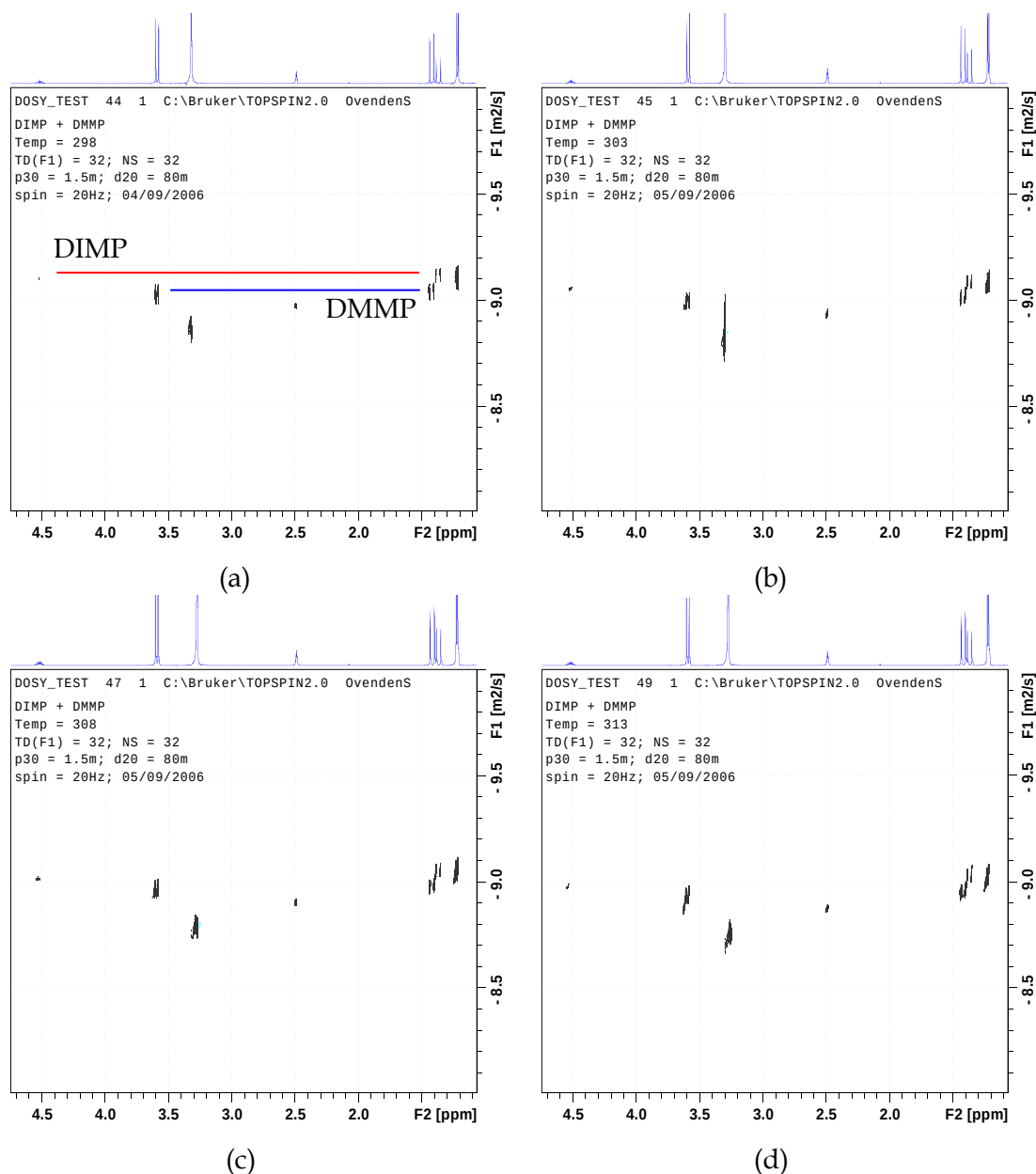


Figure 4. DOSY temperature studies on a mixture of DIMP and DMMP run at; a) 298 K; b) 303 K; c) 308 K; and d) 313 K

Considering the above data, all subsequent work in this report was performed at 298 K. In addition to this, for the purpose of sample integrity it is recommended that all experiments on the NMR are run at the lowest practicable temperature to avoid the potential for sample degradation.

Sensitivity

The solution of DIMP and DMMP were then subjected to three 1:2 dilutions, ending with final concentrations of 0.22 mg of DIMP and 0.3 mg of DMMP. The DOSY spectra of each are shown in Figure 5.

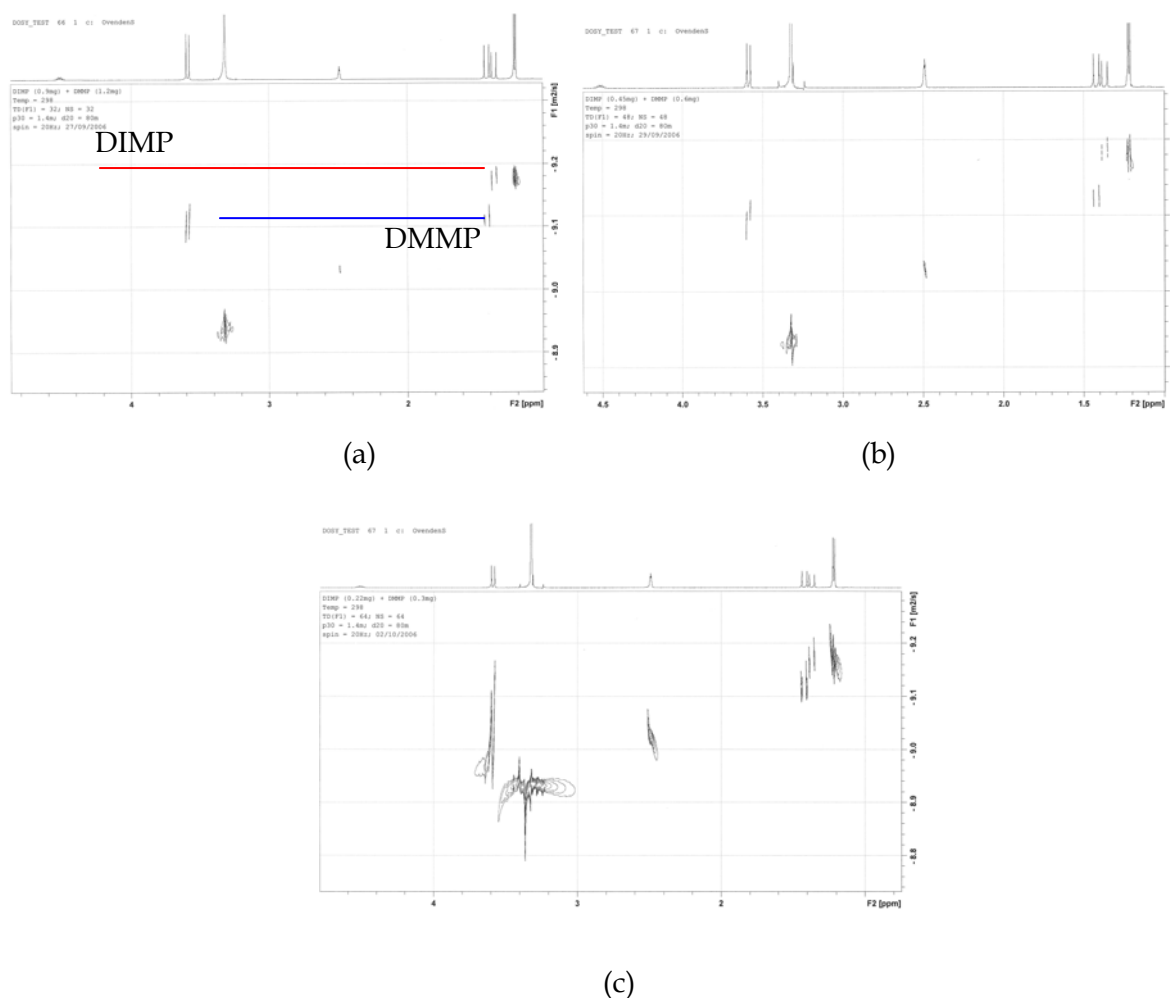


Figure 5. Dilution spectra (1:2) for a mixture of DIMP and DMMP: a) 0.9 mg DIMP and 1.2 mg DMMP; b) 0.45 mg DIMP and 0.6 mg DMMP; c) 0.22 mg DIMP and 0.3 mg DMMP

As the concentrations of the components of the mixture are diluted, more increments (td[F1]) and scans (ns) are required to obtain satisfactory spectra. Only marginal quality spectra were generated at the most dilute concentrations (Figure 5c). Despite this, it is still possible to discriminate between the components in the mixture. This analysis suggests that at concentrations less than around 300 μg in approximately 400 μL of deuterated solvent, analysis would take significant amounts of time, with the more than likely result of poor quality data. Despite this, the generated data may be useful depending on the complexity of the sample being analysed.

2.4.2 Synthetic Reaction Mixture

As an example of DOSY applied to a real sample, the previously discussed synthetic reaction mixture MLA-1-037/B was subjected to DOSY analysis. Initial ^1H and COSY data suggested that the reaction mixture was made up of two major components, as well as at least one minor component. Data was collected on three occasions with different (td[F1])

and number of scans (ns). Optimal spectral data was generated with (td[F1]) and number of scans (ns) set to 32. The generated DOSY spectrum is shown in Figure 6.

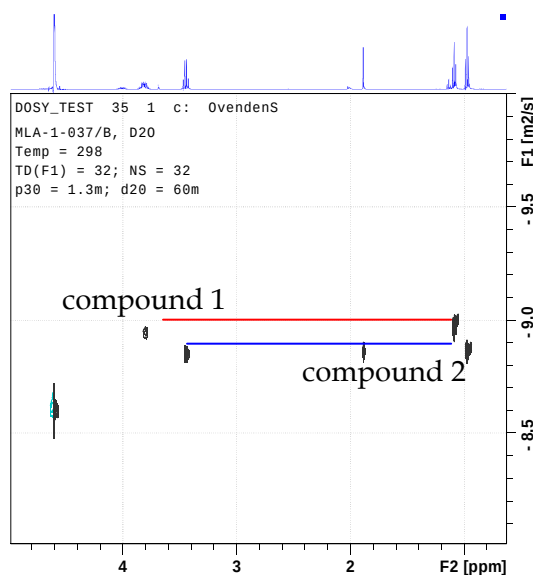


Figure 6. DOSY spectrum for the synthetic reaction mixture MLA-1-037/B

As can be seen, excellent resolution was achieved for the major components. However, the minor components were not detected, most likely as a consequence of being too dilute.

2.4.3 Bradykinin and DIMP

The final sets of experiments were conducted on a mixture of bradykinin (a peptide) and DIMP. The DOSY analysis of a mixture of compounds with a large molecular weight difference could be relevant when applied to more realistic applications (for example, analysis of a ligand diffusing to a relevant protein through a biofluid, or an analysis of a field sample containing a nerve agent).

Initially, a 1 mg sample of bradykinin in 400 μ L of D₂O was subjected to DOSY analysis to optimise conditions for the mixture analysis. The rationale behind this was that, due to bradykinin being significantly larger than DIMP, it would take longer to diffuse through a solution. This would in effect become the “rate limiting step”, and hence the diffusion parameters for this component of the mixture would be the most crucial. After several one dimensional experiments, during which time the 90° pulse, d20 (80 msec) and p30 (3.0 msec) were calibrated, data for a DOSY spectrum was collected, the spectrum of which is shown in Figure 7a. While some warping of the correlations was observed, due to any number of reasons previously outlined in section 2.2, a potentially informative spectrum was still generated.

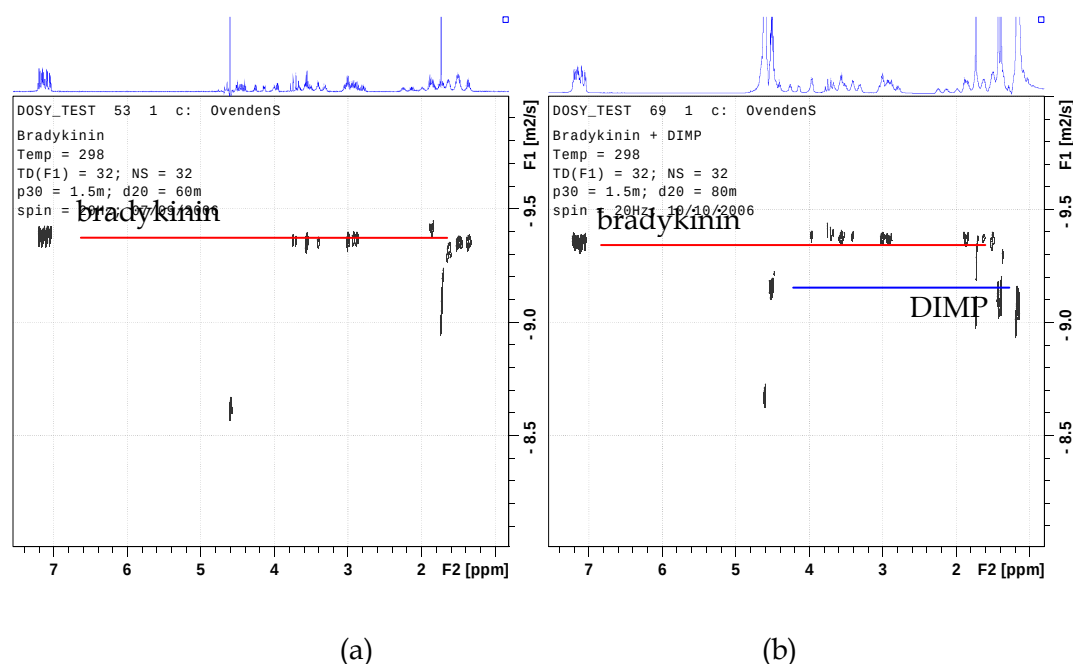


Figure 7. a) DOSY spectrum of bradykinin (1 mg) in 400 μL of D_2O ; b) DOSY spectrum of bradykinin (1 mg) and DIMP (1 mg) in 400 μL of D_2O .

With all relevant parameters optimised, a mixture of 1 mg each of bradykinin and of DIMP was dissolved in 400 μL of D_2O , and the data collected with ns and td[F1] each set to 32. The generated DOSY spectrum is shown in Figure 7b. Excellent differentiation is obtained between the two components. However, as observed for the bradykinin only spectrum, warping of the correlations is observed, this time for DIMP. Additionally, not all of the correlations associated with bradykinin are observed. This may simply be a concentration/sensitivity issue.

3. Potential Defence Applications

There are several potential applications for the DOSY NMR pulse sequence in relation to defence type scenarios. Some of these are discussed below.

3.1 Decontamination of Chemical Warfare Agents

Applications of DOSY to the *in situ* analysis of the decontamination reaction products of chemical warfare agents could be made, especially when applied during the development of new generation decontamination agents. Once essential DOSY parameters have been established, analysis of both intermediate and final reaction products can be achieved through DOSY, potentially yielding quantitative and qualitative information. Performing the reaction in an NMR tube provides several advantages over GC-MS. Firstly, DOSY can analyse a mixture of compounds that are both volatile and non-volatile, whereas for GC-MS it is necessary to make all the compounds in the mixture volatile. Secondly, utilising DOSY NMR for *in situ* analysis removes the requirement for sample work up (including derivatisation of polar compounds for GC-MS analysis as mentioned above), allowing for

the potential of faster analysis times. Thirdly, by measuring the diffusion coefficient through a decontamination solution, the efficiency of the decontamination agent could be measured. It is acknowledged that there are limitations with NMR compared with GC-MS in terms of the sensitivity of the technique, and the amount of compound required for analysis. However, despite this drawback, and on the proviso that reasonable amounts of material are available (mg amounts), significantly more structure information can be obtained through NMR analysis than GC-MS analysis.

3.2 Field Sample Analysis

The application of DOSY may be useful in field sample analysis. Following an initial clean up to remove floccular/particulate matter, samples submitted to the laboratory for analysis could initially be subjected to DOSY NMR for potential early structure class identification. Once the structure class has been identified, this information could then play a vital role in the guiding, and hence tailoring, of the purification process towards the identified structure class. Perhaps the most exciting aspect of this is that with the addition of a third dimension (ie. COSY-DOSY, HMQC-DOSY, ^{31}P -DOSY etc), there is significant potential that the components within the mixture, coupled with mass spectrometry data on the crude mixture, could potentially yield unambiguous structural information in a rapid timeframe.

One example of the above arguments is the analysis of sulphur mustard (HD) heels. On long term storage coupled with exposure to the environment (i.e. atmospheric H_2O), HD will polymerise to form HD heels.⁷ These heels form a protective layer around un-reacted HD, meaning that HD that has been exposed to environmental conditions for many years will still have significant residual activity, and as a consequence still be a significant potential risk. Current chromatographic mass spectrometry techniques don't allow for the analysis of high molecular weight polymeric material. However, application of DOSY could allow for characterisation of these heels to be performed, allowing for a convenient way forward in their analysis.

3.3 Ligand/receptor site binding in a biological fluid

The utilisation of NMR techniques for biological applications including the analysis of biofluids is an emerging field, and within this, the ability to analyse a ligand diffusing through a biological serum/fluid to a receptor site is potentially an extremely powerful tool.⁵

The molecular target for organophosphate based agents/fertilizers is acetylcholine esterase (AChE). Diffusion rates could be determined for this ligand/receptor binding, while competition between this binding effect and new therapeutics could be monitored and analysed using DOSY.

From a drug discovery perspective, the discovery of novel antibiotics is a pressing issue and a major concern worldwide due to antibiotic resistance and a chronic lack of new chemical entities entering the market. With the additional significant threat of bioterrorism, this need is ever increasing. The application of DOSY in the discovery of novel antibiotics from mixtures may help in the discovery of new novel antibiotics. Application of DOSY may aid in a rapid resolution of the binding site of the novel antibiotic to a particular mechanistic target. Through the study of chemical shift changes in

the generated NMR spectrum, specific binding site interactions as opposed to allosteric binding could be elucidated, allowing for a quick identification of false positive lead candidates. This technique could be especially useful in the discovery of novel naturally occurring antibiotics from microbes, as one of the pitfalls of this technique is the re-discovery of previously discovered antibiotics.

4. Conclusions

The PGSE technique, and DOSY in particular, is a potentially powerful technique in the NMR analysis of mixtures, as has been shown in the previously outlined examples. It allows for the separation of mixtures of compounds using the inherent diffusion properties of the individual components of the analyte in the solution the analysis is being conducted, through the collection of a series of one-dimensional experiments. Collation and processing of these experiments (and hence establishing a DOSY spectrum) allows components of the mixture to be separated through measurement of their respective diffusion coefficients.

As demonstrated above, the DOSY technique has been applied with success to both mixtures of compounds with very similar physical properties (DIMP and DMMP), to mixtures with very different physical properties (the peptide bradykinin and DIMP). Further developments to be investigated and reported for the application and method refinement for this technique include using reduced volume (3 mm) NMR tubes to increase the quality of data through the decrease of temperature gradients and eddy currents in the sample. Additionally, the application of DOSY NMR to the study of agent fate and mixture analysis in the field of decontamination, and the application of DOSY NMR in the analysis of field samples will also be investigated. The capability to run 2D DOSY NMR has now been established within HPPD, and is routinely being used in the day-to-day research of the division. This capability will be further developed to include 3D DOSY NMR pulse sequences (such as DOSY-COSY and DOSY-gHMBC) to allow for structure elucidation of compounds in situ.

5. Experimental

All experiments were performed on a Bruker Avance 500 MHz NMR spectrometer, equipped with a 5mm BBI probe. All experiments were run with a gas flow across the probes of 800 L/hr, with sample spinning, at a temperature of 298K.

DIMP (CAS 1445-75-6) was purchased in a 100 g bottle from Lancaster Chemicals, while DMMP (CAS 756-79-6) was purchased in a 100 g bottle from Sigma-Aldrich. Both chemicals were AR grade. Bradykinin was purchased in a 450 mg bottle from Auspep. The synthetic sample MLA-1-037/B was generated during synthetic studies, and was generously supplied by Dr Melissa Laws.

Samples were dissolved in deuterated NMR solvents supplied by Cambridge Isotopes. Spectra were referenced to residual protons in the NMR solvent (D₂O: δ 4.60; DMSO: δ 2.49). 5 mm glass NMR tubes were manufactured by Kontes.

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19. ABSTRACT This technical report will discuss all aspects of the DOSY two dimensional NMR experiment, including setting up the experiment, the collection of raw data, and the data processing required. Details of the DOSY experiment are explained through data collected on a variety of samples involving several chemical classes. At the end of the technical report is a discussion on the application of the DOSY pulse sequence to aspects of defence science related mixture analysis.					