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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–NUCLEAR MAGNETIC RESONANCE ON-LINE COUPLING WITH SOLVENT NON-EXCITATION

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SUMMARY

An improved type of continuous-flow nuclear magnetic resonance (NMR) probe showing excellent characteristics with respect to signal line shape and resolution is described. The stationary sensitivity in the continuous-flow cell is about 60% of that of rotating NMR tubes in a one-pulse experiment. An increase in sensitivity in the continuous-flow mode is obtained by pre-polarization of the solvent. Using the $1\bar{3}3\bar{1}$ solvent non-excitation technique a reversed-phase high-performance liquid chromatographic separation of an aromatic test mixture was demonstrated.

INTRODUCTION

Great progress has been made in recent years in the apparatus design used in high-performance liquid chromatography (HPLC). The instrument handling was simplified by the use of microprocessors, whilst automation procedures were successfully introduced by the development of autoinjection systems. On the detector side, more information as to the UV characteristics of the sample constituents were available by the introduction of variable-wavelength UV detectors. Nevertheless, the structural recognition of unknown compounds still requires a more structurally relevant detector. The superior stereochemical information available from nuclear magnetic resonance (NMR) spectroscopy justifies the current efforts at improving the sensitivity of continuous-flow NMR detection in reversed-phase separations[1–5]. In this respect, two problems must be solved: (1) improvement of the NMR characteristics such as signal line shape and signal-to-noise ratio of a continuous-flow cell of definite volume; (2) improvement of solvent suppression ratios in reversed-phase separations.

Our approach to overcome the problems is the design of a flow cell with optimum geometry together with a special detector coil which directly fits the cell. The benefit of such a development should be improved sensitivity as well as reduced signal

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linewidth. Because solvent suppression ratios depend especially on the linewidth at the base of the signal (at the height of the ^{13}C satellites) this feature is a prerequisite to overcome sensitivity problems in reversed-phase preparations. In an earlier publication[2] we have described the construction and application of 44- and 126-μl continuous-flow cells. In this work we deal with the characteristics of a new 120-μl flow cell suitable for direct HPLC–NMR coupling.

The pulse repetition time in an NMR experiment is dependent on the spin–lattice relaxation time, T_1, of the eluent. In the case of 90° pulse excitation, an equilibrium delay of $5T_1$ has to be used to obtain full equilibrium magnetization. It is shown that the solvent volume in an HPLC column may be used for pre-polarization of eluents leading to increased NMR sensitivity in the continuous-flow mode.

In reversed-phase separations the signals of protonated solvents lead to dynamic range problems. Efficient solvent suppression in the continuous-flow mode was performed by application of the $1\bar{3}3\bar{1}$ solvent non-excitation technique of Hore[6,7] in a separation of seven aromatic compounds.

EXPERIMENTAL

Continuous-flow probe design

A 3-mm I.D. glass tube tapering at both sides to the external diameters of the feed and drain PTFE tubings was silylated with dimethyldichlorosilane to avoid adsorption effects. A specially designed coil with an overall length of 18 mm was directly attached to the 4-mm O.D. glass tube resulting in a detector volume of 120 μl. PTFE tubings were attached to the glass tubing with the help of shrink-fit tubings. The cell was fixed in a glass dewar at the top of a standard narrow bore probe. The temperature was monitored by a thermocouple in the dewar. A schematic diagram of the flow cell design is shown in Fig. 1.

Apparatus

^1H NMR spectra were recorded on three NMR instruments of different magnetic field strengths: Bruker AC 300 (7.0 T), AM 400 widebore (9.4 T) and AM 500 (11.7 T). All instruments were controlled by the computer system Aspect 3000 with an array processor and hard disk. The 9.4-T instrument was equipped with a 12-bit analogue-to-digital converter (ADC), the other two instruments with a 16-bit ADC.

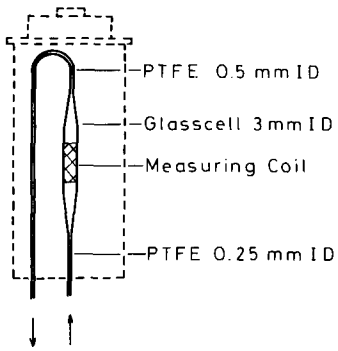

Fig. 1. Schematic diagram of the NMR flow cell design.

HPLC–NMR coupling

A stainless-steel HPLC column (ODS-Hypersil, 5 μm, 250 mm × 4.0 mm, Shandon) was fixed at the bottom of the 11.7-T magnet and connected to the continuous-flow probe by a stainless-steel capillary (Fig. 2). The injection system was fixed to one stand of the cryomagnet and connected to the HPLC column by a stainless-steel capillary. The HPLC instrument (Bruker LC 31) was located 1 m from the magnet.

Solvent systems

Test spectra were recorded of solutions of 10% chloroform in [$^2H_6$]acetone, of 0.1% ethylbenzene in deuteriochloroform and of a buffered mixture of acetonitrile–water (37.5:62.5) containing 0.15% of triethylamine at pH 3 (phosphoric acid). Solvents were circulated by the pump of the Bruker LC 31.

Separations on the ODS-Hypersil column were performed with acetonitrile–water (50:50). The distilled water in all experiments contained 3% deuterium oxide for field/frequency stabilization. All solvents were obtained from Merck (Darmstadt, F.R.G.).

Pre-polarization experiment

This experiment was performed using the decoupling coil of a dual 188.5-μl continuous-flow probe already described[3]. PTFE tubing (2 m × 1.1 mm I.D.) was coiled near the probe bottom of the 9.4-T instrument in a region of strong magnetic field, providing a pre-polarization volume of 2.0 ml. A peristaltic pump was used to circulate a 0.1% solution of ethanol in deuterium oxide. Magnetization curves were obtained by an inversion recovery sequence (180°–t–90°) with a variable delay, t, ranging between 0.1 and 2.0 s, a spectral width of 6000 Hz per 4 K and a 90° pulse of 20 μs.

Continuous-flow measurements using $1\bar{3}3\bar{1}$ solvent non-excitation

After recording a continuous-flow NMR spectrum of the pure solvent mixture of acetonitrile and water, the frequency difference between the two signals was determined. In the timing of the sequence (Fig. 3) a frequency difference of 965 Hz (AC 300) resulted in a delay, D_2, of 1 ms. The pulse carrier frequency was adjusted to the water resonance. The original 90° pulses (5.9 μs at the AC 300 and 6.0 μs at the AM

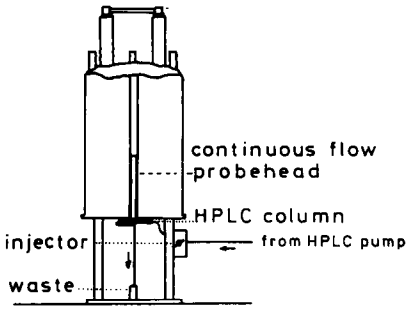

Fig. 2. Experimental arrangement for HPLC–NMR coupling.

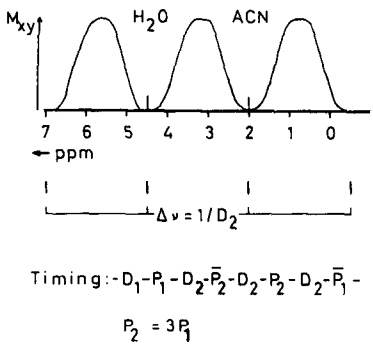

Fig. 3. Transverse magnetization excited by the $1\bar{3}3\bar{1}$ sequence as a function of the offset setting in acetonitrile–water mixtures. ACN = acetonitrile.

500) were attenuated with the help of an attenuator. According to the $1\bar{3}3\bar{1}$ solvent suppression technique, the resulting value was divided into four pulse lengths in the ratio 1:3:3:1. A spectral width of 4800 Hz (AC 300)/6000 Hz (AM 500) at a memory size of 8 K was used. For data acquisition at a flow-rate of 1 ml/min, the delay time, D_1, between scans was set to 0.1 s (AC 300) and 0.5 s (AM 500), therefore the total pulse repetition times were 0.9 s (AC 300) and 1.2 s (AM 500). In all experiments, eight scans were coadded resulting in time resolutions including the disc transfer time for data storage of 8 and 11 s. Up to 256 interferograms (files of eight scans) were recorded and stored on disk. In the data processing, magnitude calculation was performed after employing a sinebell or gaussian window function.

RESULTS AND DISCUSSION

NMR characteristics of the 120-μl continuous-flow probe

Test data for ^{1}H NMR probes are usually recorded by measuring the line shape of a chloroform signal and the signal-to-noise (S/N) ratio of a 0.1% solution of ethylbenzene. In the case of the 120-μl continuous-flow probe, tests were performed. Fig. 4 shows the spectrum of chloroform in [$^2H_6$]acetone at a flow-rate of 1 ml/min recorded on the 11.7-T instrument. The linewidth at the height of the ^{13}C satellites is 15.5 Hz, at 1/5 of this amplitude it is 23.5 Hz. In the case of the 7.0-T instrument the corresponding values are 14 and 30 Hz, whereas S/N of the CH_2 quartet of a 0.1% solution of ethylbenzene in deuteriochloroform is 75:1 (Fig. 5). In order to compare this value with sensitivity tests performed with conventional probes, one first has to consider different measuring volumes. In the case of a standard high resolution 5-mm probe at 7.0 T, a measuring volume of 250 μl yields a S/N value of 175:1. The correction factor for different volumes is[8] $\sqrt{V_2/V_1}$, therefore this value has to be corrected to 124:1. The sensitivity obtained with the present continuous-flow probe with a volume of 120 μl approaches 60% of that of the conventional probe. Nevertheless, keeping in mind that a non-rotating cell design is used, the sensitivity obtained is excellent. This is due to the increased filling factor of the measuring coil which is directly mounted around the glass containing the detection area.

The NMR cell volume employed is apparently larger than that used in routine

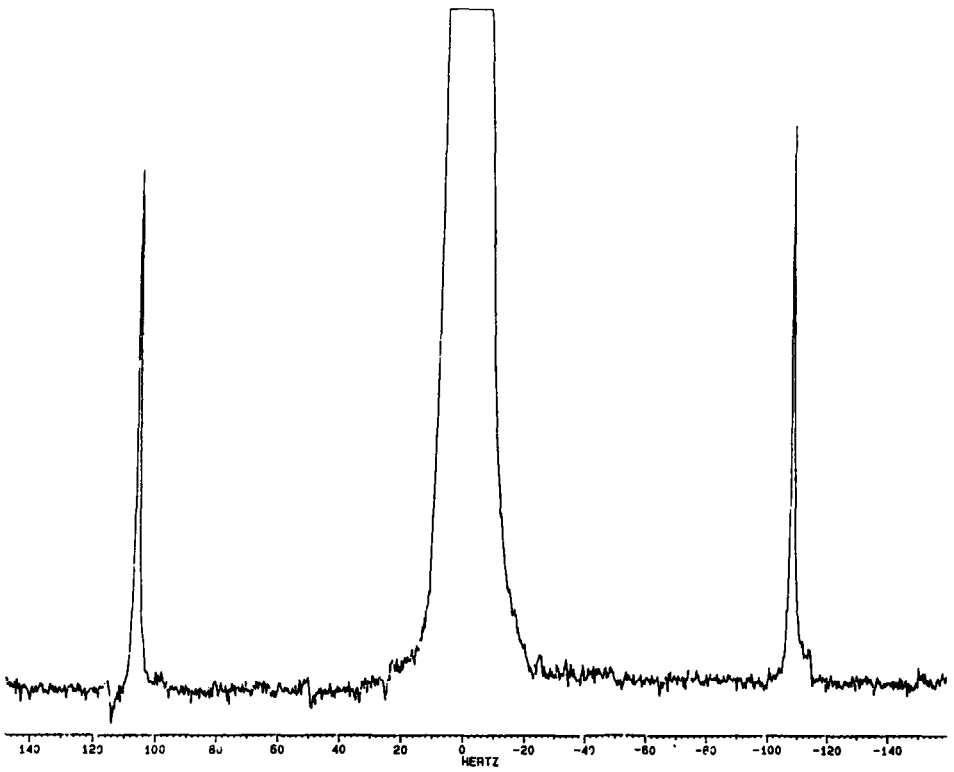

Fig. 4. ^{1}H NMR signal line shape of chloroform in $[^{2}H_{6}]$acetone (hump test), measured in a 120-μl continuous-flow probe (500 MHz) at a flow-rate of 1 ml/min.

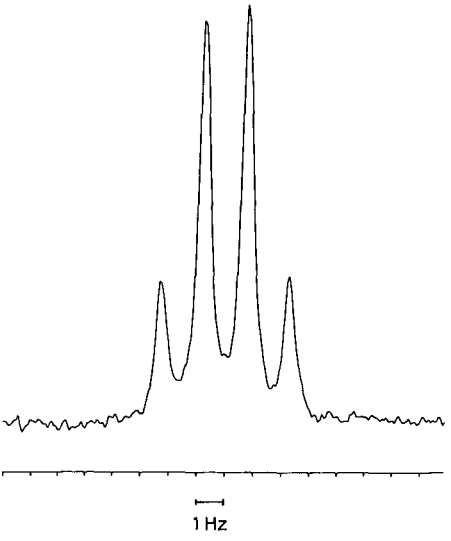

Fig. 5. ^{1}H NMR spectrum of the CH_2 protons of ethylbenzene (0.1% solution in deuteriochloroform), measured in a 120-μl continuous-flow probe (300 MHz, one scan, acquisition time 2.7 s).

UV detection. We have recently pointed out[3] that peak broadening effects due to increased NMR detection volumes are tolerable when long analytical columns (250 mm) are used. At the present state of NMR coil development, a detector volume of 40–120 μl is needed to yield good S/N values in the continuous-flow mode within a time resolution of the chromatogram of lower than 10 s.

Signal behaviour in continuous-flow NMR spectroscopy

An important feature of on-line HPLC–NMR coupling is the flow-rate dependency of NMR signals. Due to the decreased residence time, τ, of a nucleus in the flow cell, both the spin–lattice relaxation time, T_1, and the spin–spin relaxation time, T_2, are reduced[9,10]:

$$1/T_{1\ \text{flow}} = 1/T_{1\ \text{static}} + 1/\tau$$
$$1/T_{2\ \text{flow}} = 1/T_{2\ \text{static}} + 1/\tau$$

Therefore the NMR signal half width increases with increasing flow-rate[2]. On the other hand the signal intensity increases with increasing flow-rate if Boltzmann equilibrium is maintained before the nuclei enter the cell. Full polarization of nuclei may be achieved, allowing them to flow through a pre-polarization volume in the magnetic field for a time period that exceeds $5T_1$. Fig. 6 shows the magnetization curves of the CH_2 protons of ethanol at different flow-rates using a pre-polarization volume of 2 ml, equivalent to the total solvent volume in an HPLC column of 250 mm × 4 mm. The magnetization intensity, I, is plotted against the variable delay, t, between the 180° and the 90° pulse of the NMR inversion recovery sequence. The different slopes of magnetization are described by three relaxation times of 3.4 (stationary mode), 2.0 (flow-rate 2.4 ml/min) and 1.0 s (flow-rate 5.0 ml/min). Thus it is evident that a flow enhancement rate is possible, dependent on the ratio of the detector volume to the flow-rate and on the flip angle and pulse repetition time.

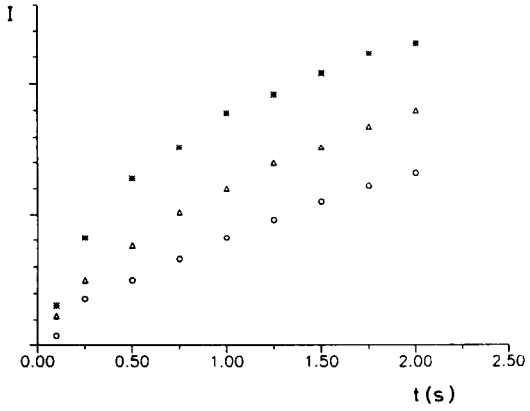

Fig. 6. Magnetization curves (400 MHz) of the CH_2 protons of ethanol in deuterium oxide at flow-rates of 0 (○), 2.4 (△) and 5.0 ml/min (*). Arbitrary intensity scale.

1$\bar{3}\bar{3}$1 Solvent non-excitation

The main problem in reversed-phase HPLC–NMR coupling is the suppression of solvent signals. Because of the time necessary for saturation of the solvent peak, the gated homodecoupling technique used in stopped-flow experiments[2] is not suitable for continuous-flow detection. Effective solvent suppression in continuous-flow acquisition is performed by application of the binomial solvent suppression techniques[6,7,11]. Laude and Wilkins[4,5] used the 11 technique of Clore *et al.*[11] whereas our group[3] takes advantage of the 1$\bar{3}$3$\bar{1}$ technique of Hore[6,7].

Fig. 7 shows the spectrum of 0.15% triethylamine in a mixture of 62.5% water (pH 3) and 37.5% acetonitrile at a flow-rate of 1 ml/min with application of the 1$\bar{3}$3$\bar{1}$ suppression technique. The intensity of the suppressed water signal is in the same range as those of the signals of the 0.15% triethylamine, indicating a solvent suppression ratio of three orders of magnitude. The disadvantage of this technique is that further nulls of the magnetization occur at intervals $1/D_2$ (Fig. 5) and that the signal phasing changes at every null. Therefore magnitude calculation has to be performed.

In Fig. 8 the contour plot of a separation of aromatic compounds (each 70 μg) at a flow-rate of 1 ml/min in acetonitrile–water (50:50) is shown. The chemical shift range between 2.6 and 10.3 ppm is plotted against the elution time between 0 and 40 min omitting the residual acetonitrile resonance at 2.0 ppm. Throughout the whole

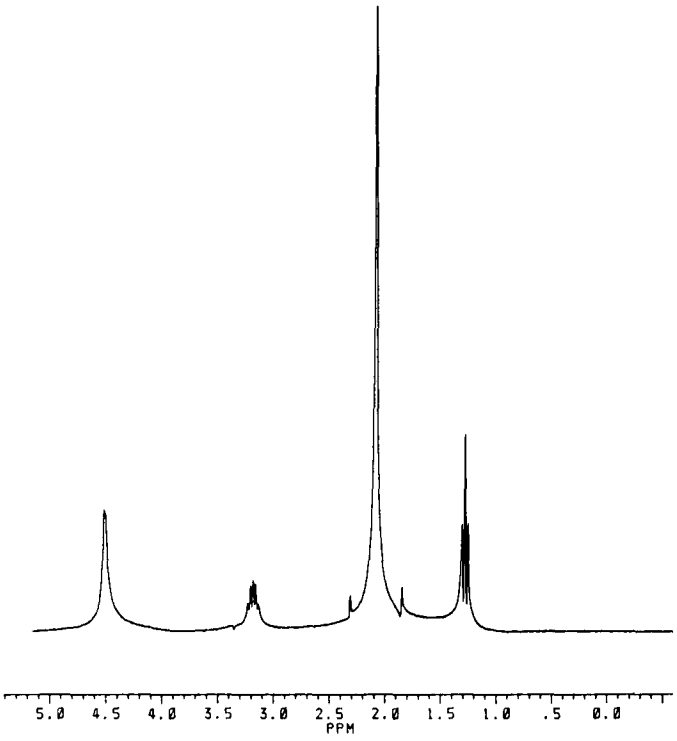

Fig. 7. ^{1}H NMR spectrum of a 0.15% solution of triethylamine in acetonitrile–water (37.5:62.5) at a flow-rate of 1 ml/min (300 MHz, eight scans, 4800 Hz per 8 K). 1$\bar{3}$3$\bar{1}$ solvent non-excitation.

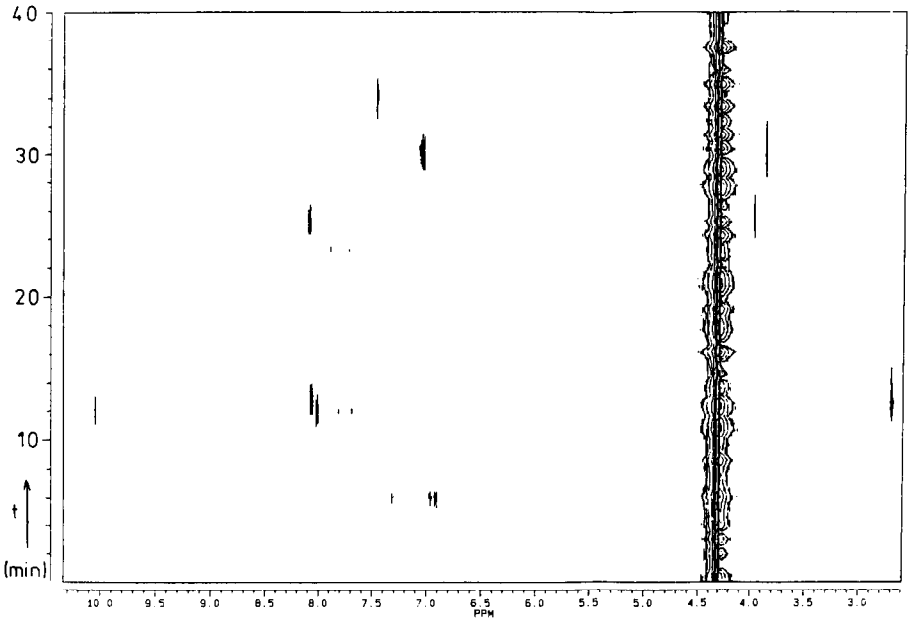

Fig. 8. ^{1}H NMR chromatogram (contour plot, 500 MHz) of a separation of phenol (t_R = 6 min), benzaldehyde (t_R = 12 min), acetophenone (t_R = 13 min), nitrobenzene (t_R = 23 min), methyl benzoate (t_R = 25 min), anisole (t_R = 30 min) and benzene (t_R = 34 min), each 70 μg, in acetonitrile–water (50:50). Flow-rate 1 ml/min. Eight scans per file; 6000 Hz per 8 K; 133T solvent non-excitation, column ODS-Hypersil 5 μm, 250 mm × 4.0 mm (Shandon).

separation the signal of the residual water is seen at 4.2–4.4 ppm. At a retention time, t_R, of 6 min the signals of phenol protons appear, followed by the signals of benzaldehyde at t_R = 12 min and of acetophenone at t_R = 13 min. The chemical shift values of the aldehyde and the alkoxy group in these compounds are strongly related to the structures of the substituents. The signals of nitrobenzene at t_R = 23 min are very weak, whereas the signal of methyl benzoate at t_R = 25 min can clearly be detected. The last two compounds are anisole at t_R = 30 min and benzene (t_R = 34 min). With the exception of nitrobenzene, the ^{1}H NMR peaks in this chromatogram reveal more structural information than any diode array detection.

CONCLUSIONS

The quality of NMR spectra obtained in continuous-flow systems is approaching more and more that of conventional stationary NMR spectra. In a one-pulse experiment, the stationary sensitivity is about 60% of that of rotating NMR tubes. In repeating multipulse experiments, the sensitivity may be equal or even better in the continuous-flow mode because of the advantage of rapid pulsing. Here pulse repetition rates are dependent on the T_1 values of the eluents.

Concerning the progress in sensitivity apparently available, another solution of the solvent problem seems to be possible. In microbore column separations, only a few ml of solvents are necessary for a separation, enabling the use of deuteriated

solvents. Using a special NMR coil arrangement, the sensitivity values reported in this paper should be obtained by continuous-flow cells using detector volumes in the range between 2 and 14 μl. Since, in microbore HPLC, flow-rates of 0.1 ml/min are used, flow broadening effects should be negligible. The application of such small cell volumes should also allow the development of solenoidal cells without changing the standard shimsystem, thus resulting in a two-fold sensitivity enhancement because of the perpendicular location of the cell relative to the magnetic field[8]. It is thus apparent that further enhancement of NMR sensitivity in the continuous-flow mode is expected within the next few years.

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