1 LC–NMR: Theory and Experiment

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1.1 INTRODUCTION

The conventional way of recording solution-state NMR spectra is by the use of a 5 mm cylindrical NMR tube in which the compound of interest is dissolved in 0.5 ml of a deuterated solvent. The sample is constantly available for an infinite time period for the registration of NMR spectra. With the commonly applied Pulse Fourier-Transform acquisition mode, a gain in signal-to-noise ratio (S/N) of the acquired NMR spectrum can be obtained by co-adding the Free Induction Decays (FIDs) resulting from pulse excitation. The FID is dependent upon the relaxation time $T_2$, which affects the line shape and the resolution of a spectrum. The recovery of equilibrium magnetization is determined by the spin–lattice relaxation time $T_1$ (Figure 1.1). After pulse excitation, it takes a time period of three to five times the $T_1$ to establish the full Boltzmann distribution, together with full magnetization of the nuclei. Then, a new excitation pulse can be applied. Thus, longer $T_1$ times, e.g. of aromatic moieties of an organic molecules, necessitate longer pulse repetition times. Whereas the S/N value is defined by the square root of the number of transients (NS), the pulse repetition time for a new excitation of fully relaxed nuclei is dependent upon the spin–lattice relaxation time $T_1$.

1.2 NMR IN A FLOWING LIQUID

In the conventional measuring mode the sample stays in the NMR tube, and thus in the radiofrequency Helmholtz coil all of the time. In the continuous-flow mode it resides within the NMR detection coil only for a distinct time of some few seconds (Figure 1.2). This residence time $\tau$ is dependent upon the volume of the detection cell and the employed flow rate (Table 1.1). For example, a detection volume of 120 μl, together with a flow rate of 0.5 ml/min, results in a residence time of 14.4 s, while with a detection volume of 8 μl the residence time is only 0.96 s. A shorter residence time $\tau$ within the NMR measuring coil results in a reduction of the effective lifetime of
the particular spin states. Thus, the effective relaxation rates, $1/T_n$, are increased by $1/\tau$:

$$\frac{1}{T_n \text{ effective}} = \sum \frac{1}{T_i} + \frac{1}{\tau}$$  \hspace{1cm} (1.1)

In a flowing system, the reciprocal relaxation rates, the relaxation times $T_{1\text{flow}}$ and $T_{2\text{flow}}$, are reduced according to the following:
Table 1.1 Variation of residence time and line broadening as a function of detection cell volume and flow rate in continuous-flow NMR spectroscopy.

<table>
<thead>
<tr>
<th>Volume (μl)</th>
<th>Flow rate 0.5 ml/min</th>
<th>Flow rate 1.0 ml/min</th>
<th>Flow rate 0.5 ml/min</th>
<th>Flow rate 1.0 ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>44.0</td>
<td>5.28</td>
<td>2.64</td>
<td>0.19</td>
<td>0.38</td>
</tr>
<tr>
<td>60.0</td>
<td>7.20</td>
<td>3.60</td>
<td>0.14</td>
<td>0.28</td>
</tr>
<tr>
<td>120.0</td>
<td>14.40</td>
<td>7.20</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td>200.0</td>
<td>24.00</td>
<td>12.00</td>
<td>0.04</td>
<td>0.08</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Volume (nl)</th>
<th>Flow rate 10 nl/s</th>
<th>Flow rate 20 nl/s</th>
<th>Flow rate 10 nl/s</th>
<th>Flow rate 20 nl/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.50</td>
<td>0.25</td>
<td>2.00</td>
<td>4.00</td>
</tr>
<tr>
<td>50</td>
<td>5.00</td>
<td>2.50</td>
<td>0.20</td>
<td>0.40</td>
</tr>
<tr>
<td>500</td>
<td>50.00</td>
<td>25.00</td>
<td>0.02</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\[
1/T_{1\text{flow}} = 1/T_{1\text{static}} + 1/\tau \quad (1.2)
\]

\[
1/T_{2\text{flow}} = 1/T_{2\text{static}} + 1/\tau \quad (1.3)
\]

In a net effect, the pulse repetition times in flowing systems can be reduced to the decrease in the apparent spin–lattice relaxation times \(T_{1\text{flow}}\), whereas at a given detection volume an increase in flow rate leads to an increase in the signal half-width \(W\) due to the decrease of \(T_{2\text{flow}}\).

\[
W = (1/\pi)T_2 \quad (1.4)
\]

\[
W_{\text{flow}} = W_{\text{stationary}} + 1/\tau \quad (1.5)
\]

Thus, the resolution of a continuous-flow \(^1\)H NMR spectrum is strongly dependent upon the flow rate/detection volume ratio.

Given a detection volume of 44 μl, a flow rate of 0.5 ml/min results in a residence time of 5.28 s and a line broadening of 0.19 min, whereas a flow rate of 1.0 ml/min leads to a line broadening of 0.38 Hz (see Table 1.1). This effect can be easily varified from Figure 1.3, which shows the signal half-width of chloroform at different flow rates. The static signal linewidth is 0.55 Hz; at a flow rate of 0.5 ml/min this is increased to 0.75 Hz (theoretical value, 0.74 Hz), and at a flow rate of 1.0 ml/min to 1.05 Hz (theoretical value, 0.93 Hz). To minimize the flow-induced broadening effect, NMR flow cells should provide residence times of the order of 5 s. Thus, the resulting line-broadening values will be about 0.2 Hz, which are tolerable for the acquisition of \(^1\)H NMR spectra.

In an on-flow NMR experiment, the excited nuclei leave the flow cell whereas ‘fresh’ nuclei enter. Due to the decrease of the apparent \(T_{1\text{flow}}\) rates, faster pulse repetition rates can be used and more transients in a distinct time-period can be accumulated (Figure 1.4). The theoretical maximum sensitivity is obtained
Figure 1.3 $^1$H NMR spectra (400 MHz) of chloroform recorded at flow rates of (a) 9 (stopped flow), (b) 0.5 and (c) 1.0 ml/min

Figure 1.4 Acquisition scheme used for a continuous-flow NMR experiment

when the pulse repetition time, PRT (The sum of the acquisition time, AQ, and relaxation the delay D1) is equal to the residence time $\tau$ in the NMR flow cell

$$PRT = (AQ + D1)_{\text{optimal}} = \tau$$  \hspace{1cm} (1.6)

If the ‘fresh’ incoming nuclei are fully magnetized upon entering the flow cell, e.g. the Boltzmann distribution is established, an increase in sensitivity can be obtained. This is demonstrated in Figure 1.5, which shows the $^1$H NMR spectra of the CH$_2$ protons of ethanol under stopped-flow and continuous-flow (2.4 ml/min) conditions.
Figure 1.5 $^1$H NMR spectra (400 MHz) of the CH$_2$ protons of ethanol recorded under (a) stopped-flow and (b) continuous-flow (2.4 ml/min) conditions

1.3 DESIGN OF CONTINUOUS-FLOW NMR PROBES

The first approach for continuously recording NMR spectra was to use the conventional existing probe for the registration of NMR spectra [1]. The latter are usually recorded under rotation of the NMR tube with a rotational speed of 20 Hz in order to remove magnetic field inhomogeneities. Thus, it should be sufficient to introduce a capillary within a rotating NMR tube and to suck off the effluent (mobile phase) with the help of a second capillary (Figure 1.6). The problem with this design is that no complete transfer of the mobile phase is guaranteed by the employment of the second capillary, and peak mixing, together with memory effects, will occur at the bottom of the rotating NMR tube. Thus, it would be more straightforward to employ a ‘bubble cell’ design of a widened glass tube. This approach was used for the registration of the first continuous-flow NMR spectra with iron magnets (Figure 1.7) [2-6] and also together with cryomagnets (Figure 1.8) [7-15]. This design, which was already introduced in the early 1980s, is still mostly used today.

Such a design combines the bubble-cell characteristics, together with an U-type design of the glass tube employed as the NMR detector. The glass tube is positioned within a glass Dewar, thus enabling temperature-dependent measurements. Another feature is the direct attachment of the NMR radiofrequency
(rf) coil to the glass tube, thus rendering any rotation of the tube impossible. However, in contrast to the conventional NMR probe design, the filling factor (ratio of sample volume to the NMR detection cell volume) is much higher (Figure 1.8). Because both the inlet and outlet of the continuous-flow detection cell are at the bottom of the cylindrical NMR probe, the whole probe body can easily be inserted into the room temperature bore of the cryomagnet. No problems with air bubbles exist because the NMR detection cell is filled from the bottom to the top against the earth’s gravity.

Due to the fact that within this design the radiofrequency coil is positioned parallel to the $z$-direction of the magnetic field of the cryomagnet, magnetic field homogeneity can be readily achieved, because the device for the correction of the magnetic field, the so-called ‘shin system’, is optimized for correcting inhomogeneities in the $z$-direction. Thus, the U-type flow cell shows very good NMR characteristics, despite the non-rotation of the cell. The quality of cryomagnets, together with the NMR probes, is checked by determining the signal linewidth of chloroform at the height of the $^{13}$C satellites and at 1/5 of this altitude. The obtained values (Figure 1.9) are very close to the conventional ones obtained without any rotation of the NMR tube. The large detection
volume of 120 μl employed leads to NMR sensitivity levels of about 100 ng for one-dimensional (1D) acquisitions, and of about 1 μg for two-dimensional (2D) NMR spectra. On the other hand, the chromatographic peaks are broadened by approximately 20%. The peak dispersion effects of several NMR flow cells were measured by directly evaluating chromatographic separations with the help of a modified fluorescence detector. These measurements lead to the conclusion that the plate height is adversely affected for capacity factors below 2.5 [15]. A further evaluation of peak dispersion effects is given later in Chapter 6 of this present book.

Better chromatographic peak performance is obtained with an NMR detection volume of 60 μl, although NMR sensitivity values suffer from the low amount of nuclei in the detection cell. Thus, despite its degraded chromatographic performance the 120 μl flow cell seems to be a good compromise.
Figure 1.8  Schematics of (a) conventional and (b) continuous-flow NMR probes suitable for cryo magnets

Figure 1.9  $^1$H NMR signal line shape of chloroform in acetone-$d_6$ (hump test), measured with a 120 $\mu$L continuous-flow probe (600 MHz)
between the NMR and chromatographic requirements. From the NMR viewpoint, it is not a problem to discriminate between the signals obtained from a major compound and a minor component, and thus the chromatographic peak broadening can be tolerated for the gain in NMR sensitivity. Major ongoing improvements in NMR sensitivity with micro-coils will lead to the development of capillary probes with superior characteristics.

1.4 EXPERIMENTAL ARRANGEMENT FOR HPLC–$^1$H NMR COUPLING

The main prerequisite for on-line LC–NMR, besides the NMR and HPLC instrumentation, are the continuous-flow probe and a valve installed before the probe for the registration of either continuous-flow or stopped-flow NMR spectra.

Due to the current development of cryomagnet technology, no benchtop-like cryomagnets will be available with a magnetic field strength between 9.4 and 14 T in the next few years. Therefore, the current available types of cryomagnets have to be used (Figure 1.10). Whereas a proton resonance frequency of 300 MHz is sufficient for GPC–NMR experiments it is advisable to use magnetic field strengths higher than 9.4 T ($^1$H resonance frequency of 400 MHz) for HPLC–NMR coupling. The position of the HPLC instrument is dependent upon the size of the stray magnetic field of the used cryomagnet.

Thus, in conventional installations the HPLC instrument is located at distances between 1.0 and 2.0 m from the cryomagnet, whereas with new available shielded cryomagnets the HPLC instrument can be directly hooked to the cryomagnet.

The analytical NMR flow-cell (see Figure 1.8) was originally developed for continuous-flow NMR acquisition, but the need for full structural assignment of unknown compounds led to major applications in the stopped-flow mode. Here, the benefits of the closed-loop separation-identification circuit, together with the possibilities to use all types of present available 2D and 3D NMR techniques in a fully automated way, has convinced a lot of application chemists [17-70]. A detailed description of the different modes for stopped-flow acquisition (e.g. time-slice mode) is found in Chapters 2 und 3.

Figure 1.10 shows an experimental arrangement of HPLC–NMR coupling which is currently employed in many analytical laboratories. In most laboratories, unshielded magnets are used at the moment, and thus the HPLC instrument, consisting of an injection device, HPLC pumps together with a gradient unit, an HPLC column (4.6 × 250 mm) and UV detector, is located at a distance of 1.5 m from the cryomagnet. The outlet of the UV detector is either connected via a stainless steel capillary (id, 0.25 mm) to a valve or to
a peak sampling unit which are connected to the continuous-flow probe in the cryomagnet. With either the valve or the peak sampling unit being under software control, stopped-flow NMR acquisition of all peaks of an HPLC separation can be performed due to the triggering of the UV signal. For a proper timing and recording of chromatographic peaks, the transfer time of peak passage between the UV detector and the NMR flow cell has to be carefully recorded.

This experimental design has the big advantage that it can be easily accomplished. The operation mode of the NMR instrument from routine NMR data acquisition to the LC–NMR mode can be easily changed by removing the routine probe from the room-temperature bore of the cryomagnet and inserting
the continuous-flow probe by fixing two screws. The magnetic field homogeneity of the continuous-flow probe can be readily adjusted by using standard reference shim files.

The transfer volume of the capillaries between the HPLC instrument and the NMR probe is about 150 µl. For minimum peak dispersion, the insertion of the HPLC column into the probe body of the continuous-flow probe would be desirable. This experimental arrangement was proposed by Wilkins and co-authors (16) and is used in (supercritical fluid chromatography) (SFC)–NMR employing immobilized free radicals (see Chapter 7.2 below). However, here column exchange is much more demanding than in the design outlined in Figure 1.8. With the increased use of shielded cryomagnets, the distance between the HPLC and NMR instruments will be reduced, thus rendering the need for inserting the HPLC column within the probe body unnecessary.

1.5 PRACTICAL CONSIDERATIONS, SOLVENT SUPPRESSION TECHNIQUES, GRADIENT ELUTION AND PURITY OF HPLC SOLVENTS

In real-life application of HPLC–NMR, three main types of data acquisition have been established, namely continuous-flow acquisition, stopped-flow acquisition, and time-sliced acquisition with the help of storage loops. For all of these acquisition techniques the major prerequisite is an optimized HPLC separation. Because sensitivity is still the crucial point of this coupling technique it is extremely important to develop a chromatographic separation where the quantity of the available separated compound is concentrated in the smallest available elution volume. This need necessitates the development of stationary phases which exhibit optimum separation characteristics, together with the capability to tolerate column overloading. The newly developed $C_{30}$ phases are typical representatives of these types of columns, which is evidenced by the separation of tocopherol isomers. Figure 1.11 clearly shows that it is possible to overload a $C_{30}$ column by a factor of 200 without loosing any chromatographic resolution.

The major amount of HPLC separations is performed with reversed-phase columns employing binary or tertiary solvent mixtures with isocratic or gradient elution. The protons of the solvents of the mobile phase cause severe problems for an adequate NMR registration. The receiver of the NMR instrument (either a 12-bit or a 16-bit analog–digital converter (ADC)) is unable to handle the intense solvent signals and the weak substance signals at the same time.
Figure 1.11 UV chromatograms (295 nm) of the separation of tocopherol isomers (1, δ-tocopherol; 2, γ-tocopherol; 3, β-tocopherol; 4, α-tocopherol; 5, α-tocopherol acetate: (a) analytical separation; (b) with a 200-fold amount of sample

Figure 1.12 shows the free induction decay (FID) and the transformed spectrum of a 0.01% sample of butylbenzylphthalate in acetonitrile (ACN) D₂O (80/20). The FID is dominated by the methyl group signal from the acetonitrile. In order to get an undistorted spectrum, a small receiver gain has to be chosen, leading to a low signal-to-noise (S/N) value for the sample signals. An increase in receiver gain does not lead to the desired result. Figure 1.13 shows the effect of overloading the receiver with solvent signals. By the ‘clipping’ of the FID, the transformed spectrum is distorted and thus useless for interpretation (see Figure 1.13) and the sensitivity of detection is severely decreased. In order to avoid this problem, the signal intensity of the solvent signals has to be reduced. Now the receiver gain can be increased and
adjusted to the smaller FID without any problems. Figure 1.14 shows the FID and the resulting $^1$H NMR spectrum of the same sample after reducing the solvent signal intensity. The remaining signals of the suppressed methyl group resonance of acetonitrile can be seen at 2.1 ppm. The signals of butylbenzylphthalate show a much higher S/N of 320:1. The 16-fold enhancement of the signal-to-noise value corresponds to a saving factor of 256.

1.5.1 SOLVENT SIGNAL SUPPRESSION

Solvent signal suppression is necessary in order to achieve a reduction of the NMR signal entering the receiver for observing small analyte signals in the
Figure 1.13 Increase in receiver gain without solvent signal suppression: (a) free induction decay; (b) resulting NMR spectrum

Figure 1.14 Optimized receiver gain with solvent signal suppression: (a) free induction decay; (b) resulting NMR spectrum

presence of much larger signals from the mobile phase. Solvent signal suppression is efficiently performed by using three techniques:

- Presaturation (NOESY presaturation)
- Soft-pulse multiple irradiation
- WET presaturation employing a $z$-gradient.
Presaturation

The principle of presaturation relies on the phenomenon that nuclei which are unable to relax, because their population in the ground state $\alpha$ and the excited state $\beta$ is the same, do not contribute to the free induction decay after pulse irradiation. Prior to data acquisition, a highly selective low-power pulse irradiates the desired solvent signals for 0.5 to 2 s, thus leading to saturation of the solvent signal frequency. During data acquisition, no irradiation should occur. NOESY-type presaturation is an effective pulse sequence of presaturation. The pulse sequence consists of three $90^\circ$ pulses (similar to the first increment of a NOESY experiment):

$$\text{RD} - 90^\circ - t_1 - 90^\circ - t_m - 90^\circ - \text{FID}$$

(1.7)

where RD is the relaxation delay, and $t_1$ and $t_m$ are the presaturation times (e.g., 0.6 and 0.08 s). The effect of NOESY-type presaturation is illustrated in Figure 1.15, which shows the $^1$H NMR spectra of 12 $\mu$g retinoic acid in ACN/D$_2$O (60/40), without and with solvent suppression. With the same number of transients as the spectrum without solvent suppression, the S/N value of the olefinic signals is much increased. In addition, the remaining $^{13}$C-satellites of the suppressed solvent signal and the baseline distortion around the presaturation frequency can be seen.

Soft Pulse Multiple Irradiation

Here, presaturation is performed with the use of shaped pulses, which have a broader excitation profile. This method is therefore better suitable for the suppression of multiplets. The advantages of this technique are that it is easy to apply, easy to implement within most NMR experiments, and multiple presaturation is possible, and that it is very effective. The disadvantages are that transfer of saturation can occur (in aqueous solutions) to slowly exchanging protons that would be detectable without saturation. Another drawback is that spins with resonances close to the solvent frequency will also be saturated and 2D cross peaks will be absent.

WET Presaturation

The WET sequence (Water Suppression Enhanced through $T_1$ Effects) uses four solvent selective pulses of variable lengths (Figure 1.16). Each selective rf pulse is followed by a dephasing field gradient pulse. By varying the tip angle of the selective rf pulse, the WET sequence can be optimized. This approach provides a fast and highly efficient saturation of multiple solvent frequencies. It can be combined with $^{13}$C decoupling to remove the $^{13}$C satellites of the solvent.
Figure 1.15  The $^1$H NMR spectra of 12 µg retinoic acid in ACN/D20 (60/40), (a) without and (b) with solvent signal suppression
Advantages and Disadvantages

The NOESY sequence proved to be very effective for the reduction of one particular signal such as the methyl group of acetonitrile. However, very often the mobile phase has a composition of several solvents, together with up to six solvent signals. Here, the application of the soft pulse multiple solvent suppression technique is advisable.

Both of these techniques, NOESY presaturation as well as soft pulse multiple solvent suppression, lead to a reduction of signal intensity of 1000:1 (see Figure 1.15). Whereas the former solvent signal results in a distortion of the baseline of the NMR spectrum, the $^{13}$C satellites which exhibit 0.55% intensity of the solvent signal, are now the most intense signals of the $^1$H NMR spectrum. Because the natural abundance of $^{13}$C nuclei is 1.1%, $^1$H, $^{13}$C heteronuclear coupling results in a doublet centered by the singlet of protons adjacent to the NMR-inactive $^{12}$C nuclei (natural abundance of 89.9%). These $^{13}$C satellites are only visible at intense signals in a routine $^1$H NMR spectrum, and it is clear that they contribute to the HPLC–$^1$H NMR spectra. With the application of the WET pulse sequence, these $^{13}$C satellites can be eliminated by a combined $^{13}$C decoupling together with a proper alignment of the proton magnetization with the help of gradient pulses.

All three suppression techniques can be used either for stopped-flow or continuous-flow acquisition. Presaturation works quite well in the stopped-flow mode, whereas the WET sequence seems to be superior in the continuous-flow mode.

However, all three techniques have the big disadvantage that compound signals lying under the solvent signal are also suppressed. Thus valuable information may have disappeared. This is also the reason why multiple solvent suppression is only useful to a limited extent because too much spectroscopic information may be lost after eliminating too many signals.
Therefore from a practical viewpoint it is advisable to use only two protonated solvents in a HPLC separation, e.g. H$_2$O and CH$_3$CN. To obtain field/frequency stabilization of the cryomagnet it is advisable to exchange H$_2$O versus D$_2$O, but even here the intense HDO signal has to be suppressed.

**Gradient Elution**

In isocratic separations the $^1$H NMR signals exhibit the same chemical shift, while in gradient separations the changing dielectric constant of the different solvent compositions leads to severe chemical shift alterations. This is outlined in Figure 1.17, which shows the proton NMR spectra of solvent mixtures of acetonitrile and water, from a 100% concentration of water to a 100% concentration of acetonitrile. With gradient separations, solvent signal suppression may be carried out by a 'scout scan' which detects the effective shift of solvent signals first, and then performing solvent suppression together with the registration of the NMR spectrum in a second step.

**Conclusions**

The choice of the suppression method to be used depends on both the solvent and sample characteristics. Therefore, HPLC–NMR suppression via presaturation

![Figure 1.17](image_url)

**Figure 1.17** $^1$H NMR contour plot of a solvent mixture over the range from 100% ACN to 100% H$_2$O
or shaped pulses has to be preferred because of the roubustness, simplicity and high suppression ratios, even for multiple solvent signals. Solvent signal suppression should be applied for as short a time as possible and with the lowest power as is necessary. A good line shape (shimming), optimal temperature control and lock stability are a prerequisite for optimal solvent signal suppression.

1.5.2 PURITY OF HPLC-GRAGE SOLVENTS

Most solvents contain a small amount of impurities, and often stabilizing chemicals have been added. The HPLC-grade solvents are supposed to be especially pure, although the criteria of purity for these solvents is their interference with the UV adsorption of the solute molecules. NMR detection is much more sensitive to smaller amounts of additional chemicals, especially since the concentration of the sample molecules is often of the order of 0.001 % (m/v).

The mostly employed solvents, such as water/D$_2$O and acetonitrile, are available with high NMR purity. For all other solvents, the amount of the impurity has to be examined by using a reference spectrum. Figure 1.18 shows a

\[ \begin{align*}
(a) \\
(b)
\end{align*} \]

\( \delta \) (ppm)

Figure 1.18 $^1$H NMR spectra of two HPLC-grade solvents, ACN (a) and THF (b). The $^{13}$C-satellites of the suppressed signals are marked with asterisks
comparison between the $^1$H NMR spectra of HPLC-grade pure acetonitrile (a) and tetrahydrofuran (b). Solvent signal suppression was carried out to eliminate the main solvent signals. In this figure, the $^{13}$C satellite signals of the suppressed resonances are marked with an asterisk. It can be seen that the spectrum of acetonitrile is free from impurity signals. In the spectrum of tetrahydrofuran, however, several additional signals occur, which are distributed over the whole spectral range. Therefore, this solvent is not advisable for use in GPC–NMR.

REFERENCES