

Basics of NMR spectroscopy

Theoretical background: P.W. Atkins: *Physical Chemistry*

Type of exercise: performed in pairs.

Goal of the experiment: Learning the fundamentals and practical basics of NMR measurements, including using a modern, bench-top NMR instrument and the preparation of liquid samples. A further goal is to learn about the relaxation mechanisms in NMR.

1 Introduction

1.1 Basics of NMR spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) is one of the most intensely used instrumental analytical techniques. NMR allows to gather both quantitative and qualitative information, and therefore it is widely used in different sectors of the chemical industry (e.g., production of organic chemicals/drugs).

The fundamental limitation of NMR spectroscopy as it only allows the investigation of atoms with nonzero nuclear spin. These are atoms in which the number of protons and/or neutrons is odd, such as in ^1H or ^{13}C - the most frequently studied atoms

The mathematical treatment of nuclear spin is analogous to that of electron spin. The angular momentum, and hence the magnetic momentum that forms in a magnetic field are both vectors, hence these can be described by a magnitude and a direction. The nuclear magnetic momentum – again analogously to the electron spin – can only possess $2\mathbf{I}+1$ discrete directions. In the absence of magnetic field, these quantum states are degenerate, their energy is identical. In the presence of a magnetic field however, the energy of the different states is given by the following equation:

$$E_m = \gamma m \hbar B_0 \quad (1)$$

where γ is the gyromagnetic constant of the given atom, \hbar is the reduced Planck's constant ($\hbar = h/2\pi$), B_0 is the magnetic induction (describing the magnetic field's strength, vector), while m is an integer value between $-I, -I+1, \dots, I-1, I$ (defining $2I+1$ different energy states). For a nucleus with $I=1/2$ magnetic momentum, 2 energy level develops (Zeeman effect):

$$E_{1/2} = \frac{1}{2} \gamma \hbar B_0 \quad (2)$$

$$E_{-1/2} = -\frac{1}{2} \gamma \hbar B_0 \quad (3)$$

The energy difference between these:

$$\Delta E = \gamma \hbar B_0 \quad (4)$$

For the transition between these states, an energy given in equation (4) should be applied in the form of electromagnetic radiation. As the energy of electromagnetic radiation is calculated as $h\nu$, we can write the following equations:

$$\begin{aligned} h\nu_0 &= \gamma \hbar B_0, \\ 2\pi\nu_0 &= \gamma B_0 \end{aligned} \quad (5)$$

As shown in Figure 1. the energy difference between the states is controlled by the strength of the magnetic field. These transitions are of low energy (in the energy range of MHz frequency radio waves), even if a large magnetic field is applied. Despite of this, the most efficient way to increase the sensitivity

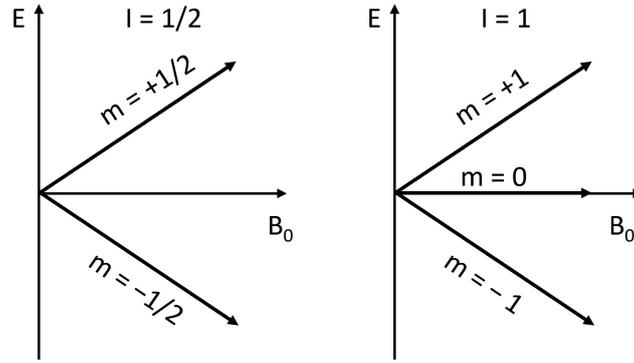


Figure 1: Different energy levels of atoms of different magnetic momentum, placed in a magnetic field - Zeeman effect.

of the instrument is to increase the strength of the magnetic field. In the instruments applied in cutting-edge scientific projects, electromagnets, forming strong magnetic fields (10–20 Tesla magnetic induction) are used. The high price of NMR spectroscopy stems from the use of these electromagnets, as the magnetic field homogeneity can only be maintained by the continuous cooling of these by using liquid helium. The energy difference between the states (i.e., sensitivity) also depends on the investigated atom (its type, abundance of its NMR active isotope and its gyromagnetic constant). Based on its abundance, the NMR measurements are notably more sensitive for ^1H atoms than for carbon atoms, as in case of the latter the 1 percent natural abundant ^{13}C is the NMR active isotope, not the dominant ^{12}C .

1.2 Generation of the NMR signal

To understand the basics of NMR signal generation, we have to consider the followings (without going into the details very deeply). When placed in a magnetic field, the magnetic momentum of a certain atom can only point towards discrete directions ($2I+1$, as explained above). Considering a magnetic field pointing towards the z-axis, the magnetic momentum vectors will be pointing to a circle, defining a cone, randomly distributed on the xy-plane (Fig2). Slightly more will be pointing towards in the direction of the magnetic field, but the population of the different energy levels will be still similar due to the small energy difference. This results in a small net magnetization along the z-axis (M_0). The interaction between the magnetic induction (B_0) and the magnetic vectors that are at a certain angle with respect to it, will cause these vectors to rotate along the surface of the cone at a constant rate - this is the Larmor-precession Figure (2.). The rate of this rotation:

$$\nu_{\text{Larmor}} = \frac{\gamma B_0}{2\pi} \quad (6)$$

where ν_{Larmor} is the Larmor-frequency (identical to the excitation frequency). Important to highlight that when a magnetic field is present, magnetic momentum can point towards specific directions, as defined by the magnetic momentum of the given nucleus (as described above). When the sample is removed from the magnetic field (or the field is turned off), the ordering of the impulse moment vectors fade away, and the net magnetization decays to 0.

To record the NMR signal, a B_1 coil is used, that is placed perpendicular to the B_0 magnetic field in the instrument. The excitation is achieved by a radio wave (electromagnetic radiation). When the frequency of this radiation matches the energy needs of the transition (as dictated by the Zeeman-effect), excitation occurs. This affects the direction of the net magnetization. Taking a typical 90° pulse excitation as example, the magnetization vector turns in the xy-plane, while precession around the z-axis still occurs. The reason behind this is that the number of spins in the different energy states will be equal due to the excitation. Another consequence of the excitation is that the spins - instead of being distributed randomly around the circle - will be crowded at some points and less crowded at others. When the excitation stops, relaxation occurs, and the original magnetization along the z-axis will be restored. The magnetization pointing in the

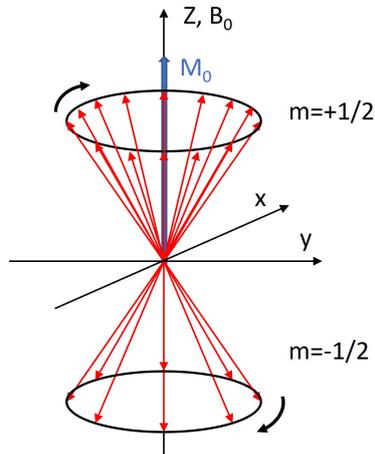


Figure 2: The ordering the the angular momentum in a magnetic field, and schematic visualization of the Larmor-precession.

xy-plane also decays, that can be detected using a suitable placed coil. The decay of the detected intensity provides the NMR signal. This is oscillating signal is the FID (**F**ree **I**nduction **D**ecay), that decays to 0 (between exponential curves) in a few seconds/minutes (Figure 3 .).

1.3 Relaxation processes in NMR spectroscopy and the used pulse-sequences for their measurement

Applying different pulses, the magnetization vector can be turned in basically any direction, not only to 90° . Different structural information can be derived using different pulse sequences – this provides the basis of modern NMR spectroscopy.

The difference between the energy levels in NMR spectroscopy is rather small (as described above). This causes the relaxation to be slow compared to other spectroscopic methods. This on one hand poses a technological challenge, while on the other hand offers the possibility to apply sophisticated pulse sequences during this time. This is very important for various applications of NMR spectroscopy, including clinical MRI measurements. Such experiments necessitate the knowledge on the relaxation process (including the relaxation times).

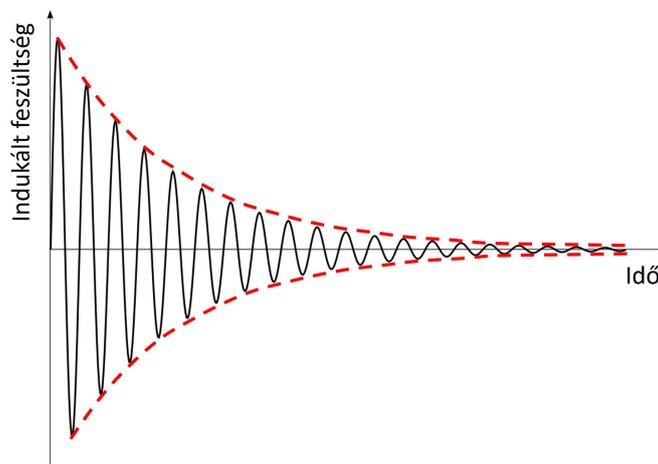


Figure 3: A typical FID signal recorded during an NMR measurements.

The relaxation occurs via two separate mechanisms:

- Longitudinal or Spin–lattice relaxation (T_1), meaning the restoration of the z-axis directed magnetization. T_1 is calculated based on the following equation:

$$I(t) = I_0 \left(1 - 2e^{-\frac{t}{T_1}} \right) \quad (7)$$

- Transverse or Spin–spin relaxation (T_2), meaning the decay of the magnetization pointing in the xy-plane. T_2 is calculated from the following equation:

$$I(t) = I_0 e^{-\frac{t}{T_2}} \quad (8)$$

Two different pulse sequence is used for the determination of T_1 and T_2 . As for T_1 , this typically means 180° pulse, hence turning the magnetization vector in the exact opposite direction. Subsequently, the magnetization in the z-direction will slowly get back to its initial value. Its magnitude is measured on the xy-plane, by turning this vector in the coil placed in the xy direction (applying a 90° pulse) after certain delay times following the pulse. To better visualize, Figure 4. shows the result of a typical experiment performed for the determination of T_1 . NMR spectra are recorded after applying different, short delay times after the excitation pulse. Applying very short delay time, negative peaks are observed on the spectrum. These negative peaks decrease with increasing delay time, and then turn into positive, growing peaks (until a saturation value). Note, that the relaxation time is affected by the chemical environment, therefore different values might be measured for the same atom (e.g., H atoms in different positions on a molecule) in a certain molecule. T_1 is determined by fitting the signal area– τ dataset based on equation (7) .

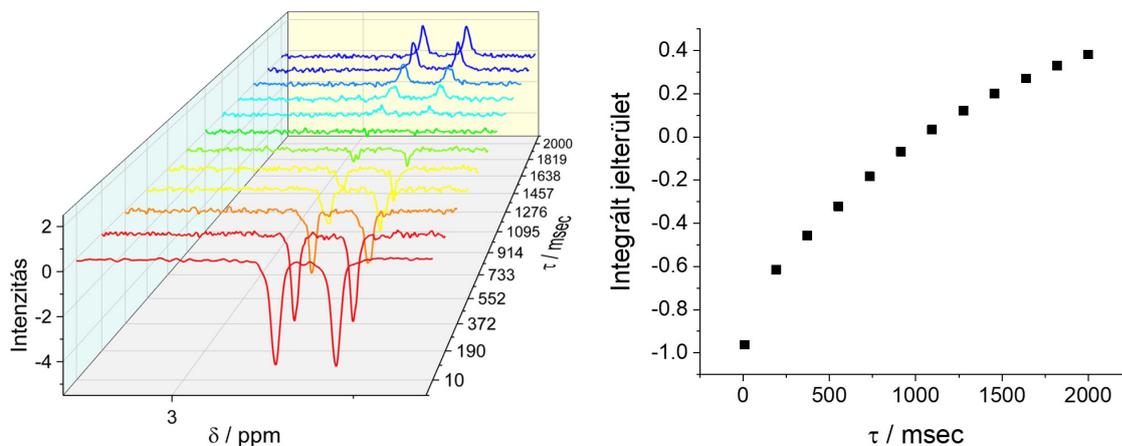


Figure 4: Results recorded during an experiment performed for the determination of T_1 , and the change of the integrated signal area in function of the delay time.

A 90° pulse is typically applied as the first step during the determination of T_2 . Following this, the spins that are focused in a certain direction in the xy-plane start to "spread" around the circle. After some delay time a 180° pulse is applied to start the re-focusing of the spins (that takes the same time as the delay time). The decay of this signal is followed to determine the value of T_2 . This is called the spin-echo experiment. The relaxation times, beyond the structure of the investigated molecule, depend on a number of parameters including the solution composition, temperature etc.. In the presence of paramagnetic compounds the relaxation is notably faster, caused by the dipolar interaction between the investigated nucleus and the paramagnetic compound.

1.4 The chemical shift scale

The widespread use of NMR spectroscopy is facilitated by the fact that this technique provides information on the chemical environment around the investigated nucleus. This is caused by the variation in the effective magnetic field (that could differ from B_0) around a certain nucleus, caused by the shielding of its surrounding. Equation (5) is still valid, but instead of B_0 , the effective magnetic field has to be considered ($B_{\text{effective}}$):

$$B_{\text{effective}} = B_0(1 - \sigma_x), \quad (9)$$

where σ_x is the dimensionless shielding coefficient describing the chemical surrounding of "X" atom. Because of this shielding, two identical atoms could be excited at different frequencies, hence these can be distinguished. The extent of shielding is affected by many factors, that are not given here at full details. In general, electron withdrawing groups in the close vicinity of the given atom will decrease the electron density, hence increasing the magnetic field strength around it. This results in higher energy (hence frequency) excitation. In case of electron donating groups the trend is opposite.

It is important to note that the NMR instruments are typically characterized by a frequency (e.g., 600 MHz) value instead of the magnetic field strength. This is the frequency at which a free H-atom can be excited in the magnetic field of the instrument. As this magnetic field is different in every single instrument, the same molecule can be excited at different frequency. Using these values it would virtually be impossible to compare results recorded at different instruments. To circumvent this, instead of the excitation frequency, chemical shift (δ) is used, defined as:

$$\delta_X(\text{ppm}) = \frac{\nu_X - \nu_{\text{ref}}}{\nu_0} 10^6 \quad (10)$$

where ν_0 is the measurement (instrument) frequency, ν_X is the excitation frequency measured for the investigated sample, ν_{ref} is the excitation frequency of the same atom (e.g., ^1H) in a certain reference molecule, as measured with the same instrument. As this difference is typically very small, the value is multiplied by 10^6 , hence the ppm (**p**arts **p**er **m**illion) unit. This scale allows to directly compare results recorded on separate instruments.

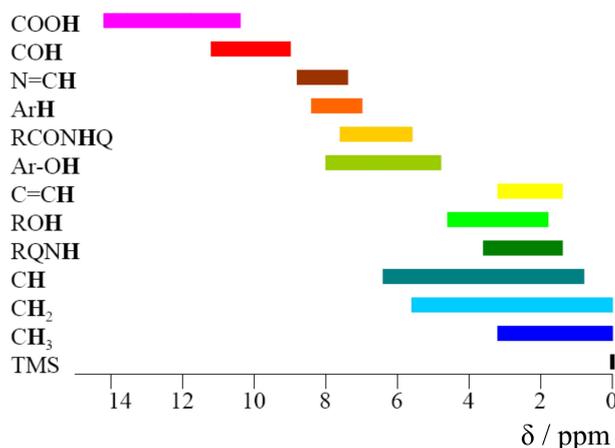


Figure 5: Typical ^1H NMR chemical shifts of different functional groups.

As reference for ^1H , ^{13}C and ^{29}Si NMR measurements, tetramethylsilane (SiMe_4 , TMS) is used. In this the 12 chemically equivalent proton provides a sharp and strong peak. Furthermore, because of the strong electron donating nature of the central Si atom, these protons are strongly shielded. Because of this, most proton signals are in the positive chemical shift range (TMS protons are at 0), as the local magnetic field is higher due to the lower shielding in these. In Figure 5, the typical chemical shift range of protons in different functional groups is visualized using TMS as reference. Important to note, that these are notably affected

by the solution compositions. However, as a first step of analyzing NMR spectra it is typical to analyze the chemical shifts of different signals/signal groups.

1.5 Signal splitting in NMR spectroscopy

The splitting of certain signals and the appearance of signal groups in the NMR spectra of even simple organic molecules can be typically observed. This is again rooted in the effective field strength around the investigated atom, that is further affected by the neighboring atoms. Considering two $I=1/2$ atoms close to each other, their spins interact with each other. In magnetic field the nuclear spins of both atom A and B can take two orientations - parallel or anti-parallel of the magnetic field. This will also affect the magnetic field around the neighboring atom, leading to the splitting of its NMR signal. The splitting is dictated by which atoms cause the splitting and on their number. This can result in different signal groups, hence we can infer to the number of chemically equivalent atoms that cause splitting. An example for this is shown in Figure 6. 3 chemically equivalent protons are located on the carbon atom on the left. These would give a single signal, as these can be excited at the same frequency. This signal however splits because of the 2 hydrogen atoms on the carbon atom next to it. As these 2 hydrogen atoms are also chemically identical, the splitting will follow the $n+1$ rule and lead to a triplet. Similarly, the signal of the two protons will split into a quartet. The H-atom in the OH group typically appears as a singlet. In such a simple example it is rather easy to establish the relationship between the NMR spectrum and the molecule structure. This is way more complex in case of larger molecules, but this task is not part of the current exercise.

With regards ^1H NMR spectra it is important to note that the area under the different signal groups is proportional with the number of protons this belongs to. This further helps the analysis of NMR spectra, as again demonstrated on the example of ethanol (Figure 6). The ratio here from left to right is 2:1:3, correlating with the number of protons in the molecule.

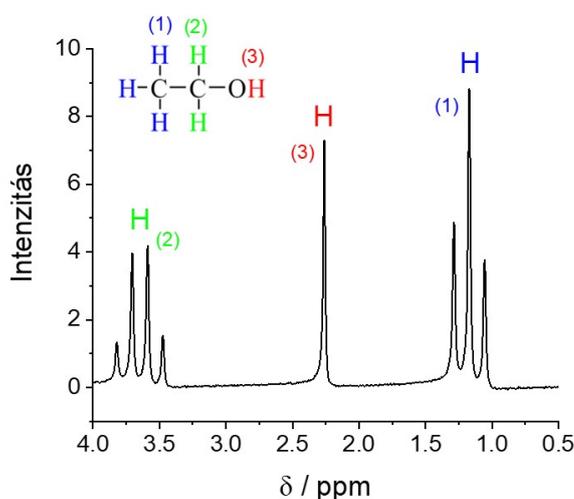


Figure 6: ^1H NMR spectrum of ethanol, recorded using the same instrument as during the current exercise.

2 Experiments performed

The goal of this exercise is to provide hands-on experience with NMR spectroscopy, and to assist the better understanding of the theoretical background. During this practice the Students will perform:

- A qualitative measurement to demonstrate the basic concepts of NMR spectrum analysis. The students will record ^1H NMR spectrum for some simple organic molecule, analyze the recorded spectra and assign it (from a list of possible compounds).

- A set of quantitative experiments to study the different relaxation processes, applying different pulse sequences. A simple model compound, caffeine will be used to study the NMR relaxation processes, hence deriving T_1 and T_2 .

Figure 7 shows the molecular structure of caffeine. In this, 3 methyl groups and a separate hydrogen atom (in a C-H bond) is seen. As the chemical environment of the different proton groups is different this results in 4 signal groups in the ^1H NMR spectrum.

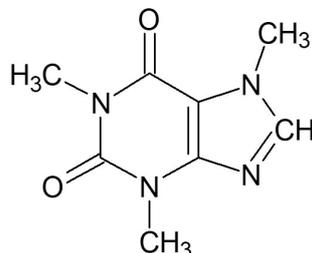


Figure 7: Molecular structure of caffeine.

2.1 Solution and NMR sample preparation

The protons in water are also NMR active, that would lead to a very high background signal, disturbing the measurement of other protons. This can be circumvented by two methods: either we keep the water protons in continuous excitation (hence there is no relaxation, no NMR signal) – this is called water suppression – or we apply water-free solvents. During this laboratory practice we apply the latter method by using D_2O as solvent. The following solutions should be prepared for this laboratory practice:

- **solution I.** $c = 70$ mM caffeine in D_2O ,
- **solution II.** $c = 70$ mM caffeine in D_2O solution, to $500 \mu\text{l}$ of which $10 \mu\text{l}$ $0,09$ mM cobalt(III)-acetylacetonate solution is added,
- **Solution III.** $c = 70$ mM caffeine in D_2O solution to $500 \mu\text{l}$ of which $10 \mu\text{l}$ $0,09$ mM gadolinium(III)-chloride solution is added.

D_2O is an expensive solvent, therefore it is typical to make small volume NMR samples (also, not much is needed to fill and NMR tube). Hence $V=1000 \mu\text{l}$ solutions should be prepared, by measuring the solid caffeine in Eppendorf tubes, and adding $1000 \mu\text{l}$ solvent using a pipette. Although the density of the solution is slightly different from that of the pure solvent, this minimal difference can be neglected during the practice.

Subsequently, $500 \mu\text{l}$ of the prepared solutions should be filled in dry new/cleaned 5mm diameter NMR tubes. The tubes should be closed by the provided airtight caps. Be careful, as the thin glass wall of the NMR tubes might break. To avoid this, place the NMR tubes in the provided plastic tube, and put the cap on the tubes in this. Importantly, entrapment of air bubbles in the NMR tubes must be avoided! Try pouring the solutions on the inner wall of the tubes using a pipette.

Aim to make the D_2O based solutions as fast as possible, as the deuterium-proton exchange is a rapid process, that can occur when the solution is subjected to air. This could hamper the collection of high quality NMR results. In this case the D_2O is directly measured from the liquid vessel using a clean pipette tip (do not pour it into a separate beaker).

Place the filled NMR tubes in the pre-heater unit that is located next to the NMR spectrometer. This ensures that the samples are at the same temperature as the inner chamber of the instrument, and that the temperature within the samples is homogeneous.

Place the NMR tube containing the investigated solution in the NMR spectrometer through the hole on the top of the instrument. Be sure that the tube is pressed down to the bottom of the measurement chamber

(but do not break them!). The measurements are started according to Appendix I., or based on the Instructor's advises.

A standard D₂O–H₂O is supplied for the calibration and shimming of the instrument. This is found in an NMR tube that is closed with a permanent, blue cap. When finishing the exercise, place this in the measurement chamber.

2.2 Experimental work

1. After learning the use of the instrument (ask the help of the Instructor and read Appendix I.) record the ¹H NMR spectrum of solution I. with the following settings: average 8 spectra, wait 5 seconds between the measurements. Use the 1D measurement method and record the spectrum on the 0–12 ppm chemical shift range. For this set the center of the spectrum at 6ppm, and the width to 12 ppm.
2. Place the standard sample in the instrument and perform the shimming of the instrument.
3. Record again the spectrum of solution I. applying the exact same conditions! Compare the two spectra!
4. Identify the different signal groups on the NMR spectrum and assign these to the respective protons in the caffeine molecule. Some help can be found in scientific publications, for example in J. Chem. Educ. 2019, 96, 786-791. This can be freely accessed from the university network. When trying to access it from home, it can be accessed using your eduID from the University of Szeged (<https://u-szeged.hu/cc/eduid>).
5. Following the Instructor's advises and Appendix I., perform the evaluation of the recorded spectrum (phase and baseline correction) and integrate separately the signal groups related to the methyl groups in caffeine (3 signal groups). The instrument will use the range used for the integration during the relaxation measurements (T₁ and T₂).
6. Perform the T₁ measurement experiment! First apply a delay time range of 10–5000 ms (τ), measuring at 12 points in linear distribution. Only the range and the number of points have to be specified in the software. Record only 1–1 spectrum. Based on this measurement, narrow the delay time range so it only contains the exponential change and not the saturation region. Repeat the measurement on this range at 12 delay times, averaging 8 spectra at each!
7. Perform T₂ measurement similarly to the T₁ measurements, using the same initial settings as their. Narrow the delay time range and perform the more precise measurements averaging 8–8 spectra.
8. Repeat steps 3–7 for solution II.-III. (the assignation of the peaks is the same here)!
9. Record the ¹H NMR spectra of the unknown samples applying the settings used for recording the 1D spectrum of caffeine!
10. Export the results in JCAMP-DX format.
11. Place the standard sample in the measurement chamber.

3 Evaluation of the measured data

Use the MestreNova software for data evaluation! Although this is not a free software, but a 45 days long evaluation licence is provided by the company upon registration. Download the software from the website: <https://mestrelab-store.myshopify.com/>. When asking for the trial license, also ask for the activation of all NMR modules.

- Plot the 1D ^1H NMR spectrum of caffeine and assign the different signal groups to the respective protons in the molecule. Compare the area under the different signal groups and try to correlate this with the number of hydrogen atoms related to the certain groups. Compare the spectra recorded before and after performing the shimming of the instrument, and explain the observed differences!
- Using a freely chosen software (online or offline, the students are advised to use search engines to identify such) compute and plot the NMR spectrum of caffeine, assuming that a 400 MHz instrument was used to record the spectrum. Compare it with that recorded during the exercise! A possible software can be found here: <https://web.chemdoodle.com/demos/simulate-nmr-and-ms#customise-template>.
- Plot the NMR spectra recorded during the relaxation experiments (T_1 and T_2) performed on the optimized delay-time range and spectra averaging number (6. measurement point) for solution I. Evaluate the changes in time considering the applied pulse sequence!
- For the relaxation measurements (both T_1 and T_2), the software generates a separate `csv` file that contains the integrated areas of the previously identified signal groups, in function of the delay time. Plot these values on a figure for a chosen signal group recorded in solution I-III (three dataset on one figure). Repeat this to the other 2 signal groups, altogether necessitating to make 3 figures. Determine the T_1 values based on equation (7). Visualize the fitted curves in the figure!
- Repeat an analysis identical to the previous point for the T_2 measurement data. Again, this results in 3 separate figures in which the fitted curves are also visualized. Fit the data according to equation (8) and determine the value of T_2 !
- Collect the results in a table (T_1 and T_2 values in different solutions, for all 3 signal groups separately).
- Analyze the changes in T_1 and T_2 values with the change in the solution composition (addition of the coordination compounds). What effect is behind the changes?
- Plot and analyze the NMR spectra of the unknown samples. Try to identify the number (or ratio) of protons belonging to the different signal groups (integration of the curves to determine the area under certain signal groups can be performed manually via numeric integration or by using any specific software). Also, try to identify the number of neighboring hydrogen atoms that cause signal splitting. Assign your unknowns to the following possible samples:

– ethyl acetate	– 2-propanol	– 1-butanol	– toluene
– ethanol	– ethyl-bromide	– acetone	
– 1-propanol	– 2-bromopropane	– benzene	

Possible test questions

1. What is the basis of NMR spectroscopy, and what is the selection rule which atoms can be investigated via NMR?
2. What is the chemical shift in NMR spectroscopy? How does it depend on the chemical structure of a the given atom?
3. What is the Zeeman effect? What determines the magnitude of energy that is needed for NMR excitation of a certain atom?
4. What is the selection rule which atoms can be investigated via NMR? Give specific examples!
5. Why is NMR spectroscopy more sensitive for the investigation of hydrogen atoms, as compared to carbon atoms? How can the sensitivity be increased for the measurement of carbon?

6. What is the spin–lattice relaxation? What kind of experiment can be used to determine its time constant?
7. What is the spin–spin relaxation? What kind of experiment can be used to determine its time constant?
8. Schematically draw the ^1H NMR spectrum of ethanol! Explain the shape, location on the ppm scale and area of these!
9. Draw the molecular structure of caffeine. How many and what shape signal groups can be found on the ^1H NMR spectrum of this molecule?
10. How would the ^1H NMR spectrum of benzene (C_6H_6) look like? Explain the number, location and shape of the different signal groups.
11. Why is D_2O applied as solvent during the exercise? How can you avoid this, but still measure high quality data?

Appendix I.: The use of the Nanalysis 60Pro instrument

Nanalysis 60Pro (Figure 8.) is a benchtop NMR instrument, that uses a permanent magnet instead of the electromagnets typically found in higher resolution instrument. For this reason this does not requires very serious cooling (as it is done using liquidified He in case of high resolution instruments), only the room temperature should be kept below 24 °C, and constant.



Figure 8: Photo of the instrument used during the exercise.

The instrument is constantly turned on during the semester, therefore **do not turn it off when finishing your experiments**. The instrument can be operated on its touch screen, or by the connected keyboard and mouse. The screen of the instrument looks like this:



Figure 9: The main menu in the software running the NMR instrument used during the exercise.

Using the 4 large buttons below the orange settings button can we choose the experimental conditions. The first is to choose the investigated atom – this is typically set to proton. The second is for choosing the type of the experiment. During the exercise, the 1D, T₁ and T₂ measurement protocols should be loaded here. In the third, "Solvent" menu the used solvent, that is D₂O should be chosen. In the "Scan" menu the number of averaged spectra is given. To adjust the specific parameters of a certain measurement, press the Settings button, that leads to the following screen:

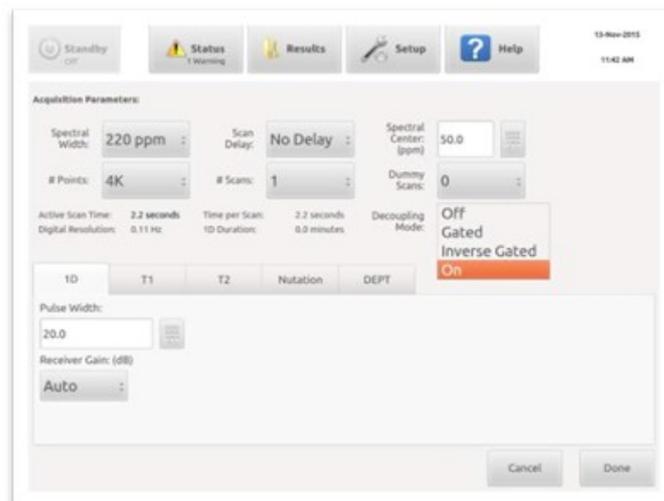


Figure 10: The settings menu of the Nanalysis software.

Here the different experimental conditions, such as the investigated chemical shift or delay time range can. When all is set, press the Done button to get back to the main menu.

The first measurement is performed without shimming. After this, press the "Status" button in the upper row and perform automatic shimming. For this, the standard sample must be placed in the measurement chamber. Perform a full shimming procedure.

Perform automatic baseline and phase correction on the spectrum recorded after shimming. Subsequently, integrate the area under the different signal groups using the manual integration function. To allow this, delete the automatically integrated values first. After this, proceed further to the relaxation experiments.

Appendix 2.: Using the MestReNova software

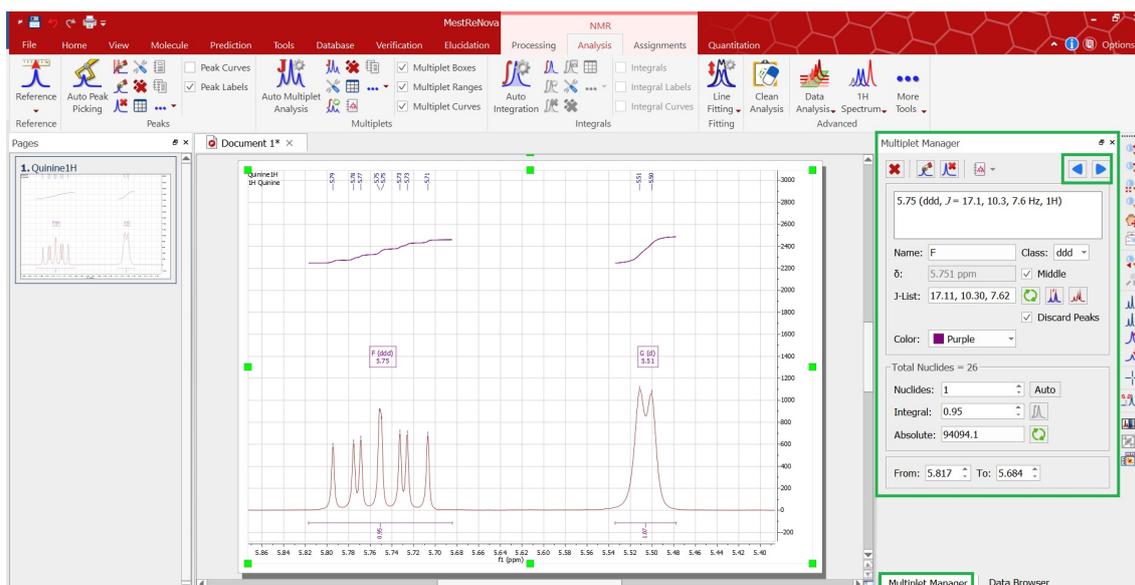


Figure 11: The main screen of the MestReNova software.

The measured data should be exported in JCAMP-DX format, that can be opened in MestReNova. Use the "File" menu "Open file" option, or simply drag and drop the file in the software window.

The imported data is readily plotted in the software. If a better formatted figure is to be prepared (that is the suggestion of the writer of this student exercise), go to the "file" menu and press "save as" to save the data in a format that can be imported in other software (e.g., Excel) – the .csv format is suggested. The spectra recorded during the T_1 and T_2 experiments are automatically shifted along the y-axis in the MestReNova figure, but not in the exported data. Therefore if an external software is used for plotting, this shift should be achieved in that (either manually or by using specific plotting options).

When exporting the results of the T_1 and T_2 experiments a .csv is also generated. This contains the area under the chosen signal groups in function of the applied delay times. These values are used for fitting for the determination of the relaxation time constants.