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ON-LINE COUPLING OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND NUCLEAR MAGNETIC RESONANCE

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SUMMARY

High-performance liquid chromatography–nuclear magnetic resonance (HPLC–NMR) on-line coupling is a possible solution to the problem of universal detectors in liquid chromatography. By use of a newly developed ^{1}H-FT-NMR* flow cell, ^{1}H-NMR spectra of flowing systems can be obtained. Although sensitivity and resolution are somewhat lower than in the conventional system, the ^{1}H-NMR spectra are of sufficient quality to enable classification of unknown compounds. The performed HPLC separation with on-line NMR-measurement shows the capabilities of the applied arrangement.

INTRODUCTION

In gas chromatography the on-line combination with mass spectrometry (MS) has been proven to be the most powerful method for the investigation of complicated mixtures and unknown compounds. Separation and structure identification are possible simultaneously. Since gas chromatography and mass spectra are both achieved in the gas phase the interface is relatively easy.

Such a powerful combination of separation and identification method is not yet possible in the case of high-performance liquid chromatography (HPLC). The combination of HPLC with different kinds of MS methods has been reported[1–3]. However, in this case, a transfer of the substance from the liquid chromatographic phase into the gas phase and the high vacuum for MS poses certain technical problems, especially connected with the large amounts of eluent. Another principal disadvantage of the combination HPLC–MS is the MS restriction in the molecular weights of the substances to be investigated. For HPLC, contrary to gas chromatography and MS, vaporization is not necessary and hence no such molecular-weight restriction exists. Therefore, a combination of HPLC with nuclear magnetic resonance (NMR) as the most powerful method for characterization of organic compounds seemed desirable to use. The development of pulse technique in NMR has overcome the restrictions of time and concentration factors in the continuous wave measurement.

* FT = Fourier Transform.

The advantages of using NMR in combination with HPLC in comparison to HPLC–MS coupling are (1) both HPLC and NMR are conducted in solution and no transfer from one phase to another is required, as from the liquid to vapour phase in HPLC–MS; (2) NMR measurements are not limited by vaporization and hence by molecular weight; (3) in many cases the structure information by NMR spectra is more extensive, especially when the stereochemistry of the molecule is also considered.

However, at present, seroius disadvantages which prevented the consideration of HPLC–NMR combination as a valuable addition to the arsenal of analytical methods had to be overcome, such as (1) very little known about the measurement of high-resolution spectra in flowing systems[4]; (2) limitation of suitable solvents due to solvent signals; (3) the relatively low sensitivity of NMR.

We have tried to overcome some of these difficulties and to develop an instrumentation for the on-line coupling of HPLC and NMR.

Problem of flow NMR

Despite some very early scattered reports on the measurement of NMR spectra in flowing systems[5–7], this method has only been used extensively for special problems. Mainly the relative change of the signal at a single frequency was monitored for the determination of flow-rates.

The following problems arise in flow NMR: the intensity of an NMR signal is dependent on the flow-rate of the solvent. Thus, when the flow-rate is low, because of steadily polarising particles, the signal amplitude increases, and with high flow-rates it decreases. However, in the flow-rate range of 0.5–2 ml/min, which is being employed in the HPLC separation process, not much change in the signal amplitude is expected. However, the half-width amplitude of the signal and thus the overall resolution of the spectrum can change with a large detector-volume. The half-width amplitude is dependent on the viscosity of the solvent, the strength of the RF* field, the non-homogeneity of the magnetic field, and above all on the time the sample dwells in the detector volume. Thus the line-broadening depends on the flow-rate/detector volume ratio. In an HPLC–NMR combination, the flow-rate is related to the separation process and therefore the detector volume to the elution volume of a peak, which amounts to *ca.* 0.2–20 ml for an analytical HPLC assay.

These considerations necessitate the following conditions for the construction of the flow-cell.

(1) It must guarantee a sufficient particle concentration for the NMR measurement; this would mean a relatively large volume. Additionally the pulse Fourier transform technique must be used, in order to overcome the problems of low concentration and to accumulate the signals during the passage of the peaks through the NMR cell.

(2) The detector volume should not exceed the volume of a separation peak. This volume is larger than that of normal detectors. However, it is sufficient if the instrument provides, besides continuous-flow measurement, the stop-flow[10] technique, and if no turbulent flow occurs in the connections between NMR cell and HPLC column.

(3) The geometry of the flow-cell must enable an optimum synchronization with the measuring coil. This requires straight and parallel cell walls in the actual measuring range.

* RF = radio frequency.

(4) An intermingling of the separated peaks should not occur in the flow-cell. This means that the geometry of the cell and the connections should be constructed in such a way that no turbulent flow arises.

Problem of the solvent

One objection raised against HPLC–NMR coupling is the solvent problem. Usually in high-resolution NMR, one employs deuterated solvents, the deuterium-signals of which are being used for field-frequency stabilization. Under the present circumstances, the problem of the application of deuterated solvents can be partially dealt with by the help of an external lock-arrangement. Thus field-frequency stabilization can be achieved with a ^2H_2O ampoule which is placed near the actual NMR cell. The use of conventional, non-deuterated solvents is possible with this arrangement.

EXPERIMENTAL

^{1}H-NMR flow-probehead

In order to obtain an optimal adjustment of the insert coil, we selected a cell construction having a detector volume of about 0.416 ml. With a flow-rate of 1 ml/min and an elution volume of 0.416 ml, the residence time in the detector cell is 25 sec.

It was found that peak mixing occurred if the PTFE capillary connection tubes (I.D. 0.23 mm) are directly connected to the cell (I.D. 7 mm). Using different dye

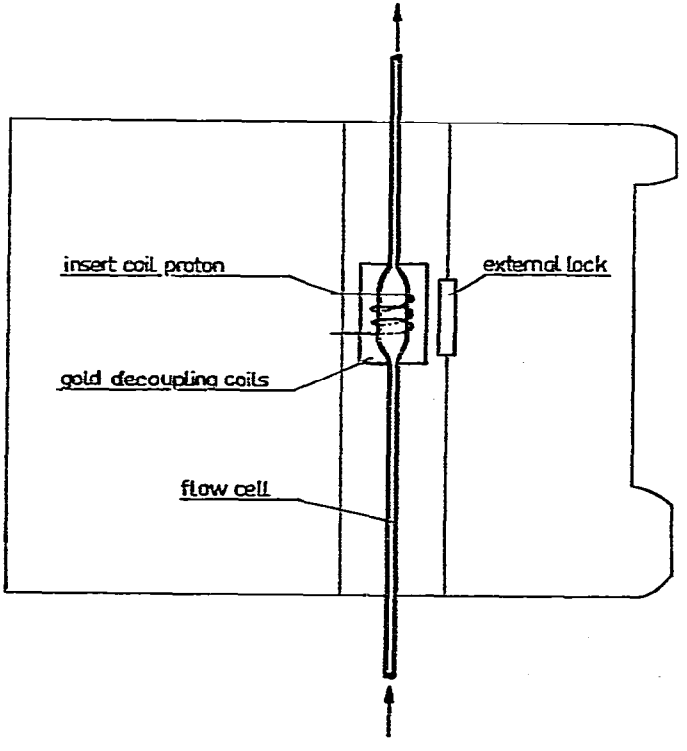

Fig. 1. Schematic diagram of ^{1}H-NMR flow-probehead.

systems, we studied flow profiles of various constructions, with the aim of obtaining a more suitable geometry. Thus we obtained an optimal geometrical arrangement, schematically represented in Fig. 1.

Apparatus for on-line HPLC–NMR coupling

The experimental arrangement used for direct HPLC–NMR on-line couplings is shown in Fig. 2.

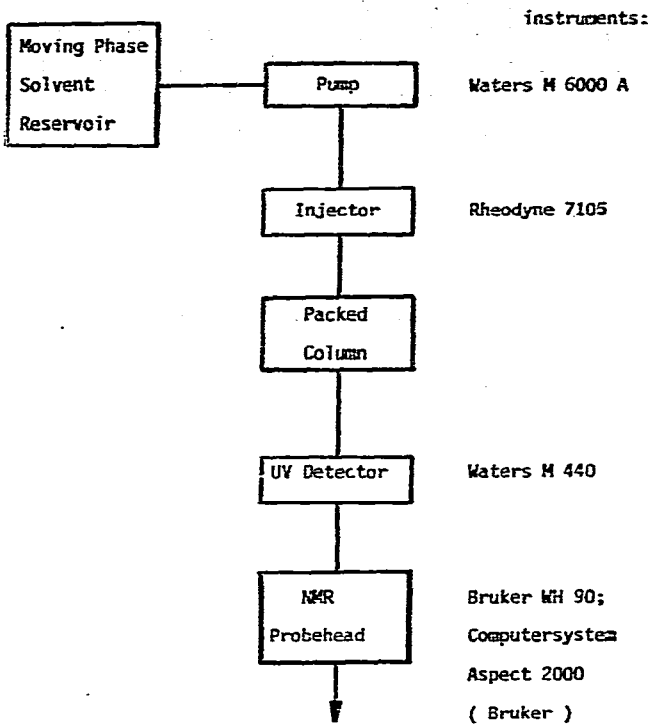

Fig. 2. Experimental arrangement for on-line HPLC–NMR coupling.

All instruments, despite our NMR probehead, are commercially available and have been proven to be suitable for our experiments. A connection between UV detector and NMR probehead was performed by PTFE capillary tubings. The NMR spectrometer was operated under normal conditions. The instrument was used with the Aspect 2000 computer system and a typical set of operation conditions is given in Table I.

RESULTS AND DISCUSSION

^{1}H-NMR flow spectra under chromatographic conditions

After dismantling the separation column and the UV detector, the test spectra were run in the HPLC–NMR system (Fig. 2). After injection through a modified feeding system, the different test mixtures were pumped into the measuring cell and then either under stop-flow or continuous-flow conditions, the spectra were measured.

TABLE I

NMR SPECTROMETER OPERATING CONDITIONS

Spectrometer frequency	90 MHz
Spectral width	3000 Hz
Memory size	4 K
Acquisition time	0.7 sec
Pulse width	4.0 μsec
Measure mode	Quadrature 4-phase sequence
Line-broadening	0.1 sec
Delay between scans	498 μsec
Temperature	300°K

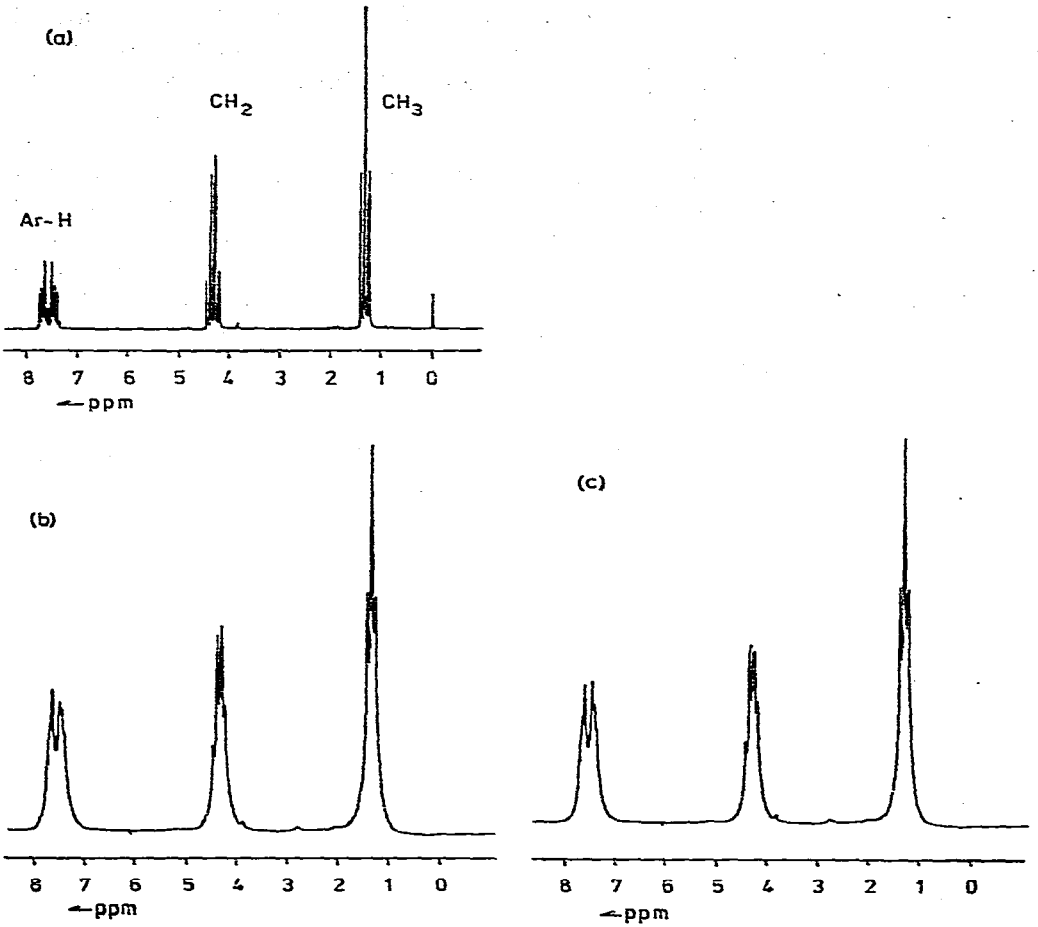

Fig. 3. ^{1}H-FT-NMR spectra (TAQT* 11.2 sec) of diethylphthalate in carbon tetrachloride (10^{-2} *m*) under the following different measuring conditions. (a) Rotation of the cell (conventional measurement), (b) stop-flow measurement (flow measure cell), (c) continuous-flow-measurement; flow-rate 1.5 ml/min (flow measure cell).

* TAQT = Total acquisition time.

In Fig. 3, the ^{1}H-FT-NMR spectra of diethylphthalate in carbon tetrachloride, obtained in a normal NMR measuring cell under optimum conditions and resolution, are compared with the NMR spectra obtained in our measuring cell designed for HPLC–NMR coupling. All measurements, also in the conventional mode, are performed with the external lock.

Without the flow of the solvent (stop-flow measurement) a resolution of 2.5 Hz (Fig. 3b) is obtained. This is lower than the resolution in the case of the measurement under conventional conditions (Fig. 3a) and is due to non-rotation of the cell. However, this suffices for recognizing the spin–spin coupling.

The change resulted by the shift of the measuring technique from stop-flow to continuous-flow was quite interesting. At a flow-rate of 1.5 ml/min the observed resolution is about 3 Hz (Fig. 3c). Hence no substantial changes occur between the measurements in the stop-flow and continuous-flow methods. This fact is confirmed from the ^{1}H flow spectra presented in Figs. 4 and 5. Fig. 4 shows the ^{1}H flow spectra of ethanol in a proton-containing solvent, chloroform. In the given concentration range, direct measurements are possible. At low substance concentrations, the solvent resonance must be suppressed and this leads to longer measuring periods. Investigations on complex molecules, such as the porphyrins, also resulted in similar observations (Fig. 5). Both spectra, the one under the conventional conditions (Fig. 5a), and the other under the continuous-flow conditions (Fig. 5b), were run under comparable instrumental conditions. The line-broading in the flow spectrum is distinctly recognizable. However, again the different protons are quite apparent and the resonance of the NH protons at —2.6 ppm, strongly broadened by the intramolecular exchange process, can easily be detected.

HPLC–NMR coupling

The chromatogram of the separation of ionol, anisol and salol, with the help

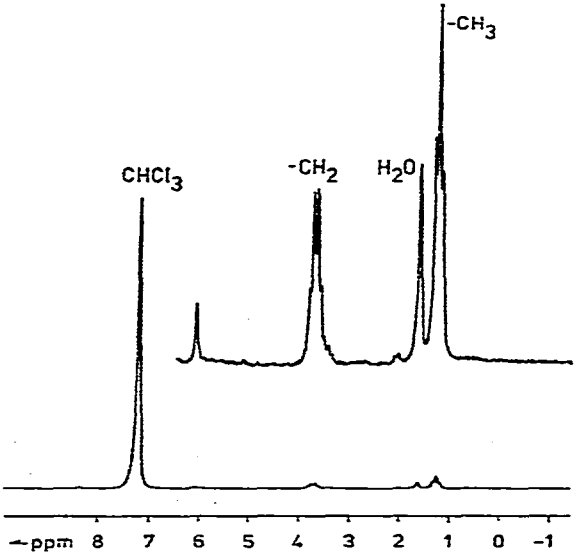

Fig. 4. ^{1}H-FT-NMR spectra (TAQT 7 sec) of ethanol in chloroform (10^{-2} *m*); flow-rate 1 ml/min.

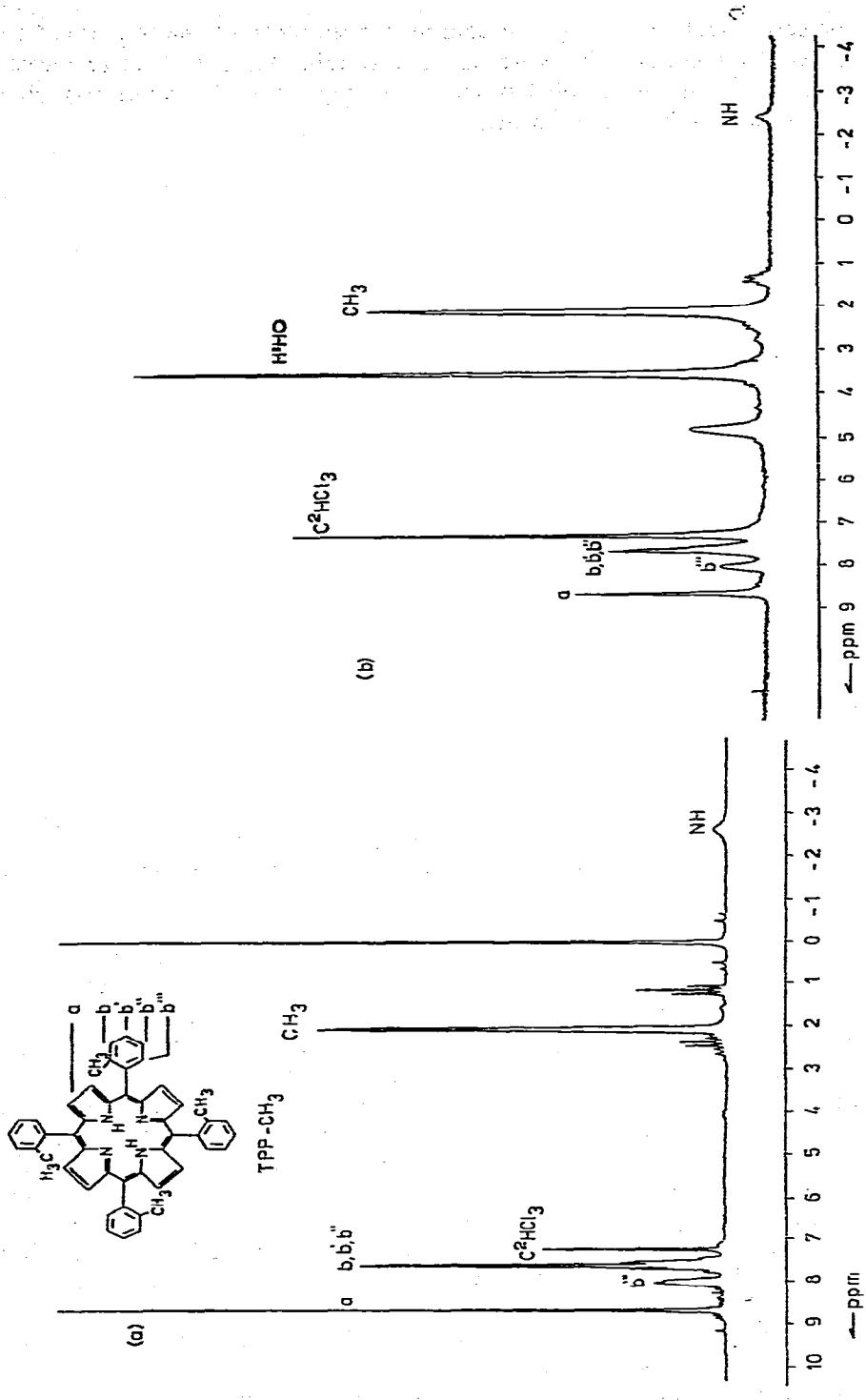

Fig. 5. ^{1}H-FT-NMR spectra (TAQT 14 sec) of tetra-*o*-tolylporphyrin (TPP-CH_3) in chloroform-d_1 (10^{-1} *m*) under the following measuring conditions. (a) Rotation of the cell (conventional measurement), (b) continuous-flow measurement, flow-rate 1 ml/min (flow measure cell).

of the UV detection technique, shows a complete resolution of the individual components (Fig. 6). This system, therefore, is quite suitable for comparative measurements with the direct on-line coupled system. The experimental arrangement shown in Fig. 2 was employed for this separation.

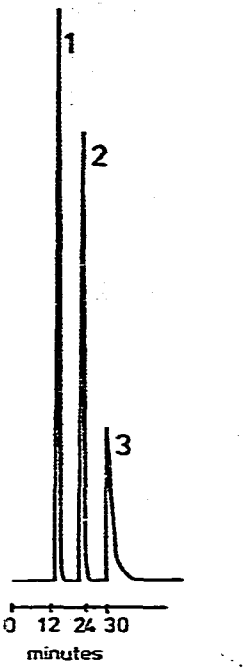

Fig. 6. HPLC separation of ionol (1), anisol (2), and salol (3). Column: 250 mm × 4 mm I.D.; packing material: LiChrosorb Si 60, 10 μm (Merck, Darmstadt, G.F.R.); mobile phase: carbon tetrachloride, LiChrosolv (Merck) (dried over Al_2O_3; ^2H_2O-saturated); flow-rate: 1 ml/min; temperature: 22°; pressure: 1000 p.s.i.; detector: UV, 280 nm.

Since the substances can be detected by the change in amplitude of the resulting interferogram under the NMR measureng conditions, the UV detector which served as the control element is, however, in principle unnecessary.

After the injection of 10 μl of an equimolar mixture (50 μmol) of ionol, anisol, and salol in carbon tetrachloride, the separation was achieved with a flow-rate of 1 ml/min within 26 min. Every 51 sec after the occurrence of peak-maximum in the UV detector, NMR measurement began. Thus different numbers of scans, depending upon the respective peak width were made. For a sweep width of 3000 Hz and the applied memory of 4 K, the acquisition time for a particular scan is 0.7 sec. The interferograms of the three separated components were stored on different memories, and evaluated after the separation. The first peak (ionol) was observed to have an elution time of 144 sec, and the measuring time for its ^{1}H-NMR spectrum amounts to 178 scans, equivalent to 121 sec.

Analogously, the elution time for anisol was 220 sec (297 scans, 202 sec) and for salol it was over 800 sec (774 scans, 526 sec).

The completed interferograms of the three components were run within their

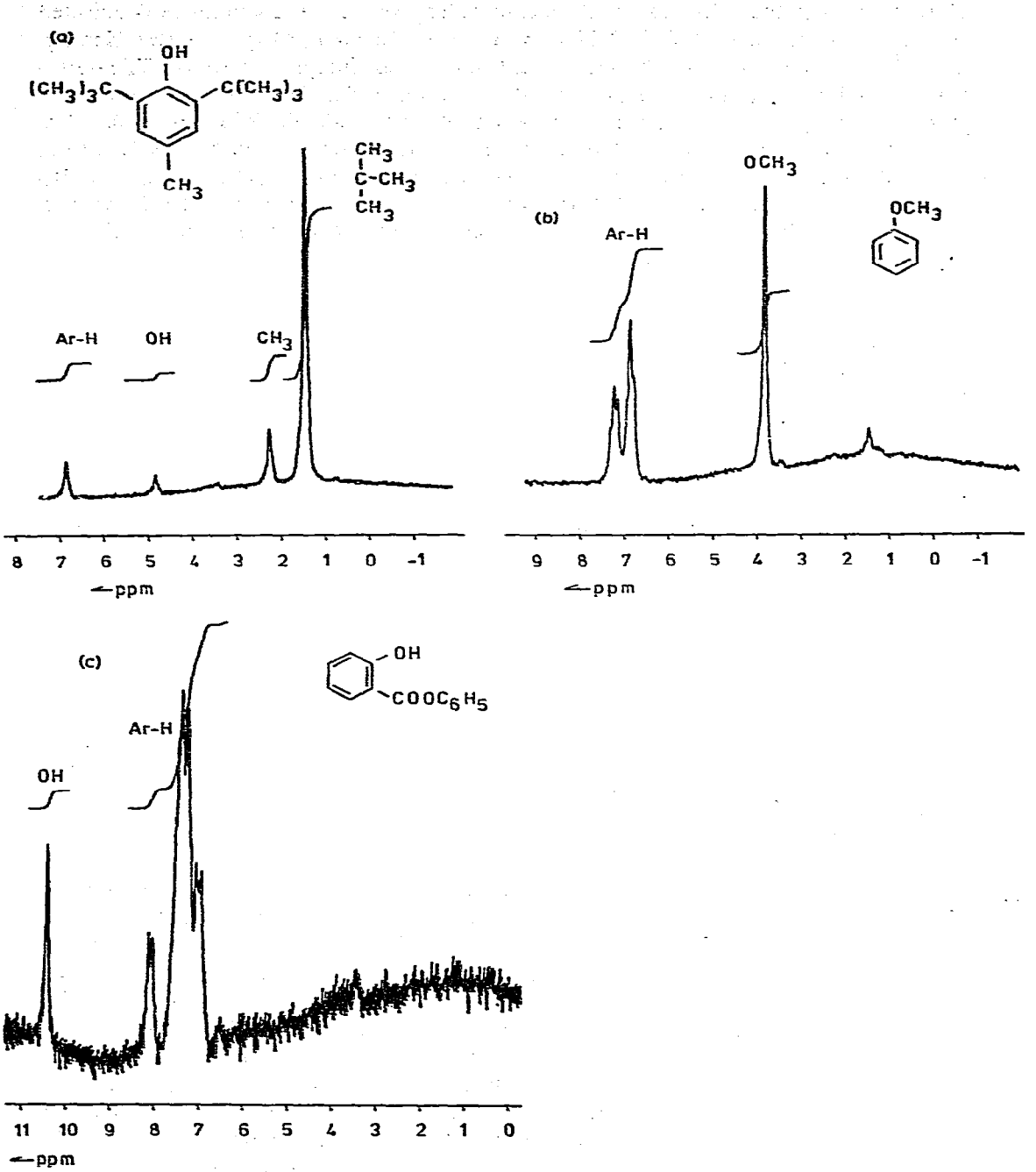

Fig. 7. ^{1}H-FT-NMR spectra of peaks separated under same conditions as in Fig. 6, with on-line NMR coupling. (a) Ionol, TAQT 124.6 sec; (b) anisol, TAQT 207.9 sec; (c) salol, TAQT 541.8 sec.

elution time and the column separation experienced no mutual disturbance as detected by the NMR. The obtained ^{1}H-NMR spectra are presented in Fig. 7. A classification is possible based upon the obtained data (ppm values, integration ratio). The detection of the intramolecular hydrogen bond formation in the ^{1}H-NMR spectrum of the third peak (Fig. 7c) demonstrates the potential of HPLC–NMR coupling for simultaneous investigations of the stereochemistry, which is not possible by MS detection.

An identification of the individual components on an analytical scale was also possible. Fig. 8 shows the ^{1}H-NMR spectrum of anisol in carbon tetrachloride (5 μmol, 4 μl injection). Even when two components are not separated, the NMR spectrum still allows for identification.

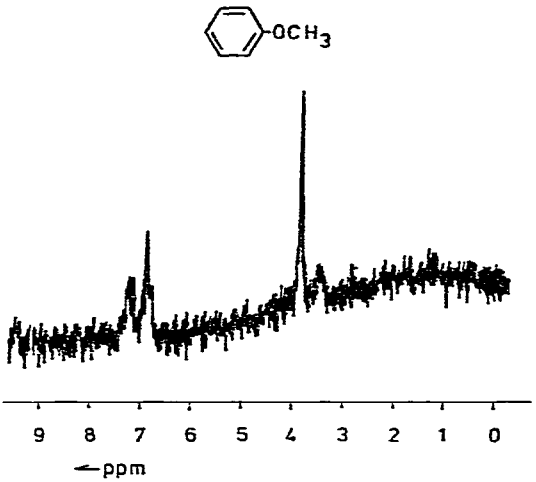

Fig. 8. ^{1}H-FT-NMR on-line spectrum of anisol (5 μmol) in carbon tetrachloride, TAQT 27 sec. Chromatographic conditions: column: Hibar column 250 × 3 mm I.D. (Merck); packing material: LiChrosorb Si 60, 7 μm (Merck); mobile phase: carbon tetrachloride LiChrosolv (Merck) (dried over Al_2O_3; ^2H_2O-saturated); flow-rate: 1 ml/min; pressure: 1200 p.s.i.; temperature: 22°.

CONCLUSIONS

From these data and the achieved separations it is clear that an HPLC–NMR coupling is feasible on an analytical scale. Stop- and continuous-flow measurements enable a large variation possibility. Since the individual components surpass the cell consecutively and no memory effect is observed (under the conditions described above) the cell construction technique used here saves complicated wash procedures. The great advantage of our cell is its compatability with conventional ^{1}H-NMR probeheads, which in principle enables the coupling of the HPLC instrument and the NMR spectrometer and demands no special NMR instrument. The examples show the large amount of information that can be obtained from the coupling of HPLC separation with NMR detection. Of the three substantial pieces of information from the ^{1}H-NMR spectrum (ppm values, integration ratio and coupling constant, J) the first two can be easily obtained and the third in the case of $J \geqslant 3$ Hz. The detection limit achievable with the instruments at our disposal lies in the μmol range and can already compete with the sensitivity of the refraction index detectors. If it succeeds in overcoming the solvent limitation through the use of less expensive chlorinated or fluor-

inated solvents, then HPLC–NMR coupling can find broader applications in comparison to HPLC–MS coupling. Sensitivity and resolution may be improved by using super-conducting NMR spectrometers, which will be the next step in our investigations.

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