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Optimization of Liquid Chromatography–NMR Spectroscopy

II—Saturation and Flow in On-Flow Liquid Chromatography–NMR Spectroscopy

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The effects of pulse interval and flow on relaxation and signal-to-noise ratio in an on-flow liquid chromatography (LC)–NMR experiment were investigated theoretically. The results led to a procedure for the optimization of on-flow LC–NMR experiments.

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The coupling of high-performance liquid chromatography (HPLC) and NMR spectroscopy was first reported as early as 1979.[1,2] Since then there has been rapid development and the area was recently reviewed comprehensively.[3]

LC–NMR can be used in either of two modes: on-flow or stopped-flow. In the stopped-flow mode, the chromatographic peak is usually located with UV detection and then stopped precisely within the NMR flow cell. Acquisition can then progress under conditions chosen specifically for NMR. In particular, the time between pulses can be tailored to suit either maximum signal-to-noise ratio (S/N) or quantitative accuracy. Alternatively, acquisition can progress until a sufficient S/N is achieved, allowing low levels of solute to be detected. Factors contributing to detection limits in LC–NMR have been examined previously.[4]

In on-flow detection, NMR acquisition takes place as the chromatography progresses. This allows more convenient characterization of every peak in a chromatogram and it allows the NMR detection of non-chromophores that could be missed in stopped-flow LC–NMR. In on-flow detection, the chromatogram is split into a series of time slices, during which individual NMR spectra are acquired. Since it is not desirable either to co-add NMR spectra between chromatographic peaks and the baseline, or to degrade chromatographic resolution, the chromatography imposes limits on the NMR conditions.

Where relaxation times are long, flow presents an opportunity to increase S/N by removing saturated material from the NMR detection coil. The theory and practice have been rigorously investigated by Sudmeier *et al.*[5] In the current work, the approach is simplified to that which affects LC–NMR only. In particular, since the sample is resident within the NMR probe for 6.8 s before it enters the detection coil (at a flow rate of 1 ml min^{-1}), the sample is considered to be completely polarized. The calculation therefore takes place only in what Sudmeier *et al.* refer to as 'Zone≠4'. However, since the flow rate can affect the chromatographic peak width, this factor has also been considered explicitly.

In a stopped-flow LC–NMR experiment, pulsing quickly with respect to the spin–spin relaxation time (i.e. pulse intervals less than three times T_2), results in finite (>5%) residual magnetization in the z-direction after the next 90° pulse. Pulsing rapidly with respect to the spin–lattice relaxation time (T_1), at intervals less than three times T_1, results in non-equilibrium magnetization in the z-direction at the start of the next 90° pulse. These residual magnetizations result in a decrease in the amount of magnetization sampled in the y-direction after several pulses compared with the situation where both T_1 and T_2 are much shorter and the sample is more fully relaxed.

In an on-flow experiment, new sample is introduced into the NMR cell and partially saturated sample removed; the effect of saturation is thus reduced. The resultant magnetization has been calculated numerically using the method described previously,[4] i.e. the extent of relaxation is the total magnetization acquired as a front of infinitesimal thickness passes through the cell divided by the number of pulses.

The calculation of S/N is more complex. If the chromatographic peak occupies a smaller volume than the NMR flow cell, the attainable S/N becomes a function of the time slice width (number of pulses × recycle time) in the pseudo-2D on-flow NMR experiment. If the time slice width is equal to the cell width, the S/N is the total magnetization acquired as the front passes through the cell divided by the square root of the total number of pulses. If the time slice width is smaller than the cell

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width, decreased S/N will result from fewer pulses acquired while the chromatographic peak is inside the cell. If the time slice width is larger than the cell width, decreased S/N will result from acquisition when the chromatographic peak is outside the cell.

In a Bruker 4 mm o.d. 140 μl flow cell, the residence time for a solute molecule is 16.8, 8.4 and 4.2 s for chromatographic flow rates of 0.5, 1.0 and 2.0 ml min^{-1}, respectively. Since the 4 mm cell is representative of most LC–NMR flow cells and HPLC peaks are usually $\geqslant 30$ s wide at a flow rate of 1.0 ml min^{-1}, the assumption is therefore made that the chromatographic peak occupies a larger volume than the NMR flow cell. In this case, the S/N is given by

$$S/N = \Sigma I/NP \times V \times 1/(PD)^{1/2} \quad (1)$$

where ΣI is the total intensity acquired as the front passes through the cell, NP is the total number of pulses acquired as the front passes through the cell, V is the volume of the cell and PD is the recycle time.

The first term in Eqn (1) accounts for the effect of relaxation and flow. The second term is included so that comparisons can be made between different-sized flow cells. This is in itself an approximation: larger cells will have a lower Q (efficiency in converting radiofrequency into magnetization), but usually have better shimming characteristics. The assumption here is that the two factors compensate and that the sensitivity per pulse is proportional to the volume sampled. The third term accounts for a faster pulse rate yielding more pulses. This term is not encoded as $(NP)^{1/2}$ since this would incorrectly add another term including the cell size.

Previously, it was suggested[4] that the time slice width should be adjusted in line with the chromatographic peak width. If this is done, Eqn (1) becomes

$$S/N = \Sigma I/NP \times V \times 1/(PD)^{1/2} \times 1/(FR)^{1/2} \quad (2)$$

where FR is the flow rate. It is therefore assumed that the time slice width is adjusted in line with the chromatographic peak width and Eqn (2) was used in our calculations.

For $T_1 = T_2 = 1$ s, the effect of flow rate and pulse interval on the extent of relaxation and the S/N are presented in Fig. 1. The S/N is a result of two competing effects. Faster pulsing results in increased saturation, decreasing the S/N. However, more pulses are possible as the chromatographic peak flows through the cell, giving rise to an increased overall S/N. The curves denoting extent of relaxation in Fig. 1 are higher for a given pulse rate at higher flow rates. Increased flow rates give rise to more complete relaxation for the same pulse interval, but the changes in extent of relaxation are small. The slope of the curves for pulse intervals greater than 1 s mean that the same extent of relaxation can be attained at smaller pulse intervals for higher flow rates, e.g. 99% relaxation is attained at a pulse interval of 4.0 s with a flow rate of 0.5 ml min^{-1}, whereas 99% relaxation is attained at 3.0 s and 2 ml min^{-1}.

The 4 mm LC–NMR cell represents a common compromise between sensitivity and chromatographic resolution within the cell; however, other cell sizes exist. Assuming similar geometry and a 1 mm wall thickness, a 3 mm cell will have a volume of 62 μl and a 5 mm cell a volume of 250 μl. The effects of pulse interval and flow rate on the extent of relaxation and S/N were calculated for 3 and 5 mm cells and are presented in Figs 2 and 3, respectively. For the 3 mm cell, the effect of flow rate on the extent of relaxation and S/N is more marked than

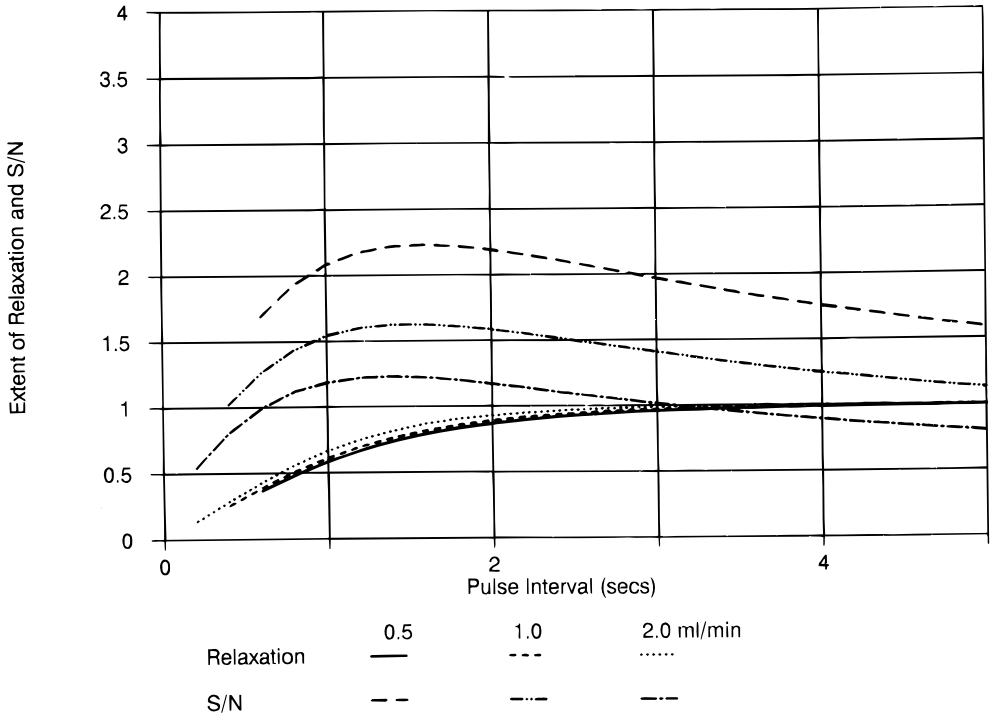

Figure 1. Effects of pulse interval and flow rate on extent of relaxation and S/N (4 mm cell). $T_1 = T_2 = 1.0$ s.

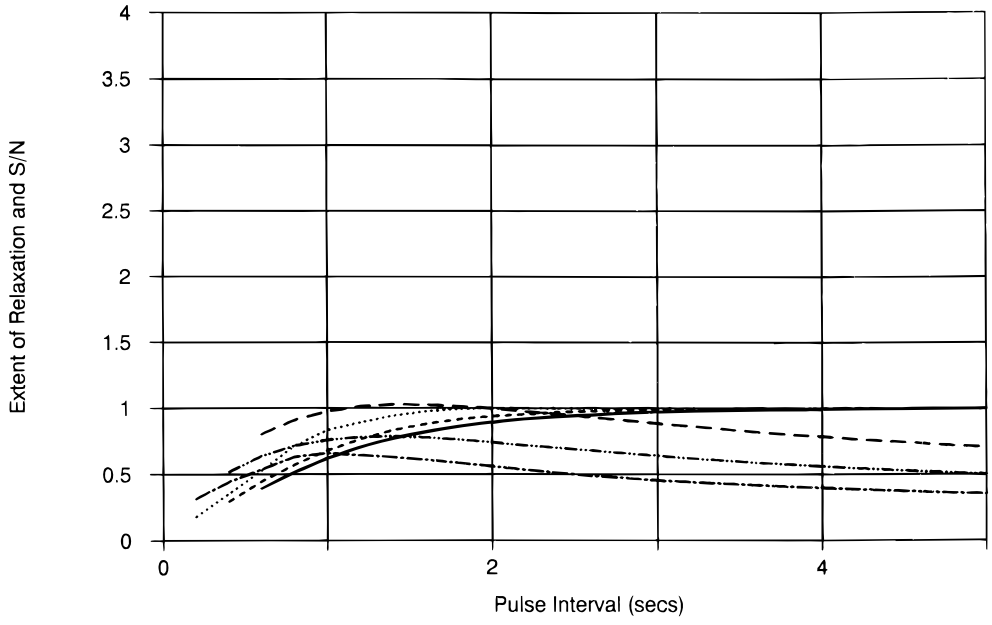

Figure 2. Effects of pulse interval and flow rate on extent of relaxation and S/N (3 mm cell). $T_1 = T_2 = 1.0$ s.

for the 4 mm cell. Lower flow rates yield smaller relative gains in S/N and much smaller absolute gains than in the 4 mm cell. In the case of the 5 mm cell, the converse is true: less effect on relaxation but larger relative and absolute gains in S/N at lower flow rates. If the calculations are extended to much smaller volumes, the number of pulses experienced by the slice approaches a limit of one. The linear interpolation of large non-linear differences in magnetization between integral numbers of pulses then breaks down. However, the effect of finite relaxation times means than S/N decreases at a lower rate than cell volume. In sample-limited on-flow applications, significant gains would therefore accrue from the use of smaller diameter columns coupled with smaller flow cells.

In all cases the optimum S/N shifts to shorter pulse intervals at higher flow rates and the shift in pulse interval is greater for the smaller cell. Furthermore, at a given flow rate, a smaller cell exhibits an S/N optimum at a shorter pulse interval.

Having considered the effect of cell size on the extent of relaxation and S/N, the effects of shorter relaxation times are now treated. Methanol–water (50:50) as an eluent has a particularly high viscosity and it is feasible that relaxation times will be reduced in this and similar systems. For comparison purposes, the effects of flow rate and pulse interval on the extent of relaxation and S/N for $T_1 = T_2 = 0.5$ s for the 4 mm cell are presented Fig. 4. The first result is that a much higher S/N is achievable. Second, the dependence of S/N on pulse

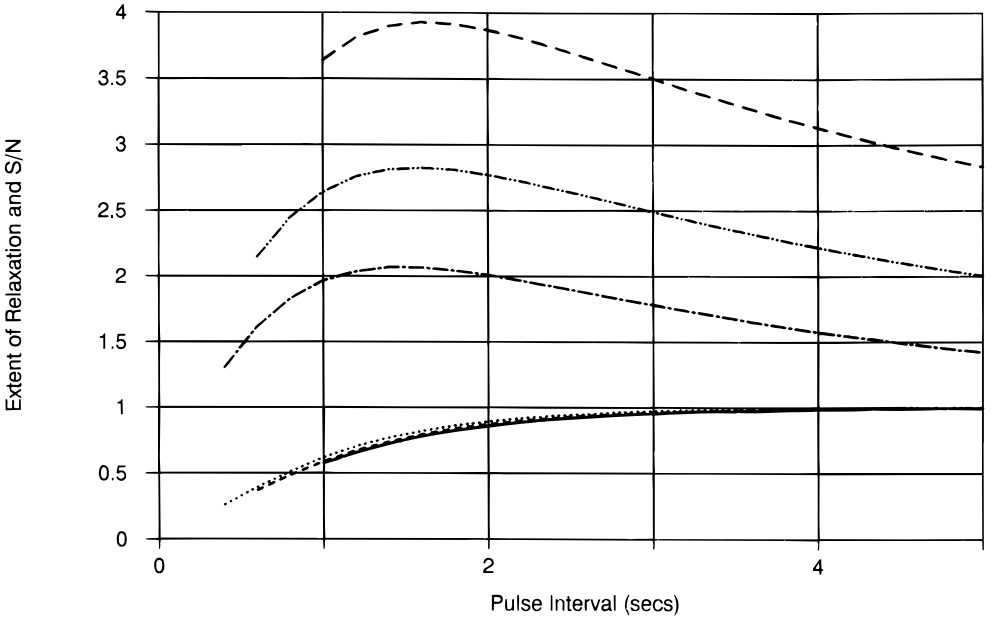

Figure 3. Effects of pulse interval and flow rate on extent of relaxation and S/N (5 mm cell). $T_1 = T_2 = 1.0$ s.

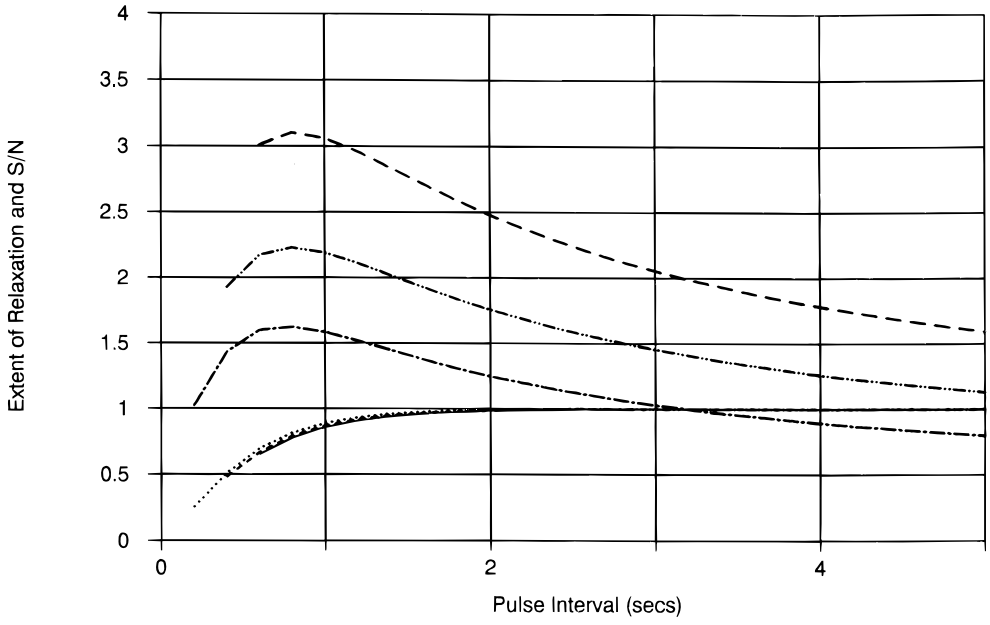

Figure 4. Effects of pulse interval and flow rate on extent of relaxation and S/N. $T_1 = T_2 = 0.5$ s.

interval is much more pronounced. Third, detailed examination of the S/N maxima reveals that the S/N is almost proportional to (flow rate)$^{1/2}$ for $T_1 = T_2 = 0.5$ s, whereas with $T_1 = T_2 = 1$ s the increase is smaller. Fourth, the effect of flow rate on the extent of relaxation is reduced. Fifth, the maximum S/N occurs at shorter pulse spacing, but the position of this maximum is less dependent on flow rate.

Conversely, the detection of aromatic compounds in a non-viscous eluent would lead to relaxation times longer than 1 s. As relaxation times increase, the dependence of the pulse interval corresponding to the maximum S/N on flow rate is greater, e.g. for $T_1 = T_2 = 1$ s the maxima is S/N occur at pulse intervals of 1.6 and 1.4 s at flow rates of 0.5 and 2 ml min^{-1}, respectively. For $T_1 = T_2 = 2$ s the corresponding maxima are at 3.0 and 2.0 s. However, the S/N attainable decreases: for $T_1 = T_2 = 1$ s, the maxima in S/N are 2.23 and 1.23 at flow rates of 0.5 and 2 ml min^{-1}, respectively, whereas for $T_1 = T_2 = 2$ s the corresponding maxima are at 1.63 and 1.01. As the relaxation times increase further, the optimum pulse interval approaches the width of the cell and the calculation breaks down.

The above calculations have assumed that the chromatographic peak volume is invariant with flow rate. This will not necessarily be the case, i.e. very low flow rates will lead to diffusion-induced band broadening on the chromatographic column.

The width of a chromatographic peak at half maximum height is given by[6]

$$W = 2.355 t_r (H/L)^{1/2} \quad (3)$$

where W = the width at half maximum height, t_r = elution time, H = plate height and L = length of the column.

To a first approximation, the elution time t_r is inversely proportional to the flow rate, so the width of the chromatographic peak (in units of time) also varies as the inverse of flow rate. The plate height H, on the other hand, can vary with the conditions (which includes flow rate) and if H is allowed to increase, the concentration of the solute in the peak will decrease. Therefore, gains made in the number of pulses that can be applied to a broader chromatographic peak are more than offset by decreases in the intensity of that peak. While the S/N decrease is directly proportional to the reduction in peak height, the gain in S/N is only proportional to the square root of the number of pulses [or 1/(peak height)$^{1/2}$, assuming the same peak shape].

The important point from the LC–NMR standpoint, however, is that the curve of W or H *vs.* linear velocity (itself proportional to flow rate) is steep and positive to low velocity of the minimum,[7] but of low slope, albeit positive, to the high velocity side of the minimum. Since the plate height increases little with increasing velocity, but the time taken to perform a chromatographic run is inversely proportional to velocity, a chromatographic method presented to the NMR spectroscopist will probably be to the high-velocity side of the H minimum rather than the low-velocity side of the minimum. If a lower flow rate is used by the NMR spectroscopist, a slightly higher concentration peak will result *and* more NMR pulses per chromatographic peak will therefore be possible. Furthermore, when the flow rate is less than optimal, it will become obvious by the sharp increase in H or decrease in solute concentration in the chromatographic peak, resulting in a loss of intensity.

The gains in S/N at lower flow rates presented in Figs 1–4 are the minimum achievable and represent a sound footing on which to base the optimization of on-flow LC–NMR experiments.

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