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Optimization of LC–NMR III—Increased Signal-to-Noise Ratio Through Column Trapping

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ABSTRACT: Significant gains in LC–NMR signal-to-noise ratio are obtained by selective concentration of chromatographic peaks on a chromatographic column using D_2O to fix the sample on the solid phase. A deuterated organic solvent is used to remove the sample from the column and into the NMR flow cell, which removes the need for presaturation of solvent resonances. Deuterated solvents unrelated to the original eluent are used in order to minimize spectral overlap between solvent and solute. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; liquid chromatogra/phy–NMR; optimization; trapping

INTRODUCTION

The minimum level of impurity that can be characterized routinely by liquid chromatography (LC)–NMR is about 1% using a 4 mm flow cell at 400 MHz. The concentration eluted by an analytical HPLC column is typically 10 μg cm^{-3}, much lower than that normally detected by NMR. A technique that offers increased sensitivity in LC–NMR would therefore allow the characterization of impurities of less than 1%.

The intrinsic low sensitivity of LC–NMR requires the spectroscopist to match the chromatographic peak volume to the volume of the NMR flow cell.[1] In the case of chromatographic peaks with long retention times, the solute volume can exceed the volume of the flow cell by an order of magnitude, thereby reducing sensitivity in direct proportion.

Increased sensitivity in LC has been achieved by using the techniques of column switching, column trapping and phase switching, e.g. the determination of drugs in serum,[2] retinoids[3] and pesticides.[4] In these cases either preconcentration of all components took place on the analytical column or concentration of components with long elution times took place on a guard column. In both cases, solvent with a higher organic composition was then used to execute chromatographic separation. Neither technique has found usage in LC–NMR since they do not give the sensitivity increases required for chromatographic peaks with long elution times, or require a guard column specific to the chromatographic method.

Kokkonen *et al.*[5] selectively introduced water into the solvent stream after the analytical column and before the guard column. This created a solvent of lower organic concentration (referred to as a 'weaker solvent' when used in conjunction with a reversed-phase column) and caused the solute to be retained and concentrated on the guard column. Subsequent reversal of the direction of flow (backflush) with a solvent of high organic content yielded a higher concentration of solute. In Kokkonen *et al.*'s application the main benefit was not in sensitivity, but in achieving the lower volume of solvent required when LC is coupled to fast atom bombardment mass spectrometry.

In this work, we modified and extended Kokkonen *et al.*'s approach to suit the needs of LC–NMR. This resulted in the following benefits:

1. Chromatographic peak volume equivalent to the NMR flow cell volume, thus removing the need for re-optimization of the chromatographic system and allowing the characterization of late-eluting peaks previously inaccessible to LC–NMR.
2. Spectral acquisition in the presence of a deuterated organic solvent, removing the need for complex multiple channel instrumentation.
3. Minimization of spectral overlap by allowing the use of a wide range of water-miscible solvents in which spectral acquisition can take place.

EXPERIMENTAL

The system for column trapping is given in Fig. 1. A Hewlett-Packard Model 1050 system was used to inject the sample and control the eluent flow (pump 1). The analytical column used was a Hichrom 25 cm × 4.6 mm i.d. Hypersil 5 μm ODS. UV detection was effected at 254 nm. Pumps 2 and 3 were Gilson Medical Electronics Models 302 and 303, respectively. Dimethyl, benzylbutyl and dicyclohexyl phthalate were supplied by Aldrich and made up to 10 mg cm^{-3} in acetonitrile. The guard column was a Hichrom HIRPB-10C5M and the D_2O was introduced via a Valco zero-volume T-

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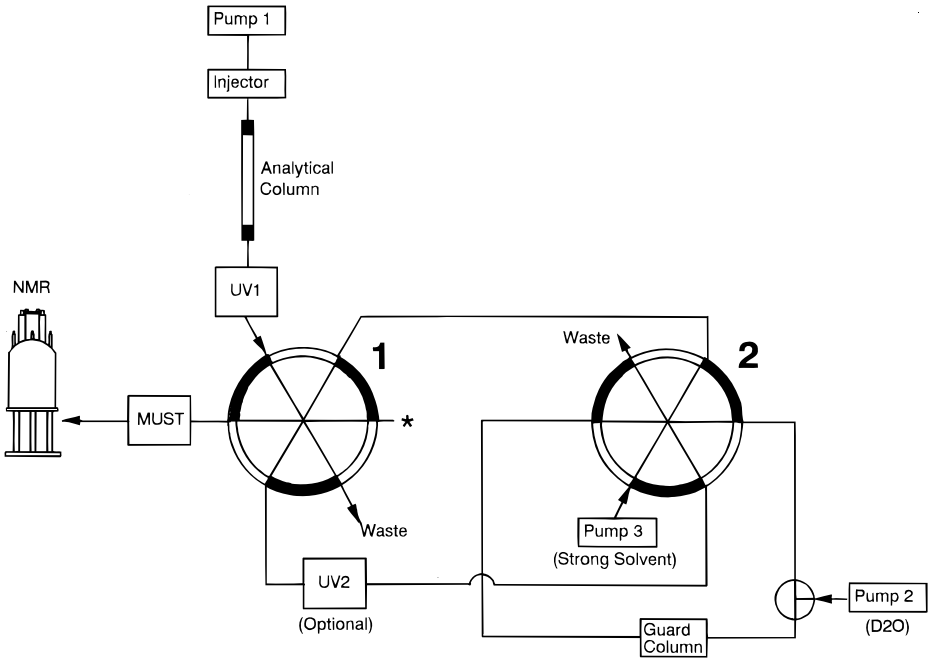

Figure 1. Column trapping system.

piece. Protic solvents were Riedel-de Haën Spectranal grade. Chromatography was conducted at an eluent flow rate of 1 $cm^3\ min^{-1}$.

Stopped-flow detection was facilitated with a Spark Holland Must column switcher, with integral timer. This unit was initiated manually at the UV peak maximum, after which the stopped-flow delay (17 s) and isolation of the NMR cell occurred automatically.

NMR spectra were obtained at 400.14 MHz on a Bruker AMX-400 spectrometer using a 4 mm LC probe. Solvent suppression was via presaturation, the carrier (O1) was set at the acetonitrile frequency and the decoupler (O2) at the residual water frequency. The signal-to-noise ratio was determined from intensity × 2.5/(peak-to-peak noise), as indicated by the aromatic protons of dicyclohexyl phthalate and the benzyl CH_2 of benzylbutyl phthalate.

The procedure employed for column trapping requires the chromatogram to be run twice. On the first occasion elution times are noted. On the second occasion (referring to Fig. 1), one proceeds as follows:

1. Place both six-port Rheodyne valves in the position indicated such that normal stopped-flow LC–NMR can proceed.
2. Before the peak of interest appears on UV detector 1 (UV1), pump 2 is switched on, pumping D_2O through the guard column and thence to waste. This is where the prior knowledge of elution times is beneficial.
3. Immediately the peak of interest appears on UV1, valve 1 is switched, diverting the eluent from the column past the T-piece into which D_2O is pumped. In this weaker solvent, the peak is then trapped on the guard column.
4. When the peak of interest has flowed through UV1, pump 1 is stopped. Switch off pump 2 after pump 1 so as to clear the leg of tubing downstream of the T-piece of organic solvent. This removes all protic solvent and helps to provide consistency of solvent delivered to the NMR flow cell and hence increases ease of magnetic field shimming.
5. Switch on pump 3 in order to establish a steady state.
6. Switch valve 2 to backflush the solute from the guard column into the NMR flow cell.
7. Initiate the Must unit to stop the peak in the middle of the NMR flow cell. The correct initiation time is established from the appearance of the peak

Table 1. Effect of peak trapping on LC–UV peak width and gain in LC–NMR signal-to-noise ratio (S/N) with acetonitrile–D_2O solvent

Solute	Solvent composition	Elution time (min)	LC peak width (s)		NMR S/N			
					Observed		After/Before	
			Before	After	Before	After	Obsd.	Calc.
Dicyclohexyl phthalate	85:15	9	10.5	2.3				
Dicyclohexyl phthalate	70:30	23	24.0	2.3	21	41	2.1	2.3
Benzylbutyl phthalate	70:30	11	12.5	2.3	123	187	1.5	1.5

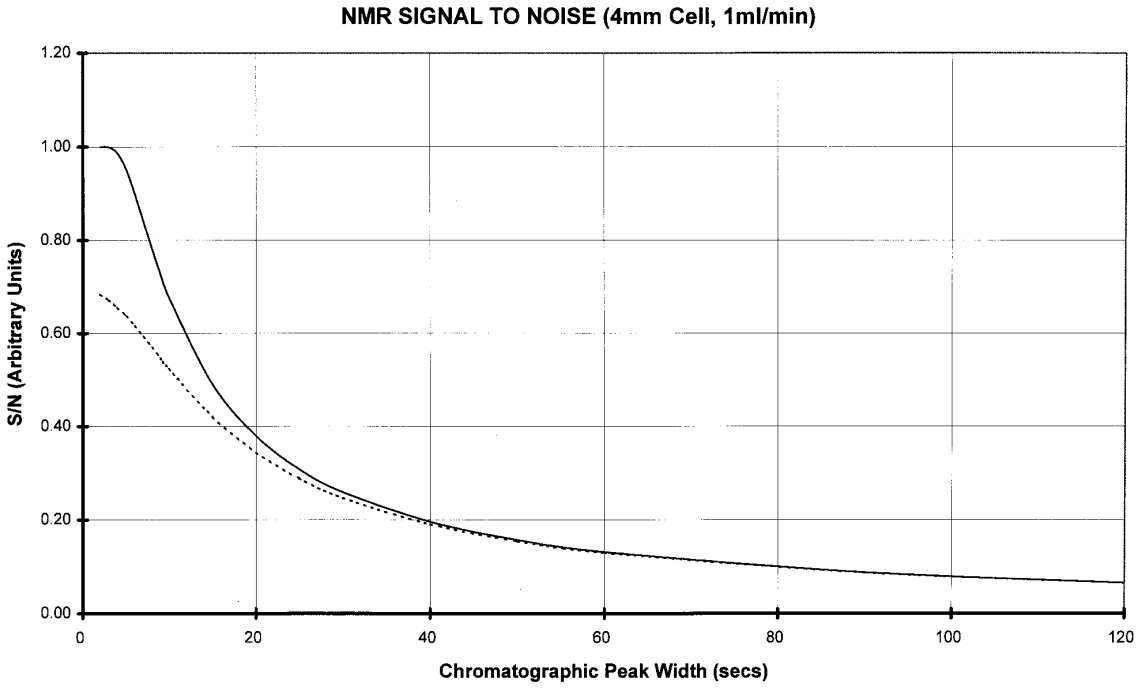

Figure 2. NMR signal-to-noise ratio as a function of chromatographic peak width.

maximum in UV2. If the backflush time has been fully characterized, the delay can be timed from the switching of valve 2 and UV2 can then be omitted, inducing less broadening of the chromatographic peak prior to the NMR flow cell.

RESULTS AND DISCUSSION

LC UV

Column trapping was first tested in our laboratory using LC–UV. Volumes of 4 μl of dicyclohexyl or benzylbutyl phthalate solutions were introduced into differing proportions of CH_3CN–D_2O eluent and thence through a Hichrom 25 cm × 4.6 mm i.d. Hypersil 5 μm ODS column. In all cases the chromatographic peak was trapped by introducing D_2O with pump 2 at a rate of 2 cm^3 min^{-1} and the solute was subsequently backflushed with CH_3CN. The elution times and chromatographic peak widths (full width at half maximum height) are given in Table 1.

The width of the trapped and backflushed chromatographic peak (as measured by UV2) was universally 2.3 s. This width did not depend on initial solvent compositions (85:15 or 70:30 CH_3CN–D_2O), or the degree to which the solute was retained by the column. The decrease in chromatographic peak width was therefore large, e.g. 10.4-fold in the case of dicyclohexyl phthalate and 70:30 CH_3CN–D_2O. When the pressure was measured before and after the analytical column with flow rates of 1–5 cm^3 min^{-1} of D_2O, there was no change in the pressure drop across the analytical column and no pulsing effects were noted. Solutes that are less well retained than even benzylbutyl phthalate will therefore be accessible by column trapping.

In a mixture of three phthalates (dimethyl, benzylbutyl and dicyclohexcyl, in order of elution), trapping initial and intermediate peaks did not affect the chromatography of subsequent peaks. Not only does this render the methodology of column trapping general but it also means that peaks could in principle be trapped in a series of guard columns in LC–NMR in an analogous way to Bruker's current loop storage.

Whilst decreases in chromatographic peak width are large, the realization of sensitivity gains is dependent on:

1. Retention of chromatographic peak shape after trapping. Using the width at 20% height/width at 50% height as a measure of peak shape in dicyclcohexyl phthalate, 85:15 CH_3CN–D_2O eluent, a minimal change from 1.57 to 1.47 was observed between normal elution and backflush, respectively. The peak shape was retained.
2. All the solute being trapped and backflushed into the NMR flow cell. The recovery for the backflushed peak in the above experiment was $\geqslant$97% as measured by UV absorption.

We therefore conclude that concentration and sensitivity gains are realized, that they are inversely proportional to peak width and are suitable for enhancing sensitivity in LC–NMR.

LC NMR

The dependence of NMR signal-to-noise ratio on chromatographic peak width (width in seconds at half maximum height) can be calculated by integrating the area under a Gaussian peak over the width of the NMR cell. The NMR cell width is given by the cell volume divided by flow rate (equal to 8.4 s in the Bruker 4 mm, 140 μl NMR flow cell with a flow rate of 1 cm^3 min^{-1}). The signal-to-noise ratio calculated for peaks of identical total area but different widths are presented in Fig. 2

as a solid line. For a chromatographic peak width of $\leqslant 4$ s (0.55 of the NMR flow cell width), $\geqslant 99\%$ of the theoretical maximum NMR signal-to-noise ratio is retained. For chromatographic peak widths >4 s, the NMR signal-to-noise ratio decreases. Large gains in NMR signal-to-noise ratio would therefore be made by decreasing the chromatographic peak width. Narrowing a peak of 30 s to 4 s would result in a gain in signal-to-noise ratio from 26% to 99% of the theoretical maximum (3.8-fold).

In practice, these gains in signal-to-noise ratio are decreased by broadening within the NMR cell and to a lesser extent the UV cell and the transfer line to the magnet.[1] The resultant attenuated signal-to-noise ratio is indicated by the dashed line in Fig. 2. The signal-to-noise ratios available are thus limited to 68% of the theoretical maximum: the 30 s wide peak mentioned previously would benefit from a gain from 25% to 68% of the theoretical maximum (2.8-fold).

In order to test these theoretical gains in practice, the same chromatographic systems as before were employed, with the exception that the column loading was increased by a factor of four (to 200 μg) in order to produce measurable signal-to-noise ratios. Reference to Table 1 shows that the chromatography was unaffected by the higher column loading. The stopped-flow LC–NMR spectrum obtained for benzylbutyl phthalate is given in Fig. 3(a). The spectrum obtained under identical NMR conditions (after trapping and backflush with pure CH_3CN) is given in Fig. 3(b). The signal-to-noise ratios were 123:1 and 187:1, respectively, a gain in signal-to-noise ratio of 1.5. This is entirely consistent with the gain predicted in Fig. 2 (dashed), i.e. 1.45.

When dicyclohexyl phthalate was run similarly, sensitivities of 21:1 and 44:1 were obtained, a gain in signal-to-noise ratio of 2.1, in agreement with the predicted gain of 2.29 (see Table 1).

This agreement means that:

1. The chromatographic sensitivity gains are translated into LC–NMR gains which we can also predict. The majority of chromatographic peak widths encountered in routine applications are equal to or greater than the 24 s of dicyclohexyl phthalate and in general, gains of $\geqslant 2.1$ are therefore to be expected.
2. The recovery of benzylbutyl phthalate (a less well retained peak than dicyclohexyl phthalate) is high.
3. Since the recovery was high and the backflushed peak was either totally contained by the NMR cell or of reproducible width, column trapping and NMR detection also offer the possibility of quantification of the number of moles of solute in one chromatographic peak relative to another.

Smaller volume chromatographic peaks can also be generated by the use of smaller bore columns. For most chromatographic peaks the volume of the peak will still exceed the volume of the NMR flow cell and it is therefore the concentration that matters rather than the volume. The maximum possible column loading is proportional to the square of the column bore. Provided that the sample is not limited, there will be (to a first approximation) no change in the concentration of the eluted chromatographic peak on going to a smaller bore column. In practice, there is a modest change in concentration, e.g. approximately a doubling can be expected on going from 4.6 to 1 mm columns. For earlier eluting peaks these gains are comparable to

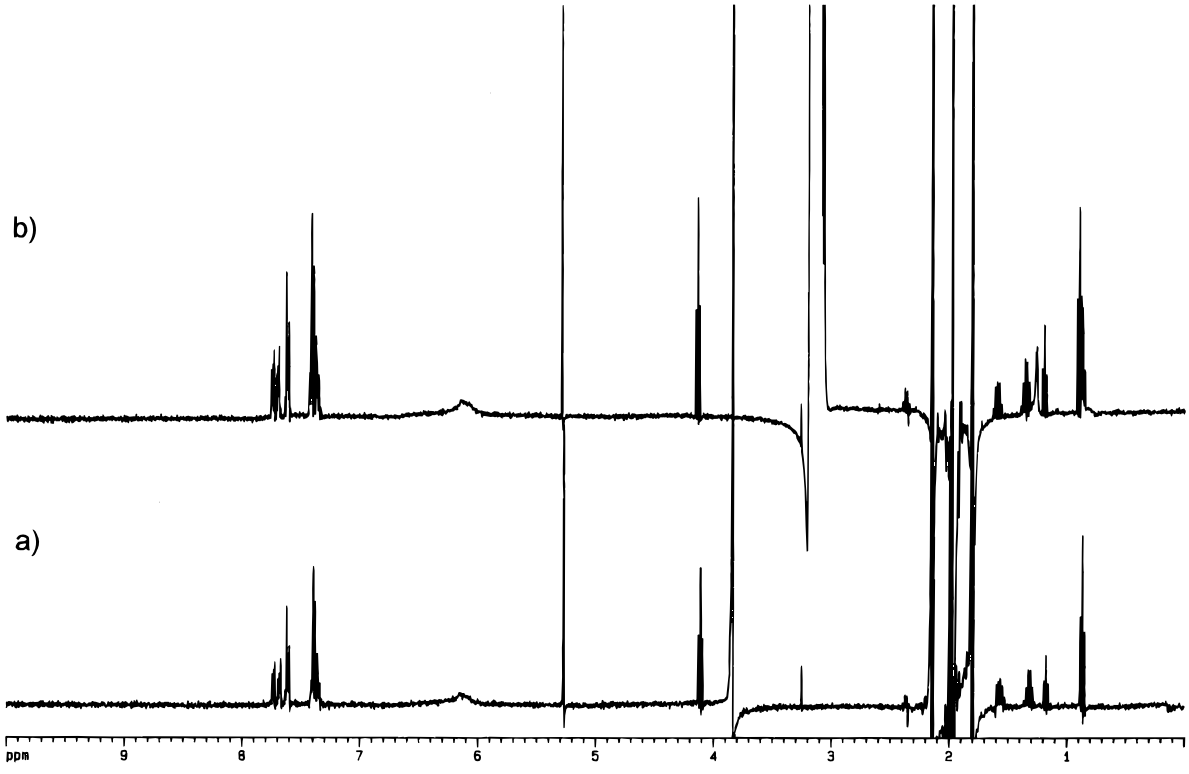

Figure 3. Stopped-flow LC–NMR spectra of benzylbutyl phthalate (a) before trapping and (b) after trapping.

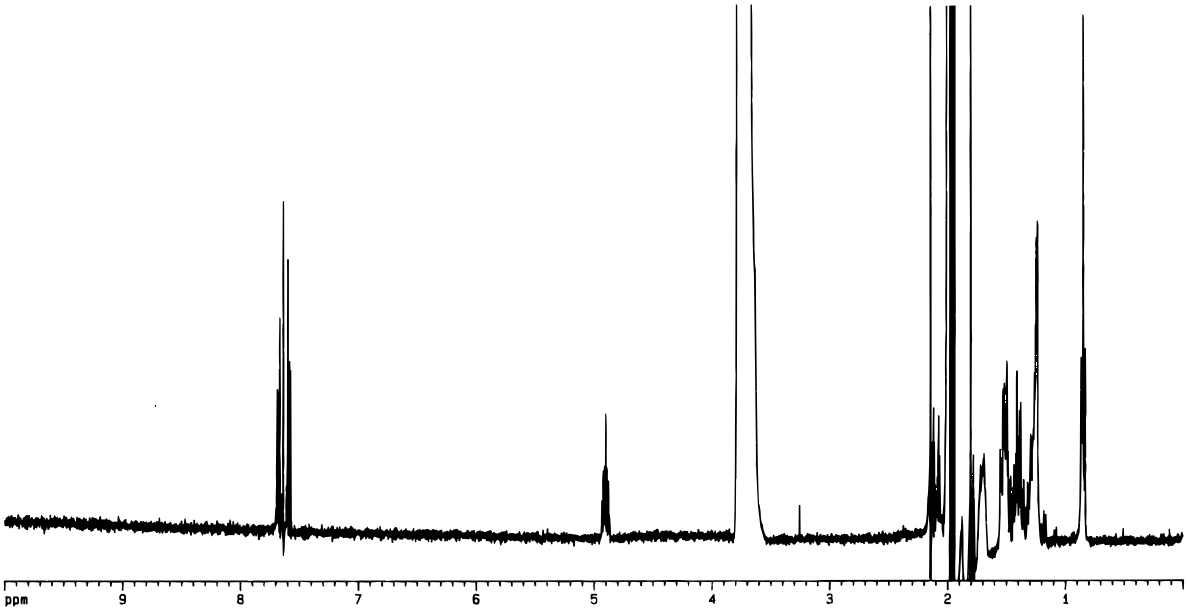

Figure 4. Stopped-flow LC–NMR spectrum of dicyclohexyl phthalate after backflush with CD_3CN.

those possible by column trapping, but for later eluting peaks column trapping offers much higher gains. Where the sample is limited, small-bore columns offer a significant advantage.

Detailed examination of the water (HOD) resonance after backflushing reveals a shift to lower frequency and a complex broad lineshape. Both effects are due to the influence of the solvent discontinuity through the cell (due to incomplete mixing) on bulk susceptibility. The backflush solvent constitutes the majority of solvent in the NMR flow cell and since it contains the majority of the solute, the spectroscopic lineshapes of both the backflush solvent and solute are unperturbed. When the chromatographic peak remained in the NMR flow cell overnight and diffusion produced a homogeneous sample, the HOD resonance reverted to a normal lineshape and position.

Deuterated solvent

In the examples studied, the solute was backflushed instantaneously ($\leqslant 5$ s). The time allowed for pump 3 to settle is currently 1 min, giving rise to a backflush volume of 1 cm^3. This volume could in principle be as small as sum of the volume of the flow cell (140 μl) and the transfer tube (360 μl). The total volume of solvent introduced by pump 3 is therefore less than or equal to the quantity of solvent used in normal NMR, facilitating the economic use of deuterated organic solvent for backflushing and subsequent spectral acquisition. If deuterated solvent is to be used for backflush, it is logical to flush the guard column thoroughly with D_2O in order to reduce the amount of protic solvent before the backflush takes place. The only relevant parts of the column trapping system that would then contain

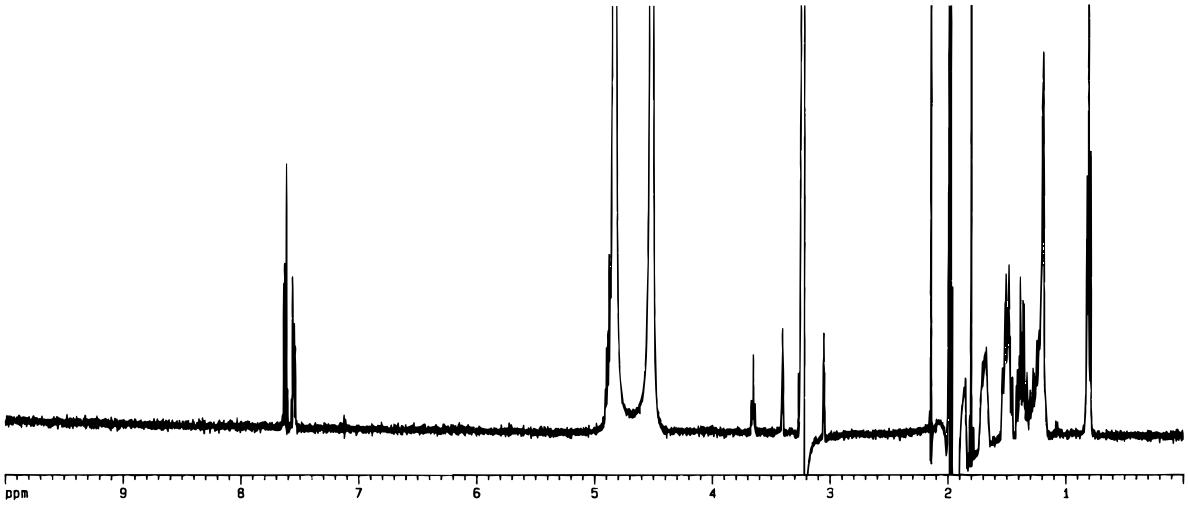

Figure 5. Stopped-flow LC–NMR spectrum of dicyclohexyl phthalate after backflush with CD_3OD.

protons are the leg of tubing between the T-piece and valve 2, and from valve 1 through to the NMR cell. The former is kept as short as possible, while the volume of the latter will be prescribed (360 μl in our apparatus). Pumping for 1 min with pump 3 prior to switching valve 2 should be adequate to remove protic material from valve 2 through to (and including) the NMR flow cell. Caution should be exercised, however, in the case of solutes poorly retained by the analytical column, since excess flushing with D_2O could partially remove the solute from the guard column to waste and reduce the recovery. When deuterated organic solvent is employed in backflushing, the field and frequency can then be locked using its deuterium resonance.

The stopped-flow LC–NMR spectrum of dicyclohexyl phthalate, run under the same LC conditions but backflushed with CD_3CN, is presented in Fig. 4. This spectrum was obtained without presaturation and the signal-to-noise ratio increased to 47:1. Further examination also reveals the presence of fewer impurities (or the presence of deuterated impurities) and improved baseline in the region of the solvent resonances due to the absence of presaturation and decreased solvent ^{13}C satellites. All the cyclohexyl resonances can be distinguished and their intensities are less perturbed than when presaturation was employed.

Other solvents

An opportunity is offered by column trapping in LC–NMR is the substitution of a backflush solvent that is unrelated to the original eluent. Provided that the backflush solvent is miscible with water and that it dissolves the trapped solute, the backflush solvent can be tailored to minimize spectral overlap. When CD_3OD was substituted for CD_3CN in backflushing dicyclohexyl phthalate it yielded the spectrum given in Fig. 5. In this case, the HOD resonance was completely split. This was probably due to diffusion-limited exchange between HOD and residual CD_3OH. A residual acetonitrile resonance was observed which yielded an integral ratio of 5.3 relative to the 99.8% deuterated methanol resonances, i.e. 1% acetonitrile remained in the flow cell. A smaller guard column and an NMR flow cell with less mixing would enable this figure to be reduced further. If the aliphatic region of the spectrum were of particular interest, pyridine-d_5 could be used as a backflush solvent.

CONCLUSIONS

Column trapping is successful at:

1. Concentrating chromatographic solute peaks. In the case of late-eluting peaks, the effect is particularly large but significant gains in concentration are still made with chromatographic peaks as narrow as 10 s.
2. Allowing solvents unrelated to the original chromatographic system to be used for backflushing, decreasing the probability of spectral overlap between solvent and solute.
3. Allowing backflush with deuterated solvent giving rise to the following additional benefits:
   - the signal to noise gains made in backflushing with protic solvent are slightly enhanced;
   - acquisition can be made without recourse to presaturation increasing the breadth of applicability of LC–NMR;
   - improved baseline and quantitation.

Column trapping, in principle, also allows the removal of polar modifiers from the sample detected by LC–NMR, yielding compatibility with a much wider range of chromatographic systems developed specifically for LC–UV.

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