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Review

On-line use of NMR detection in separation chemistry

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Abstract

The direct on-line coupling between important separation and extraction techniques such as high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), supercritical fluid extraction (SFE) and capillary electrophoresis (CE) and proton high-field nuclear magnetic resonance (NMR) spectroscopy is described. The resolution of the ^{1}H NMR spectra obtained in HPLC–NMR, SFC–NMR and SFE–NMR coupling under continuous-flow conditions is similar to conventionally recorded NMR spectra. In CE–NMR coupling signal line widths are degraded but the resolution of CE–NMR spectra is improving continuously.

The detection limit of a HPLC–NMR separation in acetonitrile–D_2O for aliphatic signals of a low-molecular-mass compound (300 Da) is 500 ng of injected compound in the continuous-flow mode at a 600 MHz NMR spectrometer with a 120 μl flow cell. A similar detection limit in the nanogram range with a 5 nl flow cell is reached in a CE–NMR separation at a 300 MHz NMR spectrometer. In SFC–NMR coupling the whole proton spectral range can be observed without any solvent windows. SFE–NMR offers the advantage of directly monitoring the extraction process.

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1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is by far the most powerful spectroscopic technique for obtaining detailed structural information about organic compounds in solution. Its particular strength lies in its ability to differentiate between most structural, conformational and optical isomers. Given a molecular mass, NMR spectroscopy can usually provide all the additional information to unambiguously identify a completely unknown compound.

The NMR detection technique is also quantitative with individual areas in spectra being proportional to the number of contributing nuclei. There are only two drawbacks of this powerful spectroscopic technique. One is the relative low sensitivity in comparison to other spectroscopic techniques such as mass spectrometry, the other is the fact that structure elucidation of mixtures of unknown organic compounds with overlapping ^{1}H NMR signals is difficult and may be nearly impossible in cases with overcrowding signals in a small chemical shift region of the NMR spectrum. Therefore separation procedures should be performed prior to the use of the NMR detection technique. For time-saving purposes a direct coupling of a separation technique with NMR detection would be the method of choice.

Despite the relative low sensitivity of NMR the use of superconducting magnets should enable the direct coupling of this powerful detection technique with different kinds of chromatographic separation techniques which are routinely employed in separation science. Whereas direct on-line HPLC–^{1}H NMR coupling is starting to become an established hyphenated technique [1–19], the direct coupling of supercritical fluid chromatography with ^{1}H NMR spectroscopy [20–22] together with the monitoring of supercritical fluid extraction [23] as well as the coupling of capillary electrophoresis and ^{1}H NMR spectroscopy [24–26] has been reported recently.

The theoretical background of continuous-flow NMR has been already described in detail [4,5,10]. This paper deals with a short overview of all four hyphenated techniques. Due to the rapid progress in this field within few years the described results may become obsolete. Nevertheless they will be considered as "classical" experiments within this fascinating analytical area.

2. HPLC–NMR coupling

The first on-line HPLC–NMR experiments using superconducting magnets have been reported in the early 80's [1–3]. Three different main types of continuous-flow cells were used, from which the approach developed in Tübingen [1,4] was further improved and commercialized by Bruker (Karlsruhe, Germany). There may have been other developments of HPLC–NMR probes but to the best knowledge of the author only the probe described below has been used in most applications described in the literature in the last years.

In contrast to conventional NMR probes (Fig. 1) where the NMR measuring tube rotates within in a glass insert (6.1 mm I.D.) to which the measuring coil is attached, the Bruker HPLC–NMR probe consists of a non-rotating glass tube to which the radiofrequency coil is directly fixed (Fig. 1), centered in the glass dewar of a conventional probe body. The internal diameter of the glass tube is either 2, 3 or 4 mm, the glass walls are parallel at least within the length of the proton detection coil (18 mm) and taper at both ends to fit to PTFE tubings (0.25 mm I.D.). PTFE tubings and glass tube are connected by shrink-fit tubings. "Inverse" continuous-flow probes contain an additional coaxial coil (matched to the ^{13}C resonance frequency) surrounding the ^{1}H detection coil for heteronuclear ^{1}H/^{13}C shift correlated experiments. A further development of this design contains a glass tube in the center of the measuring volume which can be filled with deuterated solvents for field/frequency stabilisation of the spectrometer enabling the use of non-deuterated eluents.

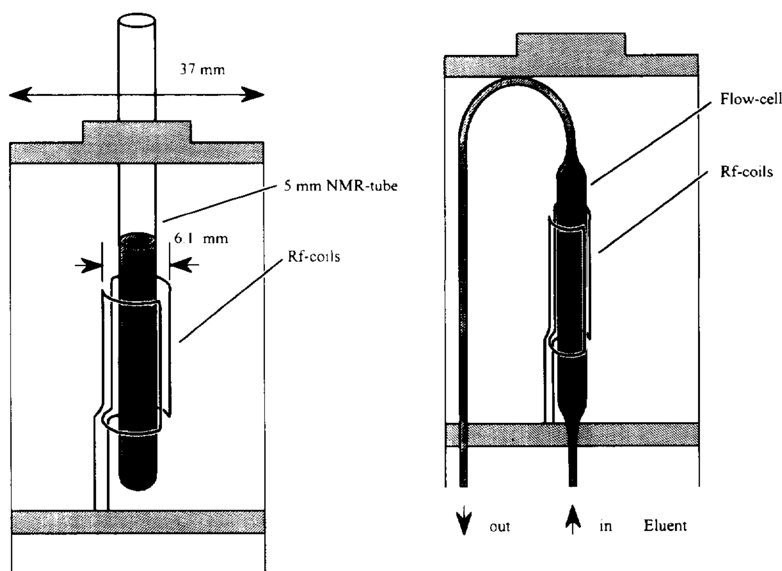

Fig. 1. Geometry of conventional and continuous-flow NMR probes.

The ratio of NMR coil volume versus sample volume, the so-called filling factor, is close to the optimum, because the radio frequency coil is directly fixed to the glass tube of the detection cell. Thus this type of NMR detection probe is in principle the most sensitive one, provided the NMR measurement is performed without rotation. In modern NMR spectroscopy two- and three-dimensional and inverse experiments are performed without sample rotation to avoid modulation problems caused by rotation of the NMR tube.

The passage of a flowing liquid through a NMR detection cell effects NMR signal intensity and signal line width [4]. At a constant flow-rate the analyte in the detection cell is completely replaced by new liquid after a period τ. Thus the ratio of detection volume to flow-rate defines the residence time τ. As a result of the limited residence time in the detection cell, the continuous-flow mode results in a decrease of the effective spin–lattice relaxation times $T_{1\ \text{flow}}$ ($1/T_{1\ \text{flow}} = 1/T_{1\ \text{static}} + 1/\tau$) and spin–spin relaxation times $T_{2\ \text{flow}}$ ($1/T_{2\ \text{flow}} = 1/T_{2\ \text{static}} + 1/\tau$) [4]. Thus faster flow-rates with shorter residence times increase the signal intensity per time increment by allowing faster pulse repetition times but may lead to signal line width degradation. A small residence time of 1 s results in a linewidth broadening of 1 Hz, whereas residence times

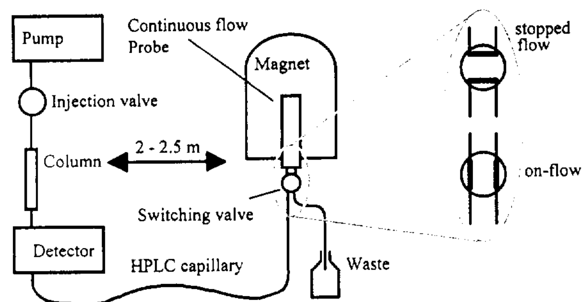

Fig. 2. Experimental arrangement for coupling a chromatographic separation system with an NMR spectrometer.

higher than 5 s lead to negligible line broadening, i.e. lower than 0.2 Hz [4].

The non-rotation of the detection cell together with the ratio between the detector volume and the employed flow-rate determine the resulting NMR signal line widths. Whereas with rotation of the NMR tube in a conventionally NMR probe the signal line width of chloroform at the height of the ^{13}C satellites in degassed acetone-d_6 is about 3–4 Hz, continuous-flow probes show values in the order of 12–16 Hz. Due to viscosity and solvent-induced effects in routine NMR spectroscopy signal half-widths mainly differ between 0.1 and 2 Hz. A practical linewidth in routine proton NMR might be 0.5 Hz.

To guarantee sufficient NMR sensitivity the inner diameter of the NMR detection cell of continuous-flow probes employed in most on-line HPLC–NMR and SFC–NMR experiments is either 2, 3 or 4 mm resulting in detection volumes in the order of 60, 120 and 180 μl. At a flow-rate of 1 ml/min, usually employed in HPLC, the residence time in all detection volumes is higher than 5 s. No difference in resolution can be observed in comparing routine and continuous-flow NMR spectra (compare Figs. 3, 5, 12, 14, 15, 16, 20, 22, 24 below).

The large detection volumes of the described flow cells result in peak dispersion effects. They have been determined by measuring the resulting peak widths of a HPLC test mixture of dansylated amino acids in flow cells with different detection volumes in the range 1–400 μl with the help of a modified fluorescence detector [4,10]. The resulting relationship between the plate height and capacity factor reveals that for analytical columns (250 × 4.6 mm I.D.) the plate height is adversely affected by capacity factors below 2.5 using detection volumes higher than 48 μl.

One feasible experimental arrangement for coupling a chromatographic separation system with a NMR spectrometer (HPLC–NMR, SFC–NMR, SFE–NMR) is outlined in Fig. 2.

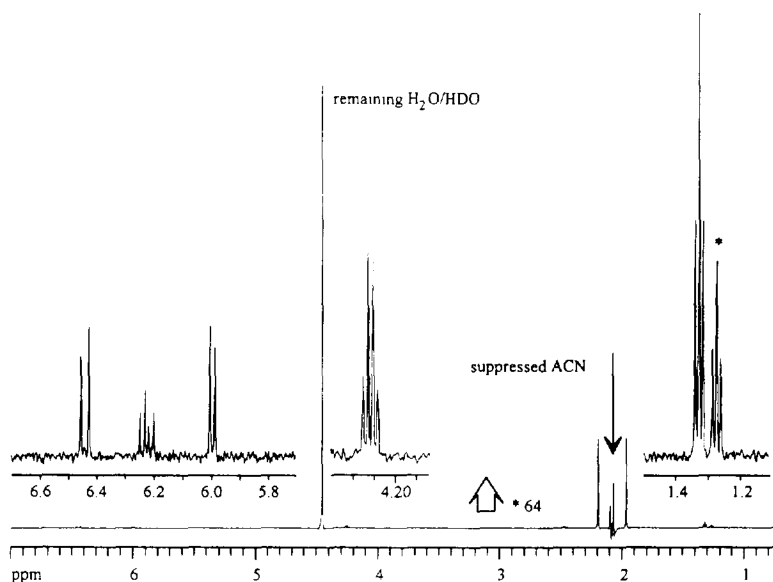

Fig. 3. Continuous-flow ^1H NMR spectrum (600 MHz) of ethylacrylate in acetonitrile–D_2O (40:60). * Denotes impurity in acetonitrile.

The chromatographic separation system, consisting of either HPLC or SFC pumps together with an injection valve, the separation column (250 × 4.6 mm I.D.) and a UV detector, is located at a distance of 2–2.5 m beside the magnet. The outlet of the UV detector is connected by polyetheretherketone (PEEK) capillaries (0.25 mm I.D.) to a valve which is fixed below the probe bottom. Due to adjustment of this valve either the acquisition of stopped-flow or continuous-flow NMR spectra is possible.

2.1. Solvent suppression

The majority of HPLC separations is performed with reversed-phase columns using binary or ternary solvent mixtures. With the exception of deuterium oxide the use of deuterated eluents is too expensive for routine analysis. Therefore proton-containing solvents such as acetonitrile and methanol are used in on-line reversed-phase HPLC–NMR experiments. To get rid of dynamic range problems of the receiver of the NMR instrument the proton NMR signals of the solvents have to be suppressed. Several possibilities exist to deal with the problem of protonated solvents. One is the use of a binomial solvent non-excitation technique, where the transmitter pulse is split in different excitation pulses. Thus no additional time delay is needed.

With a detection cell volume of 120 μl the residence time in the detection coil is 14–24 s at flow-rates of 0.3–0.5 ml/min. For instance, the acquisition time for a proton free induction decay (FID) at a 600 MHz instrument is 1 s at a memory size of 16 K and a sweep width of 7500 Hz, therefore sufficient time is available for solvent signal suppression by applying a NOESY-type presaturation scheme. As an example, in Fig. 3 the continuous-flow ^{1}H NMR spectrum (600 MHz) of ethylacrylate in acetonitrile–deuteriumoxide (40:60) is shown. The spectrum was recorded at a flow-rate of 0.3 ml/min, whereas the acetonitrile signal was suppressed by presaturation (NOESY-type presaturation). In the lower trace the proton spectrum is dominated by the residual HDO signal at 4.45 ppm and the signal of the two ^{13}C satellites together with the residual signal of the methyl group of acetonitrile. The main center signal of the methyl group is suppressed by using presaturation delays of 643 ms and 80 ms before data acquisition. The intensity of the acrylate signals is far below the ^{13}C satellites, they are clearly visible after increasing the vertical scale by a factor of 64 (upper trace). This example demonstrates that the proton signal intensity ratio between signals of the eluent and of the eluted compound is in the order of 8000–10 000. Modern NMR spectrometers exhibit constant solvent suppression performance without any intensity and phase distortions throughout the whole chromatographic separation run. Because solvent signal suppression is only performed at the solvent windows the remaining parts of the NMR spectra are not affected and the signals of the separated compounds can be quantified.

At a 400 MHz instrument continuous-flow data are usually accumulated at a number of 8192 data points using a sweep width of 4000–5000 Hz; 8 to 16 scans are co-added defining a time resolution of 12–25 s. The Fourier-transformed spectrum results in a "row" in the two-dimensional plot of ^{1}H chemical shifts versus retention times. Within one separation run up to 256 or 512 rows are accumulated. In the stopped-

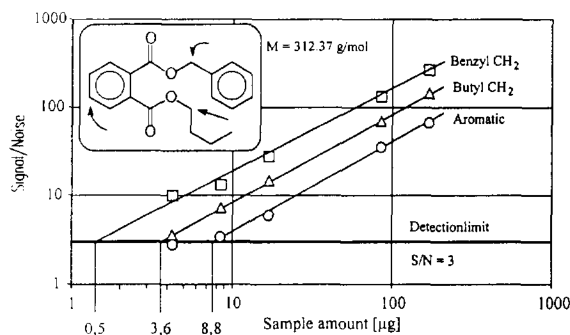

Fig. 4. Detection limits for different signal groups of *n*-butyl benzylphthalate at a 600 MHz NMR instrument.

flow mode the entrance valve is switched as soon as the peak maximum is in the flow cell. The peak maximum is indicated by the UV detector (Fig. 2). For stopped-flow measurements the delay time between UV detector and NMR flow cell must be carefully determined.

2.2. Detection limit

The major drawback of the NMR detection technique is the low sensitivity. The NMR signal-to-noise (S/N) ratio is dependent on the number of (equivalent) nuclei within the detection vol-

Fig. 5. ^{1}H NMR chromatogram (stacked plot, 400 MHz) of a separation of two plastifiers in acetonitrile.

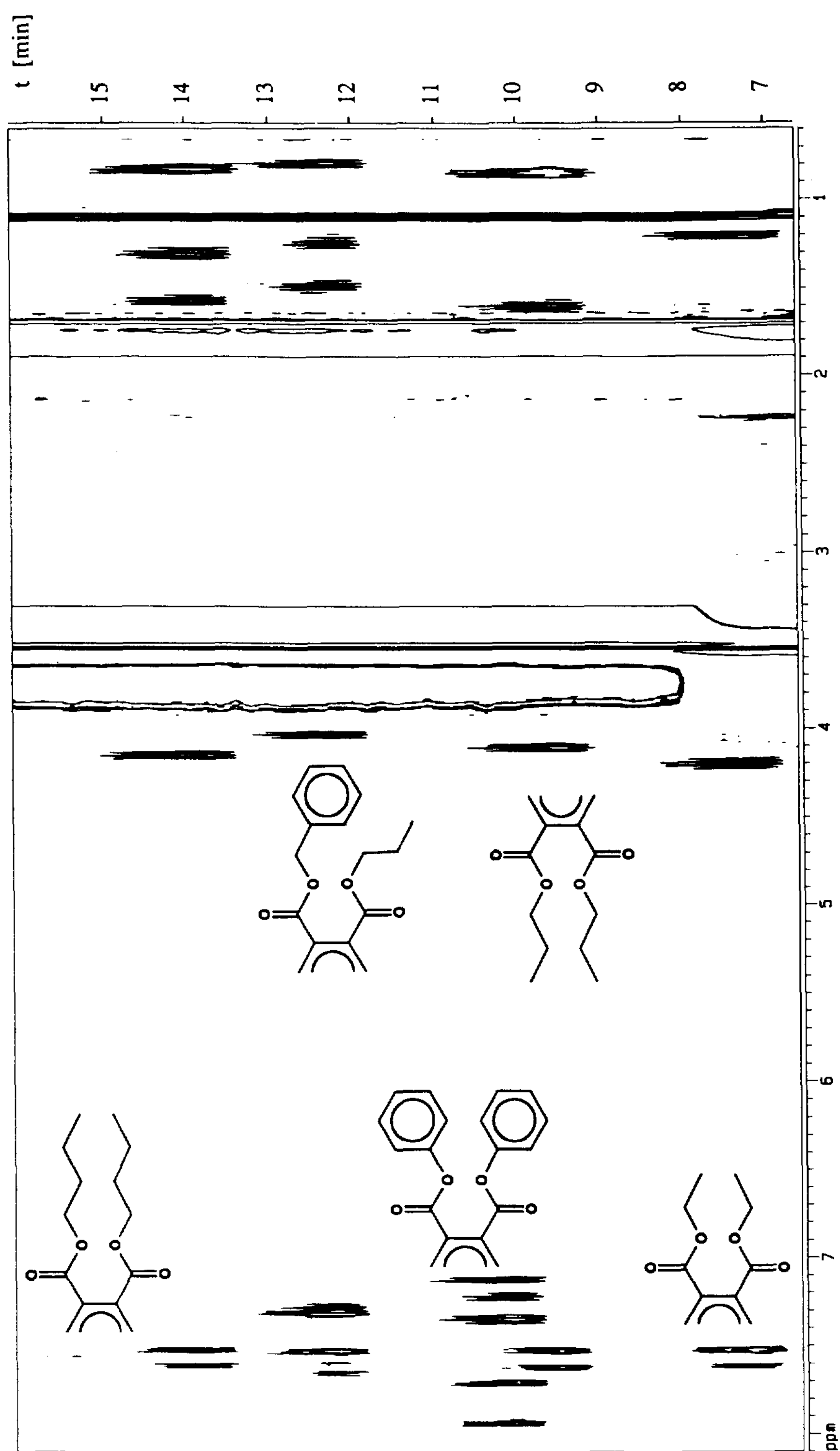

Fig. 6. ^{1}H NMR chromatogram (contour plot, 400 MHz) of a separation of five plastifiers in acetonitrile–water–D_2O (80:15:5).

ume, the magnetic field strength, the filling factor of the NMR detection coil and the spin–lattice relaxation times of the detected protons. Fig. 4 shows signal-to-noise values for different sample amounts of injected *n*-butyl benzylphthalate recorded in a 120-μl flow cell (600 MHz) after the passage through a 250 × 4.6 mm I.D. column. The *S/N* values were obtained by co-adding 8 scans per row and adding all rows together (4–6) which correspond to one chromatographic peak. Acetonitrile–D_2O (40:60) was used as eluent at a flow-rate of 0.3 ml/min. Assuming a signal-to-noise ratio of 3/1 to be sufficient for detection, the aromatic protons of *n*-butyl benzylphthalate with a spin–lattice relaxation time T_1 in the range of 4 s and their signal intensities split in a multiplet, show a higher detection limit compared to the benzyl CH_2 and butyl CH_2 protons with T_1 in the range of 2.5 s. The low detection limit of the benzyl CH_2 group of 500 ng is obtained because the two protons of this signal group show a singlet.

In the stopped-flow mode the detection limit is dependent upon the number of co-added trans-

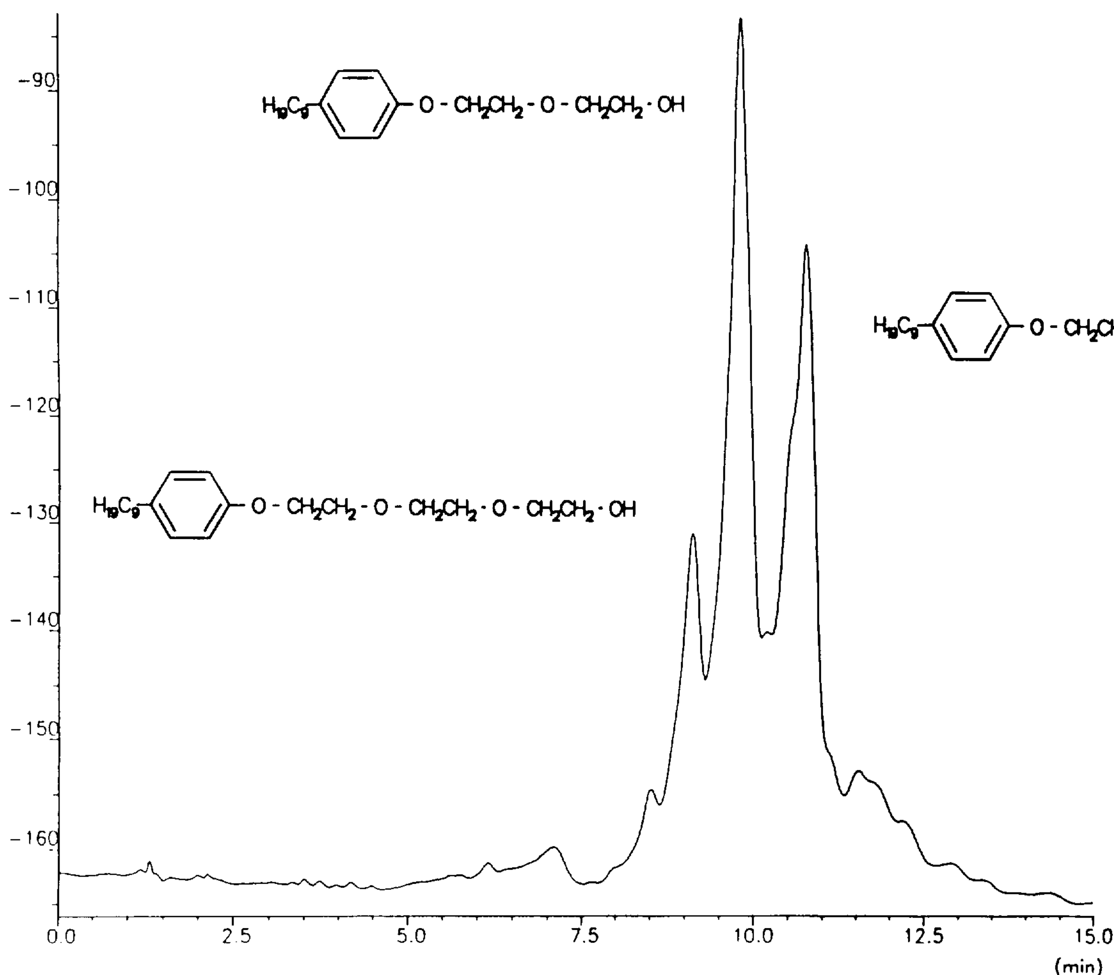

Fig. 7. UV chromatogram of a separation of surfactants in acetonitrile–water (65:35).

ients. Because an increase in S/N by a factor of two corresponds to a four-fold increase in measuring time, practical reasons imply a ten-fold smaller detection limit with respect to values obtained in the continuous-flow mode.

2.3. Continuous-flow measurements

2.3.1. Analysis of monomeric compounds

A "classical" example for the on-line NMR detection of a reversed-phase separation is the ^{1}H NMR chromatogram of two plastifiers recorded in acetonitrile (Fig. 5). In the stacked plot of Fig. 5 the proton chemical shift horizontal axis together with the vertical axis of the chromatographic retention times compose a two-dimensional frame which clearly enhances the possibilities for compound identification: Overlapping peaks in the chromatographic dimension can be separated due to the larger dispersion of the proton chemical shifts in the second dimension. A superior display of the ^{1}H NMR chromatogram is the contour plot (Fig. 6) with the same axes as the stacked plot but with the signal lines (contour) at a distinct signal height level.

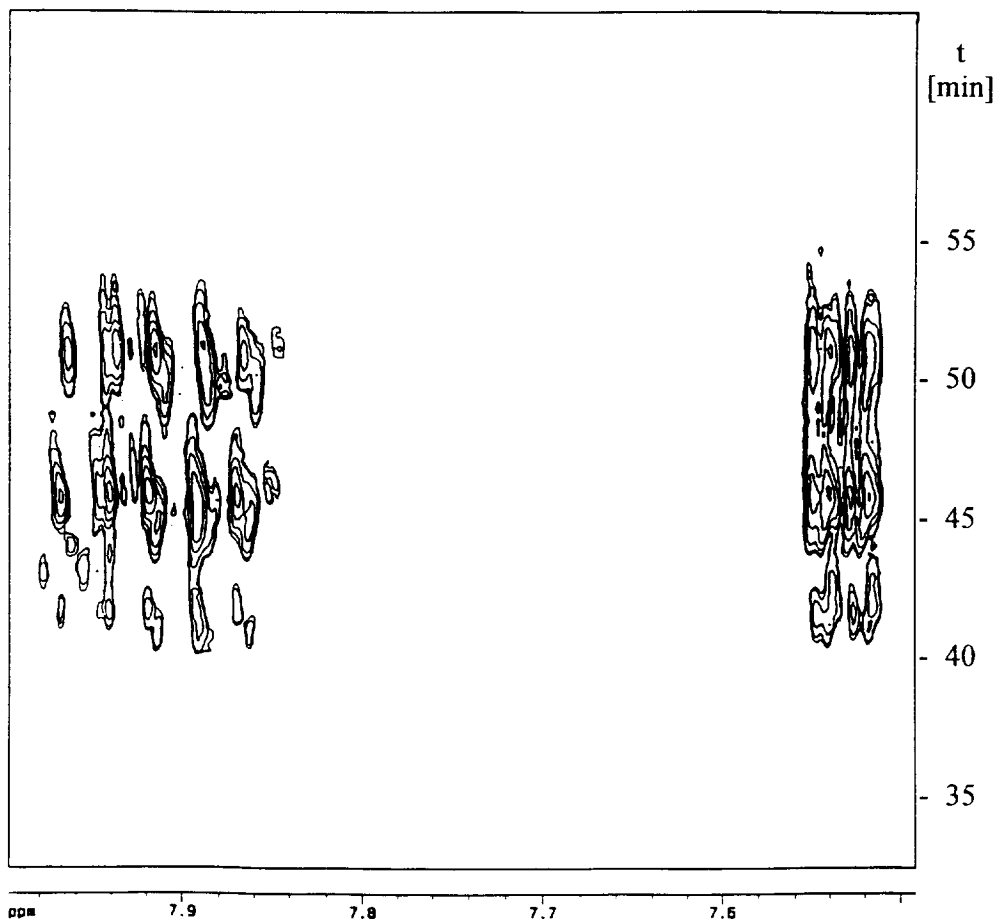

Fig. 8. ^{1}H NMR chromatogram (stacked plot of the aromatic region, 400 MHz) of the separation shown in Fig. 7.

Fig. 6 shows the separation of five plastifiers, recorded at a flow-rate of 0.5 ml/min in acetonitrile–water–D_2O (80:15:5) [20]. In contrast to the separation shown in Fig. 5 the amount of injected compound is reduced from 2–4 mg to 50 μg. Whereas the two compound separation has been performed without any deuterated solvent, practical experience in the lab has proved that field/frequency stabilisation is preferably throughout the whole chromatographic run to guarantee highest resolution. Field/frequency stabilisation can either be performed by substituting water by deuteriumoxide in case of reversed-phase separations or by the use of the already mentioned probe which contains a capillary with a deuterated solvent in the center of the detection cell.

Comparing the NMR spectra of Figs. 5 and 6 the ^{1}H NMR signals of the different plastifiers in the aliphatic, ester and aromatic region can be easily assigned to the corresponding structure. Despite the fact that the signals of residual HDO and acetonitrile are seen throughout the whole separation, the proton NMR signals of coeluting di-*n*-propyl and diphenylphthalate are clearly separated.

Fig. 7 shows the UV chromatogram (detected at 254 nm) of a reversed-phase separation (C_{18} column) of a surfactant mixture in acetonitrile–water (65:35) recorded at a flow-rate of 1.0 ml/min: The analogous ^{1}H NMR chromatogram of the aromatic region is shown in Fig. 8. Whereas in the UV chromatogram only three main peaks can be observed, the ^{1}H NMR

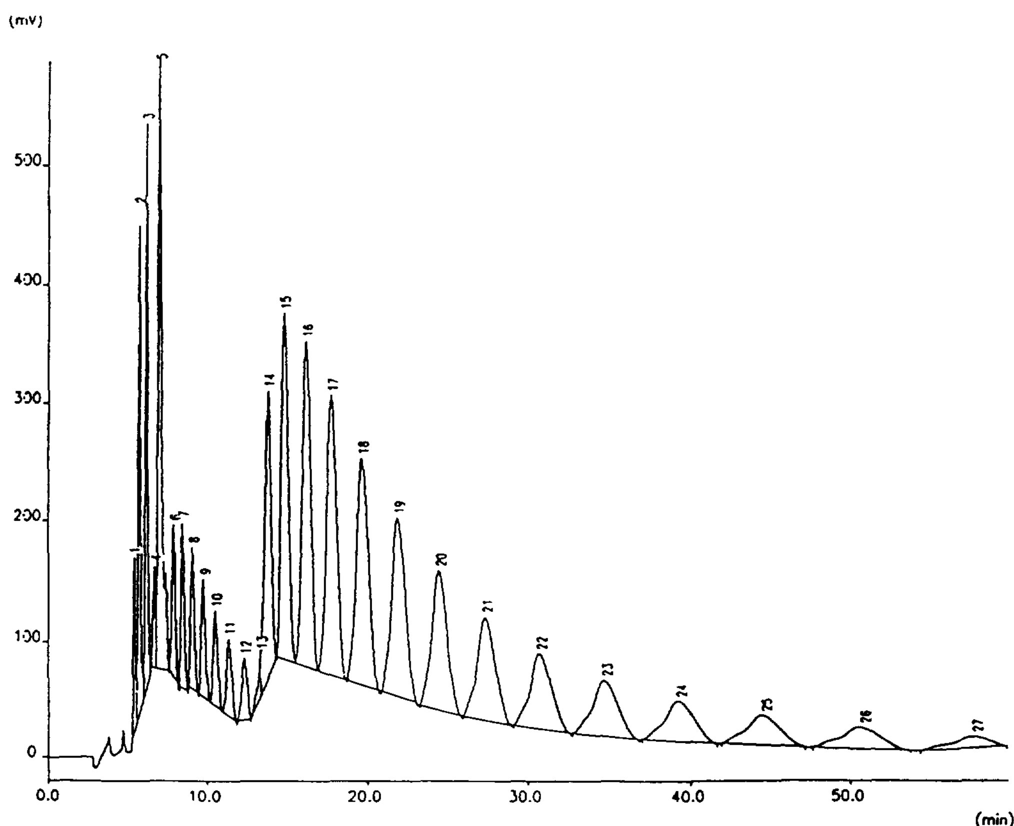

Fig. 9. UV chromatogram of a separation of a polyester mixture in methanol–D_2O (95:5).

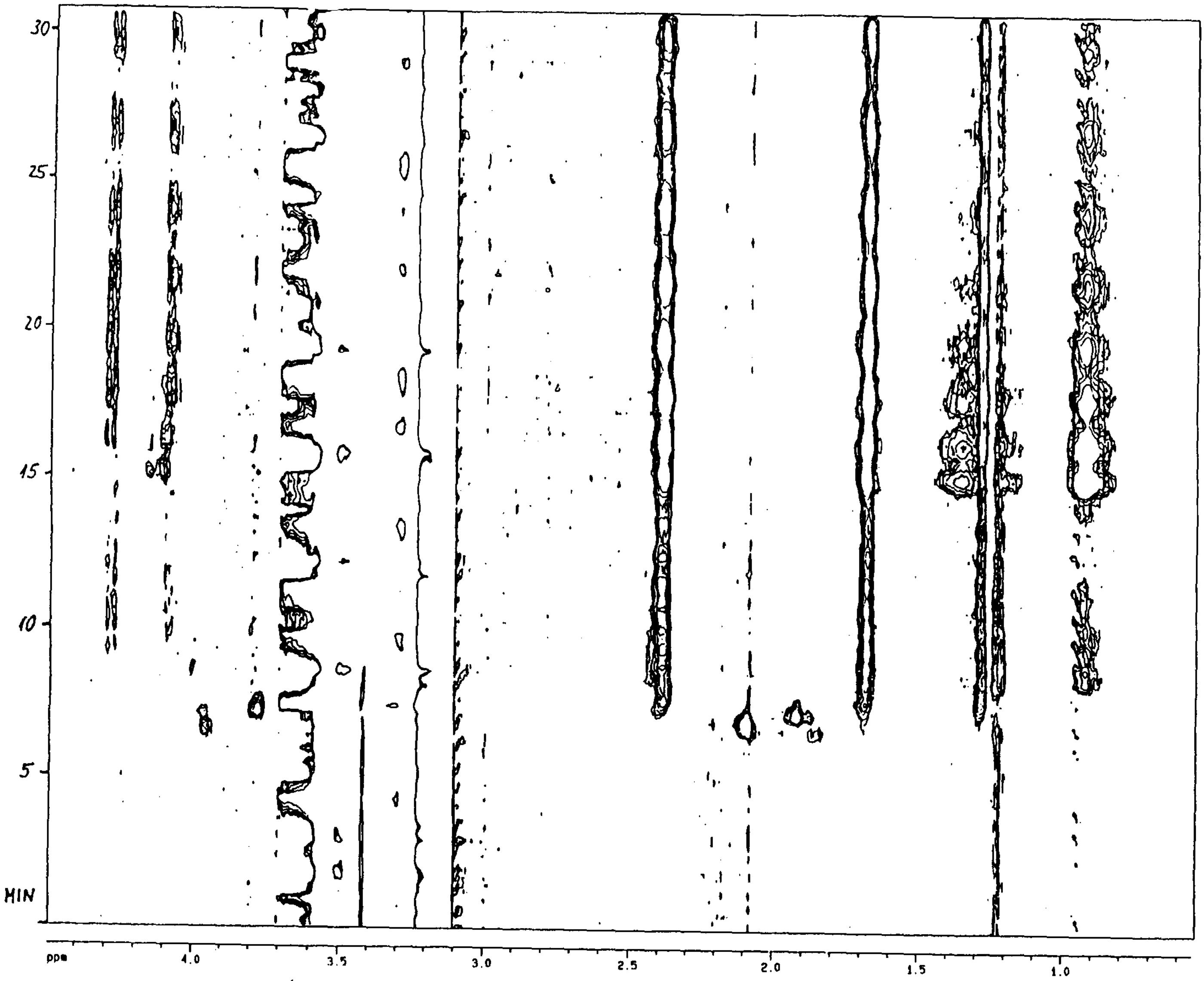

Fig. 10. ^{1}H NMR chromatogram (contour plot, 500 MHz) of the separation shown in Fig. 9.

chromatogram reveals that each of the three UV peaks consists at least of three different isomers which give the complex pattern between 7.8 and 8.0 ppm [12]. A different sterical arrangement of the aliphatic chain of nonylphenylethoxylate results in different chemical shifts of the aromatic protons in the ortho position to the aliphatic chain.

2.3.2. *Analysis of polymeric compounds*

Using direct HPLC–NMR and GPC–NMR coupling the determination of the chemical composition of polymers and blockcopolymers is possible. Investigations have been performed with epoxy resins [11] and with emulsion polymerized latices from styrene and *n*-butylacrylate.

Fig. 9 displays the UV chromatogram (detected at 210 nm) of a reversed-phase separation (phenyl column) of a polyester mixture (polypropylene–adipate endcapped with a variety of C_{10} alcohols) in methanol–D_2O (95:5) recorded at a flow-rate of 0.5 ml/min [8]. The corresponding HPLC–NMR separation of a 2 mg polyester mixture recorded at a 500 MHz instrument using a 3-mm inverse probe is shown in Fig. 10; 128 experiments have been performed with 16 transients each resulting in a time resolution of 21 s. Whereas in the UV chromatogram 27 peaks can be observed, the ^{1}H NMR chromatogram reveals a lot of structural information on the different reaction products. Besides the signals of the methyl group of polypropylene at 0.95 ppm and the methylene groups of adipic acid at 1.65 and 2.38 ppm additional signals between 1.1 and 1.4 ppm can be attributed to the methylene groups of the endcapped alcohols. By extracting the corresponding spectra from the pseudo-2D data matrix a detailed structure determination of the different reaction isomers is possible.

2.3.3. *Analysis of drugs and vitamins*

Coupled reversed-phase HPLC–NMR has been used for the detection and identification of the urinary metabolites of ibuprofen [13,14], flurbiprofen [15], antipyrine [16] and paracetamol [17]. It has been shown that spectra on impurities in drug compounds at around the 3% level can be determined in the continuous-flow and in the stopped-flow mode with 300 MHz and 400 MHz NMR spectrometers [7,18]. The potential of LC–NMR in pharmaceutical research can be demonstrated with the LC–NMR profile of an ibuprofen metabolite containing urine [13,14]. Ibuprofen (2-[4-isobutylphenyl]propionic acid) is a widely used anti-inflammatory drug in

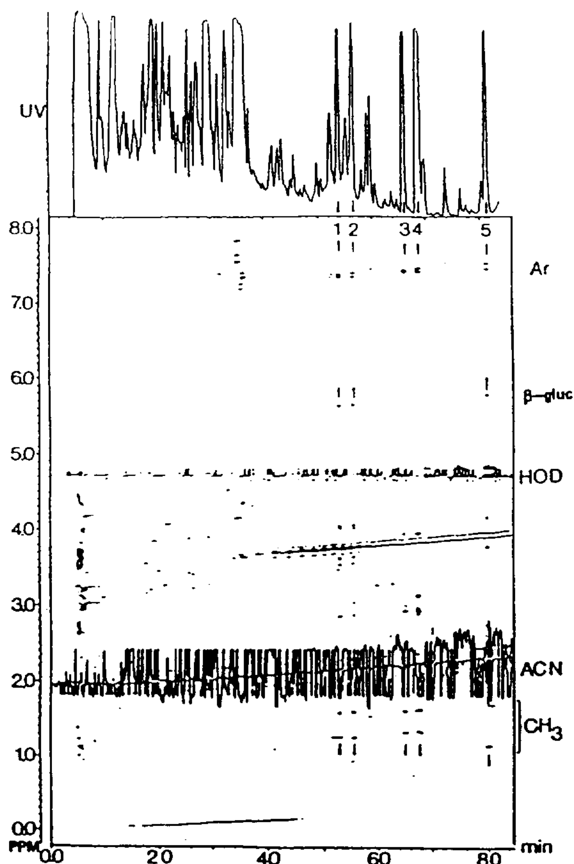

Fig. 11. ^{1}H NMR /UV chromatogram (contour plot, 500 MHz/210 nm) of the separation of ibuprofen metabolite containing human urine.

medical therapy. In vivo conjugation of the drug and its main metabolites 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (HMPPA), 2-[4-(2-carboxy-2-methylpropyl)phenyl]propionic acid (CMPPP) results in the formation of the corresponding glucuronides. Fig. 11 shows the contour plot of a HPLC–NMR separation of a freeze-dried human urine recorded with a 500 MHz instrument. The separation is performed with a linear gradient from 2% to 45% acetonitrile over 70 min. Continuous-flow spectra were obtained following a preliminary run required to obtain the correct presaturation frequencies. The ^{1}H NMR signals of HMPPA glucuronide (1), CMPPP glucuronide (2), HMPPA (3), CMPPP (4), and ibuprofen glucuronide (5) can clearly be differentiated from signals of endogenous compounds. High-quality 1D spectra were obtained by stopped-flow measurements. For instance, the structure of HMPPA glucuronide can easily be

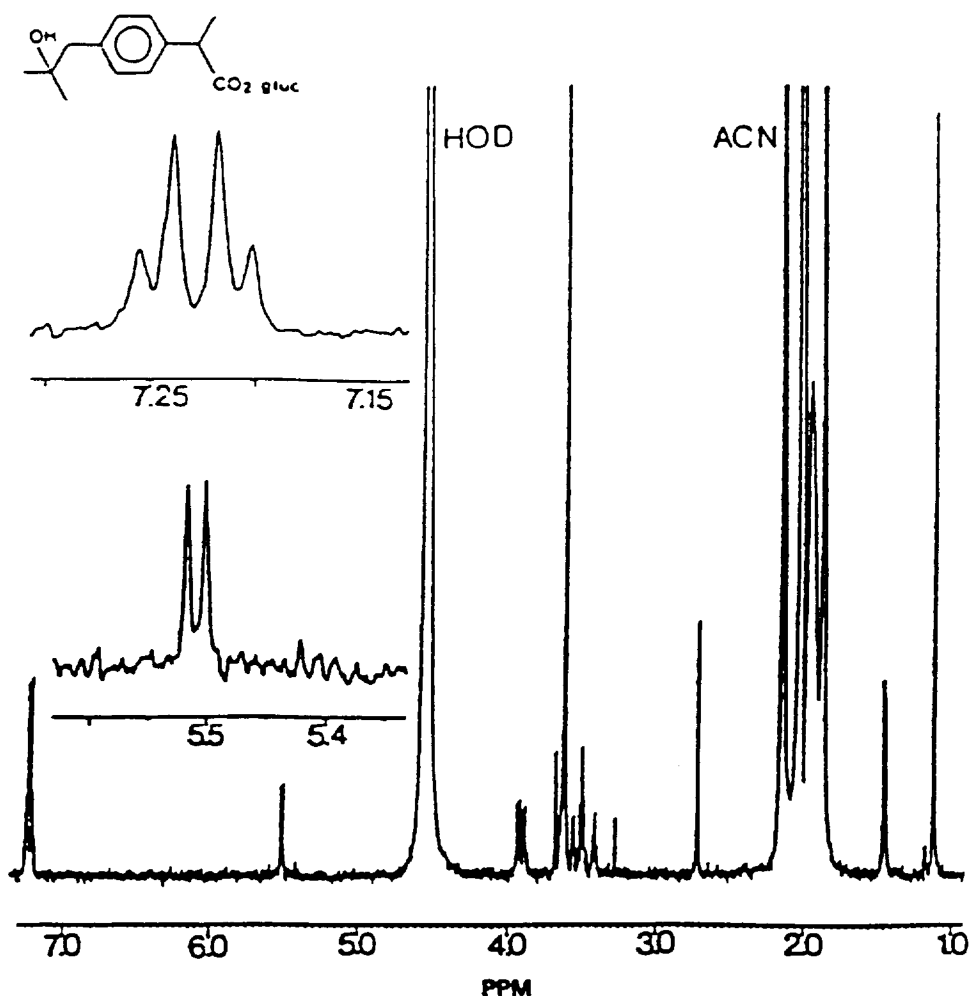

Fig. 12. Stopped-flow ^{1}H NMR spectrum (500 MHz) of 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid glucuronide in human urine.

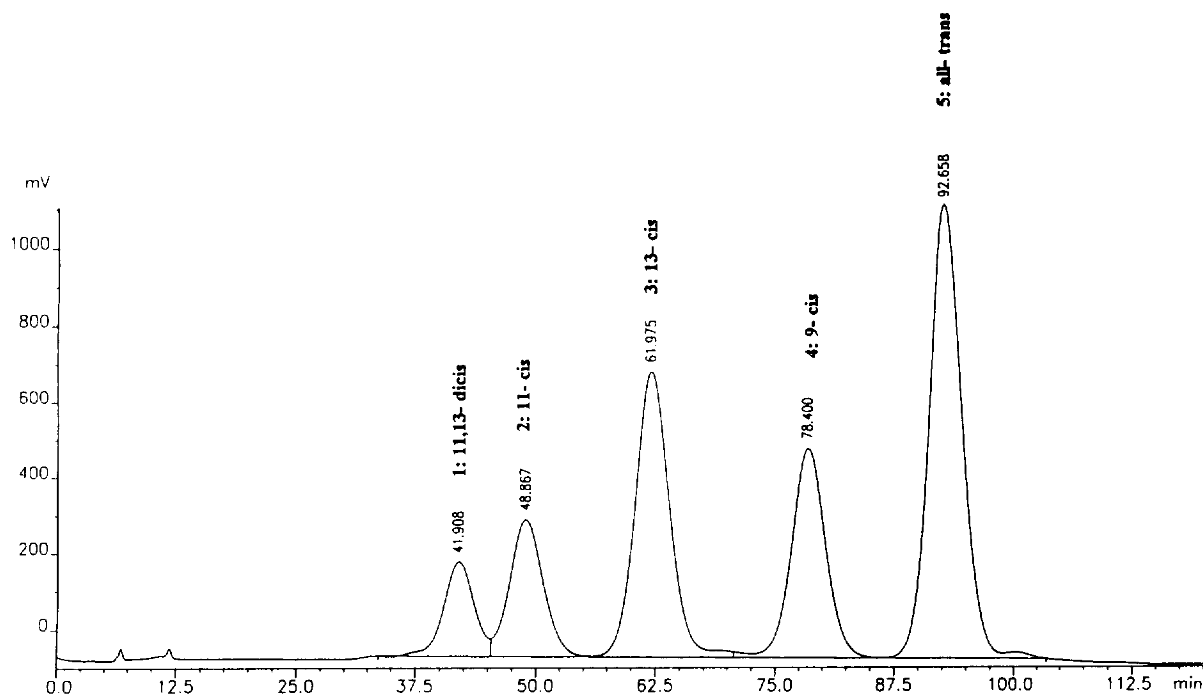

Fig. 13. UV chromatogram of a separation of vitamin A acetate isomers in *n*-heptane.

derived from the stopped-flow ^{1}H NMR spectrum displayed in Fig. 12.

Fig. 13 shows the UV chromatogram (325 nm) of a separation of 5 vitamin A acetate isomers on a cyanopropyl column in *n*-heptane recorded at a flow-rate of 0.2 ml/min [19]. Whereas peak 3 (13-*cis* vitamin A acetate), peak 4 (9-*cis* vitamin A acetate) and peak 5 (all-*trans* vitamin A acetate) are well separated, peak 1 (11,13-di-*cis* vitamin A acetate) and peak 2 (11-*cis* vitamin A acetate) coelute. In the HPLC–NMR on-line experiment, recorded at a flow-rate of 0.2 ml/min by co-adding 48 scans (time resolution 62 s), due to the different ^{1}H chemical shifts of the 11-13-di-*cis* and the 11-*cis* isomer, a clear separation of both compounds is possible (Fig. 14). This is substantiated in Fig. 15 showing the continuous-flow ^{1}H NMR spectra of the original vitamin A mixture and the separated isomers. Each row corresponds to a distinct retention time in which the peak maximum of the separated compound is passing through the flow cell. For instance, row 25 corresponds to the peak 3 with a retention time of 62 min. Whereas structure elucidation is impossible considering the ^{1}H NMR spectrum of the vitamin A mixture, the continuous-flow NMR spectra clearly yield the parameters chemical shift, coupling constant, and integration ratio for interpretation purposes. This is demonstrated in Fig. 16 displaying the assigned olefinic signal regions of all-*trans* and 11-*cis* vitamin A acetate. Fig. 16 further proves that the resolution of the continuous-flow ^{1}H NMR spectra is not degraded and is equal to the resolution of conventionally recorded NMR spectra.

By coupling a chromatographic separation technique with NMR spectroscopy the retention times are correlated to the proton chemical shifts (two-dimensional correlation) and an increased

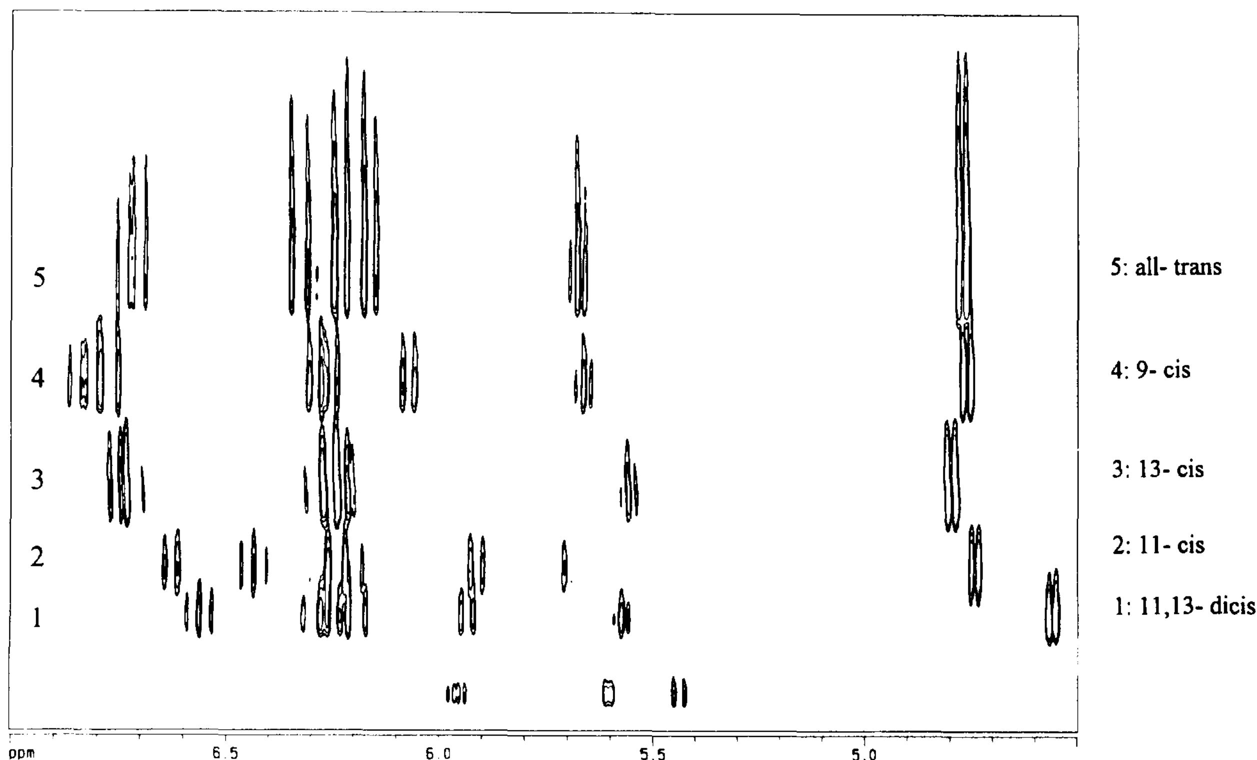

Fig. 14. ^{1}H NMR chromatogram (contour plot of the olefinic region, 400 MHz) of the separation shown in Fig. 13.

peak dispersion in the retention axis is more than compensated by the gain in spectroscopic information (proton chemical shifts, coupling constants) of nearly coeluting peaks.

2.4. Stopped-flow measurements

As outlined in the introduction, for structure elucidation of unknown compounds knowledge of the molecular mass as well as of the connectivities between different kinds of protons and carbons is necessary. Modern NMR spectroscopic techniques use elaborated two- and three-dimensional assignment techniques which are performed in an conventional probe without rotation of the NMR tube to avoid modulation problems. Because continuous-flow probes can also be used in the stopped-flow mode all two- and three-dimensional assignment techniques can be performed. Here solvent suppression has to be performed prior to the 2D experiment. Fig. 17 shows an example of a homonuclear shift correlated experiment (COSY 45) of the olefinic region of a vitamin A acetate reaction product recorded within a measuring time of 120 min.

Heteronuclear ^{1}H,^{13}C-shift correlated experiments can be performed with the already described inverse continuous-flow probes which contain an additional coaxial coil (matched to the ^{13}C resonance frequency). Because of the low natural abundance of 1.1% of the ^{13}C isotope the acquisition of ^{1}H{^{13}C} NMR spectra (Fig. 18) can only be performed in measuring times of 6–18 h. It is obvious that other peaks separated during this time would be unacceptably

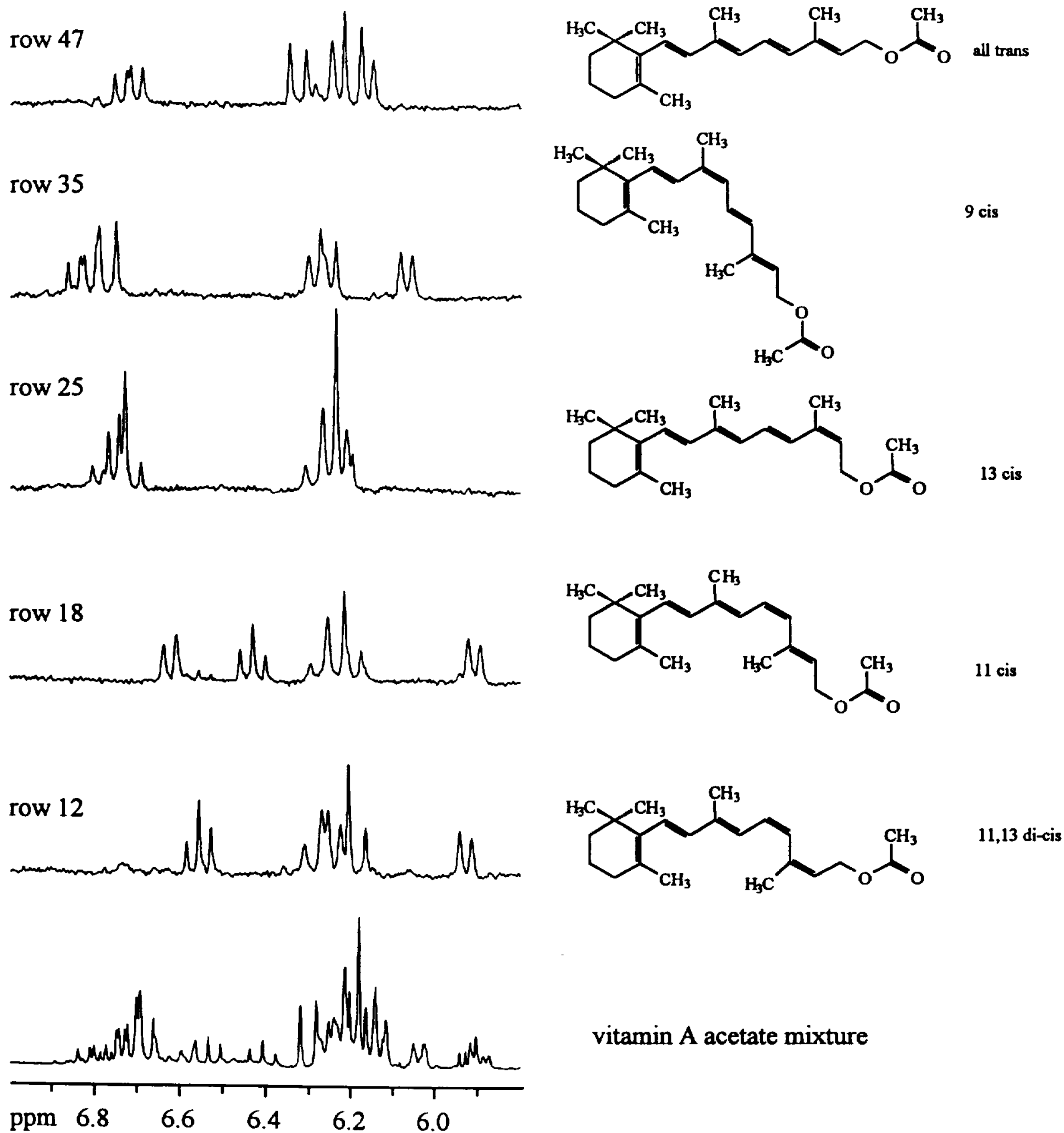

Fig. 15. Comparison of the olefinic region of the ^{1}H NMR spectra (400 MHz) of a reaction mixture of vitamin A acetate and the separated isomers.

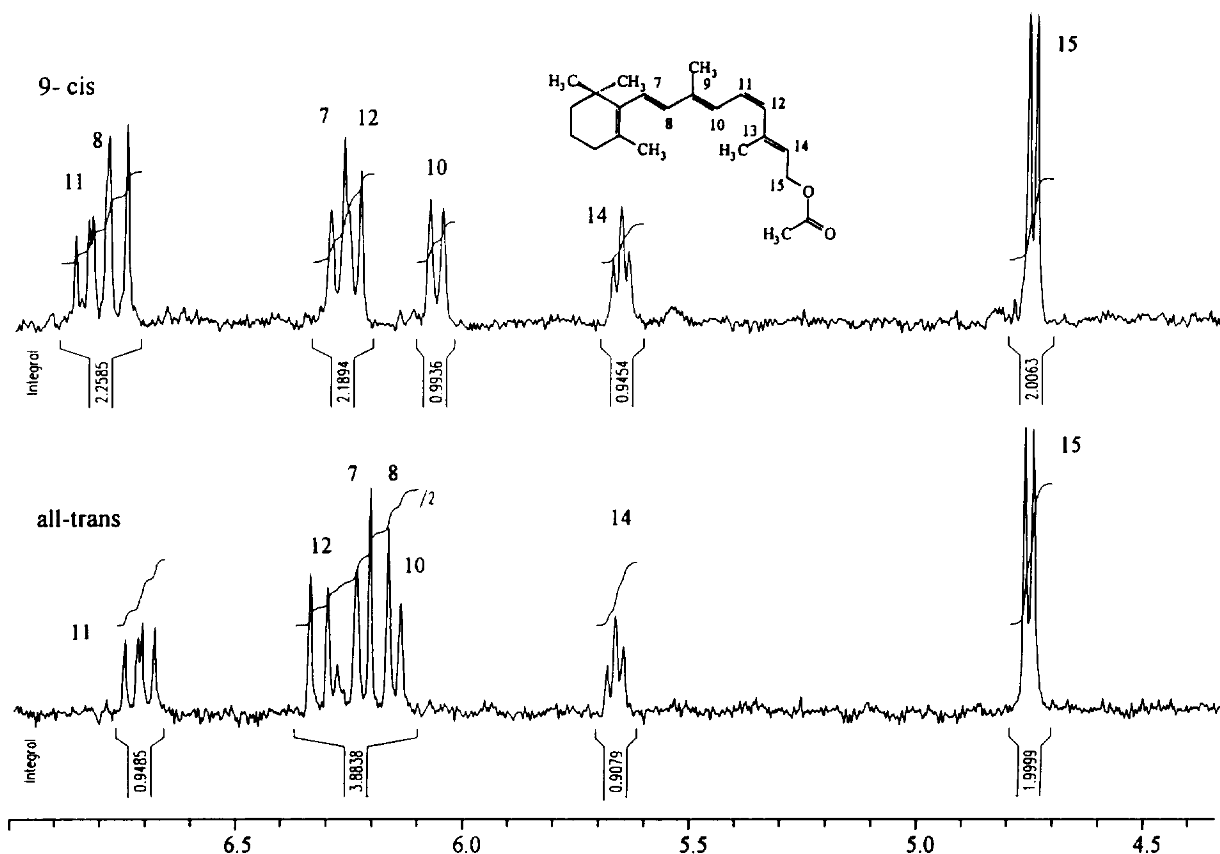

Fig. 16. Continuous-flow ^{1}H NMR spectra (400 MHz) of the olefinic region of two vitamin A acetate isomers (all-*trans* and 9-*cis* vitamin A acetate).

broadened due to self-diffusion. If these peaks also have to be subjected to NMR investigation, they could be stored in a sample loop.

3. SFC–NMR coupling

One of the major drawbacks of currently performed HPLC–NMR coupling experiments is the occurrence of solvent windows throughout a separation (compare Fig. 6). One way to solve this problem is the use of a separation technique which exhibits similar separation power as HPLC and uses non-protonated solvents. Therefore on-line coupling of supercritical fluid chromatography and NMR with supercritical CO_2 as eluent could be an alternative to HPLC–NMR coupling with protonated solvents. Because separations in supercritical CO_2 are only feasible at temperatures higher than 31.3°C and pressures higher than 72.9 bar a pressure-stable probe was developed. Fig. 19 shows the schematic diagram of a continuous-flow ^{1}H NMR probe suitable for supercritical state detection. The design is derived from continuous-flow probes used for liquid-state detection (Fig. 1). The inner glass tube of the HPLC–NMR probe is substituted with a

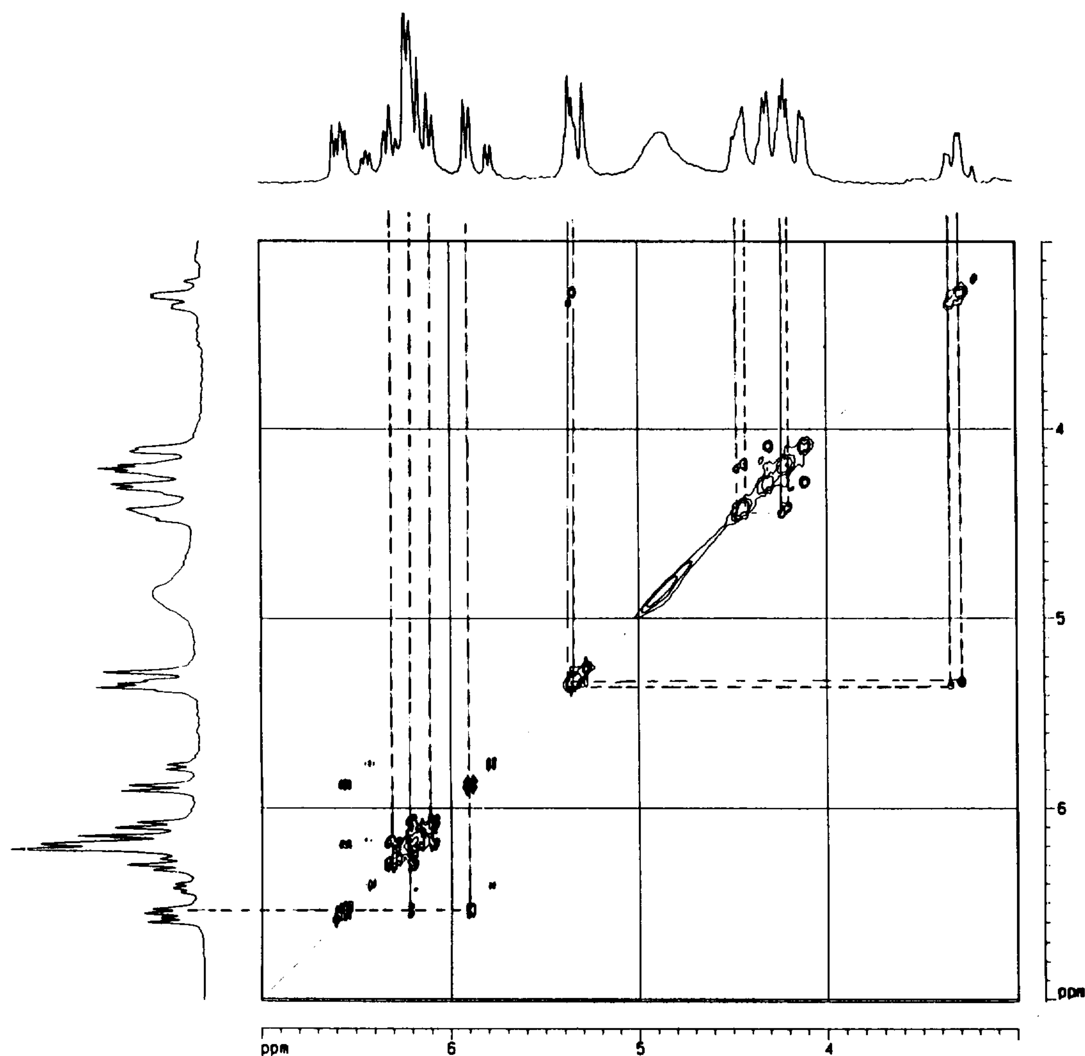

Fig. 17. Contour plot (600 MHz) of a ^{1}H homonuclear shift correlated experiment (COSY 45) of the olefinic region of a reaction product of vitamin A acetate.

sapphire tube (O.D. 5 mm, I.D. 3 mm, detector volume 120 μl) whereas the PEEK capillaries used in the HPLC–NMR probe are replaced by Titan tubings. To maintain supercritical conditions within the sapphire cell a backpressure regulator was connected to the outlet of the SFC probe and the flow cell was kept at a temperature of 321 K using preheated air. The stopped-flow ^{1}H NMR spectrum of ethylbenzene (Fig. 20) recorded in supercritical CO_2 at a temperature of 323 K and a pressure of 161 bar proves that no degradation in resolution and thus in the spin–spin relaxation time T_2 can be observed in the supercritical state. In contrast to the T_2 the spin–lattice relaxation times, T_1 are increased two to three times because of lower viscosity in

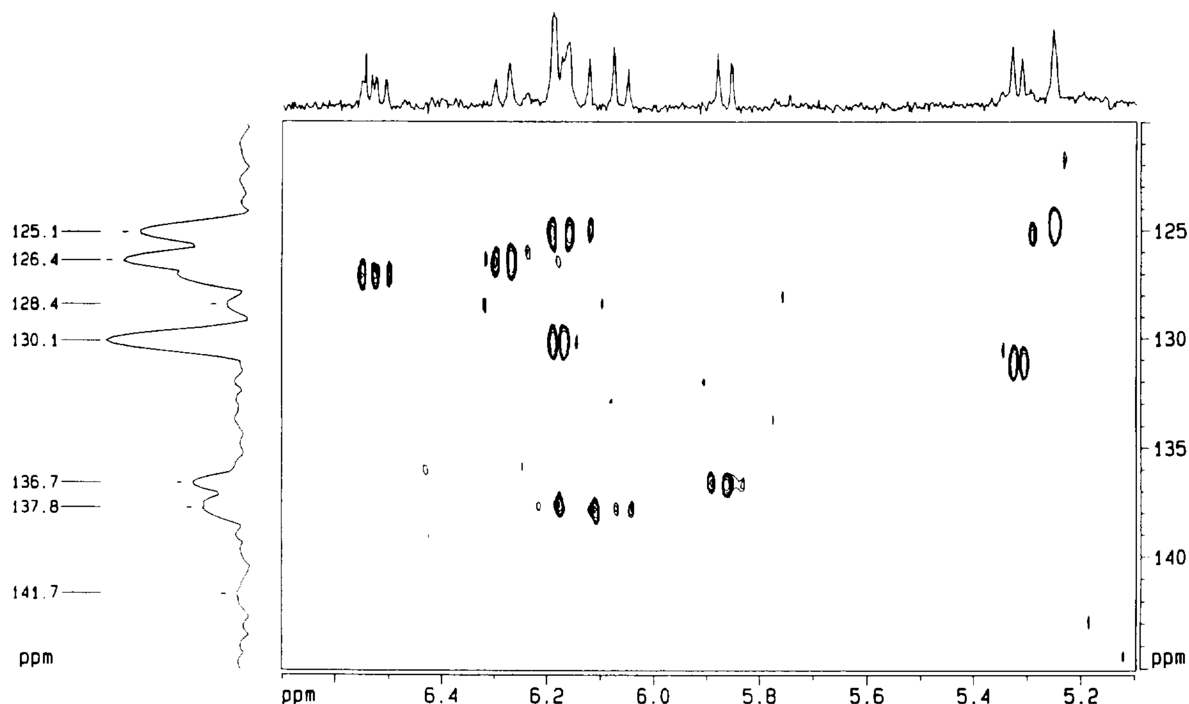

Fig. 18. Contour plot (600 MHz) of an inverse $^{1}H/^{13}C$ correlation of the olefinic region of a reation product of vitamin A acetate.

the supercritical versus the liquid state [20]. Another problem arises from the pressure dependence of ^{1}H chemical shifts. With increasing pressure the ^{1}H NMR signals shift upfield.

3.1. Continuous-flow measurements

Supercritical fluid separations are performed using pressure gradients and often by adding a polar modifier (e.g. methanol) to the supercritical eluent. At low pressures (< 100 bar) supercritical CO_2 has the solvating power of aliphatic hydrocarbons, at higher pressures (about 400 bar) its solvating power is similar to that of dichloromethane.

In an on-line SFC–NMR separation with a pressure gradient, normally up to 16 scans can be co-added without affecting the NMR resolution [20]. In an isobaric separation the number of acquisitions is dependent upon the concentration of the separated compound. Fig. 21 shows the separation of 4 acrylates in supercritical CO_2 with 1% of methanol-d_4 used as polar modifier. A 250 × 4.6 mm I.D. aminopropyl column was used with a flow-rate of 1.0 ml/min employing isobaric conditions (80 bar) for the first 7 min of the separation, a succeeding pressure ramp of 20 bar/min and final isobaric conditions at 100 bar. Throughout the separation, the temperature of the oven was kept constant at 60°C. Eight transients per row were co-added resulting in a time resolution of 6.4 s. The residual ^{1}H NMR signals of methanol at 0.8 and 3.45 ppm show the prescribed high field shift during the pressure ramp between retention times of 7–8 min.

At the left vertical axis of the contour plot a one-dimensional chromatogram is reconstructed by co-adding all appearing ^{1}H NMR signals. This NMR mass chromatogram corresponds to a one-

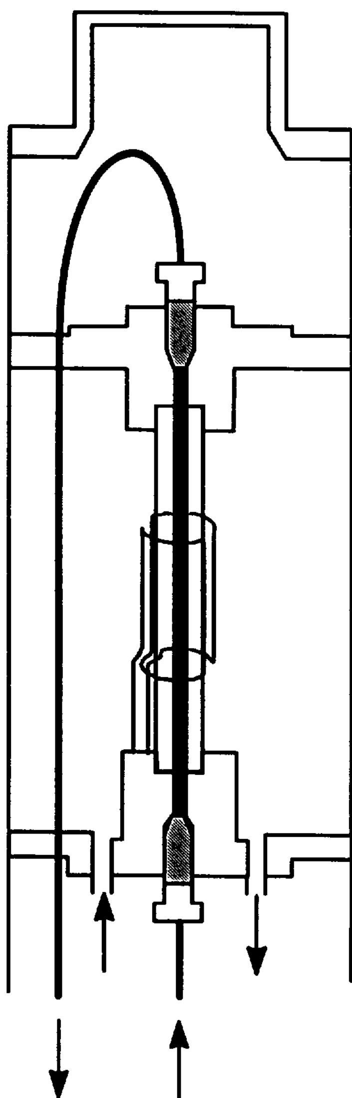

Fig. 19. Schematic diagram of an continuous-flow ^{1}H NMR probe suitable for supercritical state detection.

dimensional UV- or refractive index chromatogram. The second peak consists of methylmethacrylate and ethylacrylate which is evident from the different chemical shifts provided from the on-line experiment. A clear differentiation is available from the chemical shift of the methyl groups of both compounds. The chemical shift at 1.9 ppm is indicated for the methyl group at the double bond of methylmethacrylate (peak a) whereas the signal at 1.22 ppm is from the terminal methyl group of ethylacrylate (peak b). Thus once again the second dimension of the SFC–NMR run, provided by the ^{1}H chemical shifts, enables the separation of coeluting compounds.

The spectral quality of the continuous-flow spectra in the supercritical state obtained in an on-line separation is demonstrated in Fig. 22. With the exception that the terminal methyl group signal of *n*-butylacrylate is hidden under the signal of the modifier, the ^{1}H NMR spectrum clearly shows that all essential features for structure determination can be derived.

3.2. Stopped-flow measurements

The acquisition of 2D spectra is also possible in the supercritical state. With the present experimental set-up the pressure in the flow cell can be kept constant for several hours. Thus no resolution degradation due to pressure drops may occur. Fig. 23 displays the contour plot of a ^{1}H homo-nuclear shift correlated experiment of *n*-butyl benzylphthalate in supercritical CO_2. The one-dimensional ^{1}H NMR spectra of *n*-butyl benzylphthalate is displayed in both horizontal and vertical axes together with the diagonal from the lower left to the upper right. The connectivities between neighbouring proton groups are indicated by cross peaks in the contour plot. For example the ester OCH_2 protons at 4 ppm show a cross peak to the adjacent CH_2 protons of the butyl chain at 1.6 ppm, which are connected to the CH_2 protons at 1.3 ppm. These protons finally show a cross peak to the terminal methyl protons at 0.8 ppm.

4. SFE–NMR coupling

Extraction with supercritical CO_2 is a technical process with increasing importance. It provides a

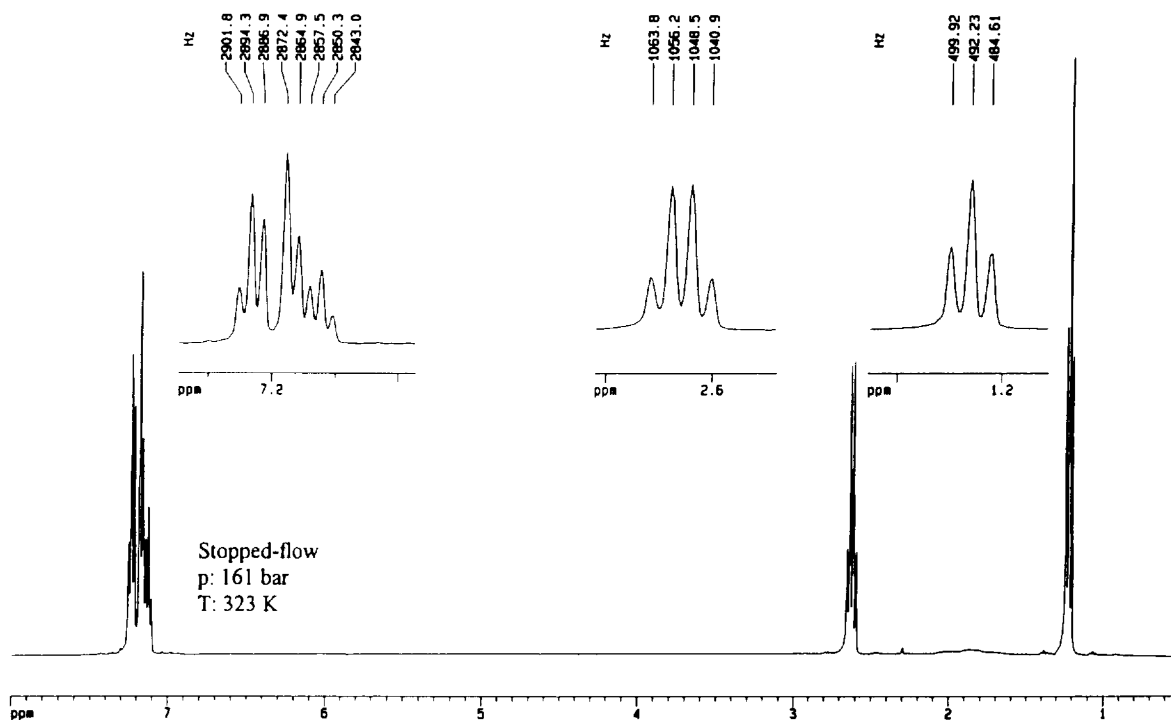

Fig. 20. ^{1}H NMR spectrum of ethylbenzene (400 MHz) in supercritical carbondioxide.

mild rapid technique of extraction of low or medium polar substances. As a lot of applications are performed in pharmaceutical, polymer, environmental and nutritional fields direct on-line SFE–NMR would be an ideal tool to monitor the extraction process. This has already been successfully performed for the extraction of caffeine and triglycerides from roasted coffee and several compounds including piperine from black pepper [23]. For the on-line SFE–NMR experiment the apparatus shown in Fig. 2 was modified. As "pump" served a Hewlett-Packard supercritical fluid chromatograph G1205A. Analytical HPLC columns (125 × 4.6 mm) were used as extraction cells. The continuous-flow NMR cell was connected between the column outlet and the back pressure regulator. The continuous-flow NMR spectrum of the extraction of black pepper is shown in Fig. 24. By comparing the obtained NMR data with those available from literature the structure of the extraction product piperine could be unequivocally assigned.

5. CE–NMR coupling

The NMR detection cell for CE–NMR was built by wrapping a radio-frequency microcoil directly around a 75 μm I.D. fused-silica capillary, creating a solenoid detection cell with a volume of 5 nl (24–26). With a 350 μm O.D. silica capillary, signal line widths lower than 7 Hz could be obtained. An on-line CE–NMR separation (300 MHz) was performed of arginine, glycine and cysteine at an injection level of 20 nl (0.7–0.8 *M* compound concentrations) with a time resolution of 16 s. Optimized cell geometry

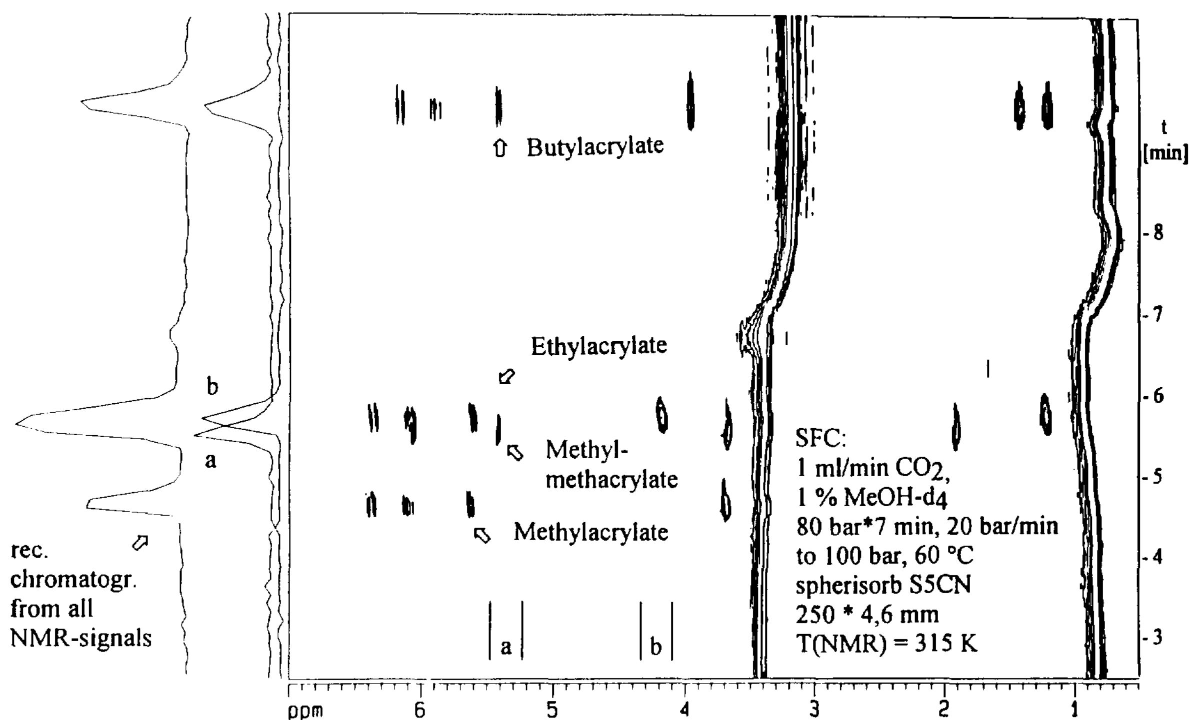

Fig. 21. ^1H NMR chromatogram (contour plot, 400 MHz) of a SFC separation of 4 acrylates in CO_2 with 1% methanol-d_4.

together with the use of exactly wound microcoils is expected to further minimize the observed signal line widths.

6. Conclusions

The obtained data clearly show that on-line HPLC–NMR coupling is able to solve real problems in different fields of pharmaceutical, polymer and biomedical research. Continuous-flow detection offers the advantage of using the ^1H NMR chemical shifts as second dimension whereas with stopped-flow detection 2D assignment techniques can be used. Whereas no loss of spectral quality is obtained in this hyphenated technique, a saving of analysis time of at least 40% is obtained with respect to off-line analysis. This can be easily concluded from the analysis of vitamin A acetates shown in Figs. 13–16. On-line HPLC–NMR analysis of the five isomers was performed within 2 h. Off-line HPLC separation (40 min) performed with a lower concentration to allow the collection of fractions of the separation, solvent evaporation and sample uptake in [^{2}H]chloroform (75 min) and NMR measurement of the five different samples (95 min) results in an over-all handling time of 3.5 h.

Further improvements in solvent suppression by the use of field gradient techniques as well as the improvement of the continuous-flow probe design will further lower the detection limit. A flow cell design using a solenoidal detection coil could be an alternative to the present flow cell design employed in LC–NMR, SFC–NMR and SFE–NMR if a resolution in the order of 0.5 Hz is obtained.

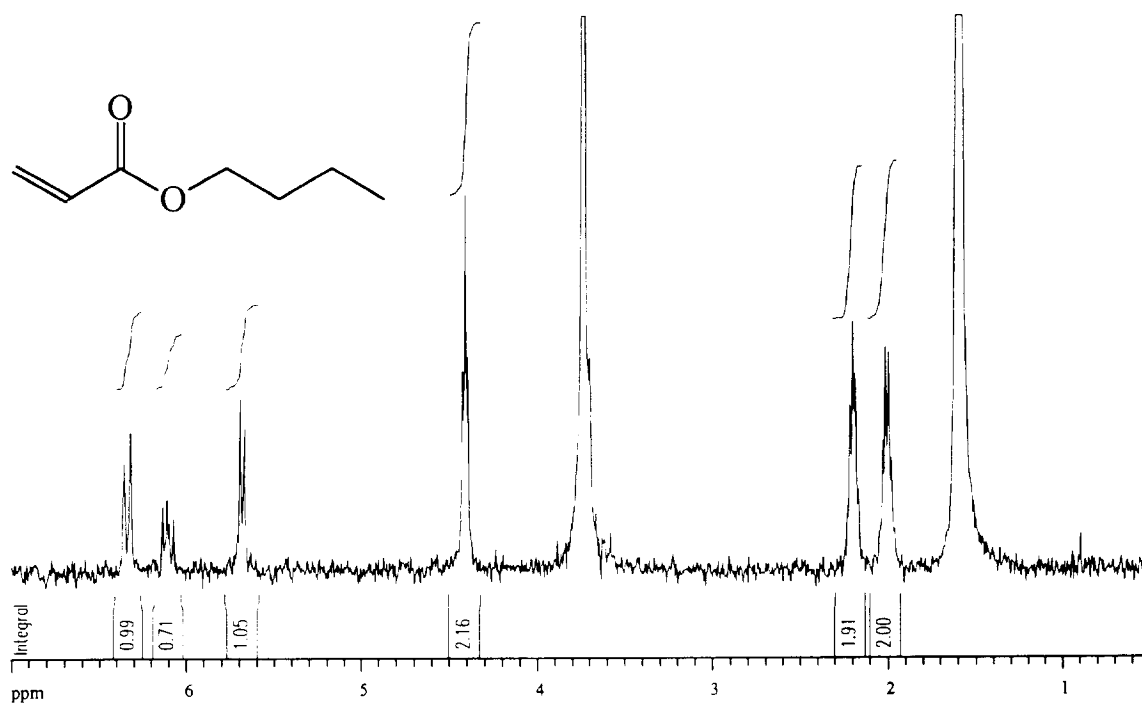

Fig. 22. Continuous-flow ^{1}H NMR spectrum (400 MHz) of *n*-butylacrylate in supercritical CO_2.

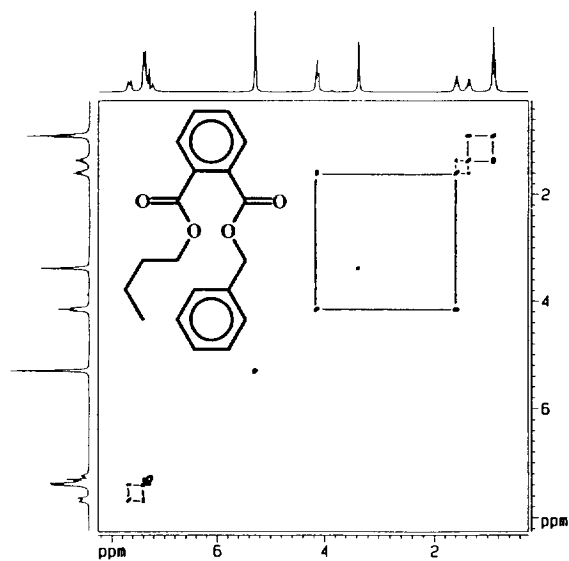

Fig. 23. Contour plot (400 MHz) of a ^{1}H homonuclear shift correlated experiment (COSY 45) of *n*-butyl benzylphthalate in supercritical CO_2.

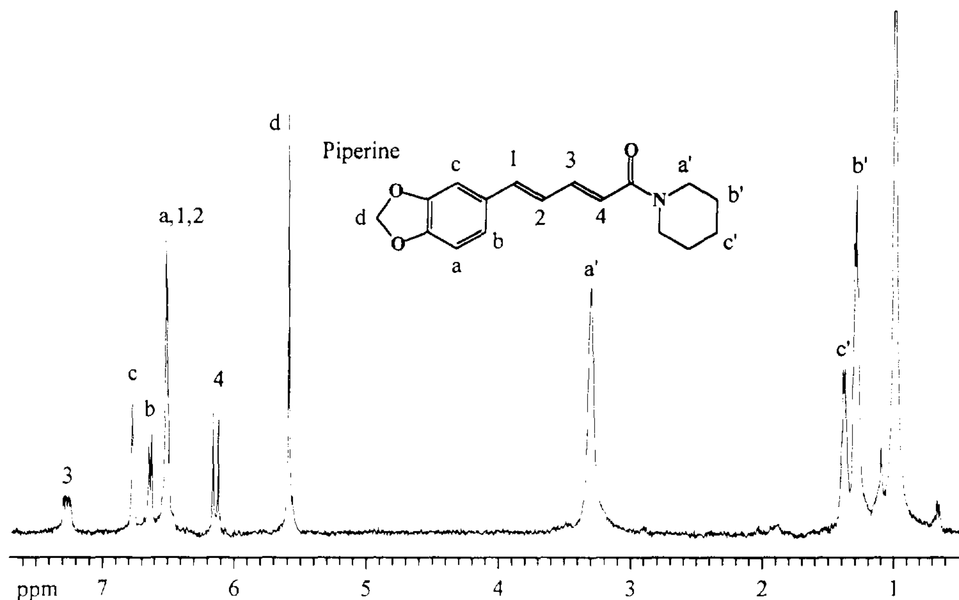

Fig. 24. Continuous-flow ^{1}H NMR spectrum (400 MHz) of piperine extracted from pepper with supercritical CO_2.

SFC–NMR may play an important role in pharmaceutical and polymer research whereas numerous applications can be foreseen for SFE–NMR and CE–NMR. With the further improvement of the sensitivity of the NMR detection cell [27] the monitoring of the extraction process by on-line NMR detection may become a routine diagnostic tool in the future.

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References

[1] E. Bayer, K. Albert, M. Nieder, E. Grom, G. Wolff and M. Rindlisbacher, *Anal. Chem.*, 54 (1982) 1747.

[2] H.C. Dorn, *Anal. Chem.*, 56 (1984) 747A.

[3] D.A. Laude Jr. and C.L. Wilkins, *Trends Anal. Chem.*, 5 (1986) 230.

[4] K. Albert, *Habilitationsschrift*, Tübingen University, 1988.

[5] K. Albert and E. Bayer, *Trends Anal. Chem.*, 7 (1988) 288.

[6] K. Albert, M. Kunst, E. Bayer, M. Spraul and W. Bermel, *J. Chromatogr.*, 463 (1989) 355.

[7] K. Albert, M. Kunst, E. Bayer, H.J. de Jong, P. Genissel, M. Spraul and W. Bermel, *Anal. Chem.*, 61 (1989) 772.

[8] M. Spraul, M. Hofmann, H. Glauner, J. Gans and K. Albert, *Bruker Report*, 2 (1990) 12.

[9] M. Spraul, P. Dvortsak, M. Hofmann and H. Glauner, *Bruker Report*, 139 (1993) 23.

[10] K. Albert and E. Bayer, in G. Patonay (Editor), *HPLC Detection Newer Methods*, VCH Publishers, New York, 1992, p. 197.

[11] M. Hofmann, M. Spraul, R. Streck, I.D. Wilson and A. Rapp, *Labor Praxis*, 10 (1993) 36.

[12] H. Händel, A. Bunn, U. Braumann, C. Dowle and K. Albert, in preparation.

[13] M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson and I.D. Wilson, *J. Pharm. Biomed. Anal.*, 10 (1992) 601.

[14] M. Spraul, H. Hofmann, P. Dvortsak, J.K. Nicholson and I.D. Wilson, *Anal. Chem.* 65 (1993) 327.

[15] M. Spraul, M. Hofmann, I.D. Wilson, E. Lenz, J.K. Nicholson and J.C. Lindon, *J. Pharm. Biomed. Anal.*, 11 (1993) 1009.

[16] I.D. Wilson, J.K. Nicholson, M. Hofmann, M. Spraul and J.C. Lindon, *J. Chromatogr.*, 617 (1993) 324.

[17] M. Spraul, M. Hofmann, J.C. Lindon, J.K. Nicholson and I.D. Wilson, *Anal. Proc.*, 30 (1993) 390.

[18] J.K. Roberts and R.J. Smith, *J. Chromatogr. A*, 677 (1994) 385.

[19] K. Albert, G. Schlotterbeck, U. Braumann, H. Händel, M. Spraul and G. Krack, *Angew. Chem.*, in press.

[20] K. Albert, U. Braumann, L.-H. Tseng, G. Nicholson, E. Bayer, M. Spraul, M. Hofmann, C. Dowle and M. Chippendale, *Anal. Chem.*, 66 (1994) 3042.

[21] K. Albert and U. Braumann, in *Frontiers in Analytical Spectroscopy*, in press.

[22] K. Albert, U. Braumann, R. Streck, M. Spraul and R. Ecker, *Fresenius J. Anal. Chem.*, in press.

[23] U. Braumann, H. Händel, K. Albert, R. Ecker and M. Spraul, *Anal. Chem.*, 67 (1995) 930.

[24] N. Wu, T.L. Peck, A.G. Webb, R.L. Magin and J.V. Sweedler, *J. Am. Chem. Soc.*, 116 (1994) 7929.

[25] N. Wu, T.L. Peck, A.G. Webb, R.L. Magin and J.V. Sweedler, *Anal. Chem.*, 22 (1994) 3849.

[26] J.V. Sweedler, Lecture at the Seventh International Symposium on High Performace Capillary Electrophoresis, 29 Jan.–2 Feb. 95, Würzburg, Germany.

[27] S. Stevenson and H.C. Dorn, *Anal. Chem.*, 66 (1994) 2993.