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REVIEW

From single to multiple microcoil flow probe NMR and related capillary techniques: a review

Ozan Gökay · Klaus Albert

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Abstract Nuclear magnetic resonance (NMR) spectroscopy is one of the most important and powerful instrumental analytical techniques for structural elucidation of unknown small and large (complex) isolated and synthesized compounds in organic and inorganic chemistry. X-ray crystallography, neutron scattering (neutron diffraction), and NMR spectroscopy are the only suitable methods for three-dimensional structure determination at atomic resolution. Moreover, these methods are complementary. However, by means of NMR spectroscopy, reaction dynamics and interaction processes can also be investigated. Unfortunately, this technique is very insensitive in comparison with other spectrometric (e.g., mass spectrometry) and spectroscopic (e.g., infrared spectroscopy) methods. Mainly through the development of stronger magnets and more sensitive solenoidal microcoil flow probes, this drawback has been successfully counteracted. Capillary NMR spectroscopy increases the mass-based sensitivity of the NMR spectroscopic analysis up to 100-fold compared with conventional 5-mm NMR probes, and thus can be coupled online and off-line with other microseparation and detection techniques. It offers not only higher sensitivity, but in many cases provides better quality spectra than traditional methods. Owing to the immense number of compounds (e.g., of natural product extracts and compound libraries) to be examined, single microcoil flow probe NMR spectroscopy will soon be far from being sufficiently effective as a screening method. For this reason, an inevitable trend towards coupled microseparation–multiple microcoil flow probe NMR techniques, which allow simultaneous online and off-line detection of several compounds, will occur. In this review we describe the current status and possible future developments of single and multiple microcoil capillary flow probe NMR spectroscopy and its application as a high-throughput tool for the analysis of a large number of mass-limited samples. The advantages and drawbacks of different coupled microseparation–capillary NMR spectroscopy techniques, such as capillary high-performance liquid chromatography–NMR spectroscopy, capillary electrophoresis–NMR spectroscopy, and capillary gas chromatography–NMR spectroscopy, are discussed and demonstrated by specific applications. Another subject of discussion is the progress in parallel NMR detection techniques. Furthermore, the applicability and mixing capability of tiny reactor systems, termed "microreactors" or "micromixers," implemented in NMR probes is demonstrated by carbamate- and imine-forming reactions.

Keywords Microcoil NMR · Capillary techniques · Multiple microprobe · Solenoidal microcoil · Parallel detection · Microseparation · Microreactor

Introduction

In recent decades, many attempts have been made to enhance the sensitivity of nuclear magnetic resonance (NMR) spectroscopy. The use of strong magnetic fields [1], polarization transfer techniques [2], cryogenic probes [3, 4], and advanced data processing contributed significantly to the improvement of the signal-to-noise ratio (SNR) in NMR spectroscopy. Presently, NMR spectroscopy of small sample volumes is performed mainly by means of small solenoidal-type microcoils wrapped around a capil-

Dedicated to Prof. Klaus Albert on the occasion of his 65th birthday

O. Gökay (✉) · K. Albert
Institute of Organic Chemistry, University of Tübingen,
Auf der Morgenstelle 18,
72076 Tübingen, Germany
e-mail: ozan.goekay@uni-tuebingen.de

lary [5], or planar coils on glass chips containing microfluidic channels [6–8]. The fact that the nearby copper windings of the radio-frequency (RF) coil induce static field distortions that limit the resolution and the SNR required the development of other alternative micro-NMR detection systems. Therefore, RF stripline-based microfluidic microchip NMR spectroscopy was developed, which according to various studies provides (owing to its simpler copper geometry) a better SNR [8, 9]. However, this article reports mainly on the currently more widespread microcoil probe and its most commonly utilized coupled systems. The main reason for the development of NMR microcoil probes is a physical law that states that for a given mass of an analyte, a reduction of the diameter of the receiving coil results in an increase of the SNR [10–14]. Since a reduction of the coil diameter unavoidably leads to a reduction of the sample volume, the use of NMR microcoil probes is only advantageous if the mass-limited sample is fully soluble in small solvent volumes. Therefore, microcoil capillaries offer no advantages in the measurement of poorly soluble (low-concentration) samples because the low sample concentration and the low sample capacity of the detection capillary lead to a strong decrease of the SNR. In such a case, conventional 5-mm probes provide better results [12]. The SNR can be expressed mathematically as follows:

$$SNR \propto \frac{k_0 \frac{B_1}{i} VsN\gamma \frac{\hbar^2}{4\pi^2} I(I+1) \frac{{\omega_0}^2}{kT3\sqrt{2}}}{\sqrt{4kT\,\Delta f\left(R_{Coil}+R_{Sample}\right)}} \quad (1)$$

where k_0 is a constant which accounts for spatial inhomogeneities in the $\boldsymbol{B}_1$ field produced by the probe, V_s is the sample volume, N is the number of nuclei within the detection volume, ω_0 is the Larmor frequency, γ is the gyromagnetic ratio of the nucleus, T is the temperature, k is the Boltzmann constant, Δf is the measurement bandwidth, and R_{Coil} and R_{Sample} are the coil and sample resistances, respectively [6]. The factor $\boldsymbol{B}_1/i$, the magnetic field per unit current, is defined as the coil sensitivity. Hoult and Richards [11] demonstrated that the value of $\boldsymbol{B}_1/i$ for both saddle and solenoid coil geometries is inversely proportional to the diameter of the coil.

The smaller the coil diameter, the smaller must be the distance between the sample and the coil. The evidently most mass-sensitive microcoil measuring cells have a volume of 5.0 μL and an active detection volume of only about 1.5-2.5 μL [14]. At both ends of the flow capillary there are fused-silica capillaries or fluorinated ethylene propylene tubing (75-100 μm inner diameter, ID) which serve as the inlet and the outlet. The sample can be injected through the inlet line into the cell either manually by using a microsyringe or automatically by employing an automated liquid handling system. Compared with measurements in NMR capillary tubes, the utilization of flow capillaries offers decisive advantages. By use of flow cells, slightly higher filling factors can be achieved. Through the use of very narrow capillaries, mixing within the inlet and outlet is almost entirely eliminated. Hence, problems such as peak diffusion, which are usually counteracted by the use of flow cells with larger volumes, are reduced [14]. Moreover, the inlet and outlet lines can be easily connected with other analytical systems, e.g., capillary high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), Fourier transform infrared (FTIR) spectroscopy, and capillary gas chromatography (GC) online mode applications. The compatibility of microprobe NMR spectroscopy with these analytical platforms enables its use in high-throughput analysis of compound libraries.

Especially in the past two decades, great advances in magnetic and RF probe technology were made. The RF coil diameter has been reduced significantly, resulting in a considerable increase in mass sensitivity of NMR detection [15]. Particularly microprobes, equipped with a solenoidal-type coil and an active detection volume of 1.5 μL, proved, with detection limits in the low nanomolar range, very sensitive, and thus greatly exceed the mass sensitivity of the conventional saddle-shaped Helmholtz NMR probes. Since then, these microcoil probes, usually applied with capillary separation techniques (mainly with capillary HPLC), have been used. In all online-coupled microseparation–NMR methods, the best compromise between chromatographic resolution and NMR sensitivity must be reached by choosing the most appropriate flow rate [16].

Short residence times of the nuclei in the measuring cell lead to short spin–lattice (T_1) and spin–spin (T_2) relaxation times and therefore cause line broadening of signals. A high flow rate, however, also has a SNR-enhancing effect, as a slow pulsing and too strong saturation effects can be circumvented by setting higher flow rates. Maximum sensitivity is achieved when the sample spends a certain residence time in the magnetic field before it enters the cell for nuclear spin polarization. The design and spatial dimensions of the flow cell influence the extent of dispersion effects, the quality of laminar flow, and the chromatographic separation quality. In recent years great progress has been made in the development of NMR flow cells [17, 18]. Continuous-flow probes have been manufactured in which a microcoil is directly attached to the cell [10]. By this arrangement, the filling factor ($FF = V_{\mathrm{Sample}}/V_{\mathrm{Coil}}$, where V_{Sample} is the sample volume and V_{Coil} is the volume of the NMR coil) was optimized [19], which led to improved sensitivity. Thanks to advanced sensors and modern shim systems, it is now possible to record high-resolution solenoidal microcoil NMR spectra.

Coupling of single microcoil flow probe NMR spectroscopy with microseparation techniques

Capillary HPLC–NMR spectroscopy

Mainly owing to its popularity and applicability, HPLC is the method of choice for a wide range of separation problems [20]. For this reason capillary HPLC is the most common separation technique which is coupled with capillary probe NMR spectroscopy [21–23]. The online coupling of HPLC systems to high-resolution NMR spectrometers offers a powerful tool for analyzing and characterizing unknown molecules and complex chemical mixtures without the need for time-consuming preparation procedures (e.g., fraction collection, evaporation of solvents). In principle, three types of liquid chromatography (LC)–NMR spectroscopy coupling are distinguished: the continuous-flow LC–NMR technique, the stopped-flow LC–NMR technique, and the highly effective LC–diode-array detector (DAD)–solid-phase extraction (SPE)–NMR technique. The LC-DAD-SPE-NMR technique [24] allows the use of the so-called postcolumn peak trapping, the selective enrichment of individual substance peaks, and thus a structural analysis of minor components in complex mixtures such as natural product extracts or samples obtained by stress tests with pharmaceutical drugs.

The drawback of the analytical-scale NMR technique is the low sensitivity when compared with other spectroscopic methods. In contrast to the traditional LC-NMR technique, in which relatively large amounts of expensive solvents are used, the capillary HPLC–microcoil NMR spectroscopy coupling is performed with small amounts of deuterated solvents. Conventional NMR probes and a special solenoidal microcoil capillary flow probe with enhanced mass sensitivity are illustrated in Fig. 1a. Under continuous-flow conditions, the NMR line width, signal intensity, and the effective longitudinal (T_1) and transverse (T_2) relaxation times can all be affected by the flow rate, and thus this can be altered to achieve better results. Equations 2, 3 and 4 describe the relation between the dwell time (τ), detection volume, flow rate, and longitudinal (T_1) and transverse (T_2) relaxation times in a flow probe (Fig. 1b):

$$\text{dwell time } \tau = \frac{\text{detection volume}}{\text{flow rate}} \tag{2}$$

$$\frac{1}{T_{1\,flow}} = \frac{1}{T_{1\,static}} + \frac{1}{\tau} \tag{3}$$

$$\frac{1}{T_{2\,flow}} = \frac{1}{T_{2\,static}} + \frac{1}{\tau} \tag{4}$$

For capillary HPLC-NMR spectroscopy coupling, a flow probe with an active detection volume of 1.5 μL proved particularly useful [20]. The measuring capillary, wrapped with a microcoil, is aligned horizontally to the external magnetic field and is located in a perfluorocarbon (FC-43) (Fluorinert) magnetic-field-based susceptibility-matching solution [19] (Fig. 2). This solenoidal measuring arrangement provides high sensitivity for the detection of small analyte amounts in the lower nanomole range [25, 26].

In the continuous-flow measuring method, the chromatographic separation is consecutively registered in characteristic time intervals, which are determined by the number of pulse repetitions. To achieve an acceptable SNR (at least 3), 1-2 μg (for analytical separation at least 100 μg) of the sample is necessary for capillary separations.

For microcoil flow probe NMR studies, deuterated solvents of high purity are required, as impurities would lead to the appearance of additional signals in the spectra.

Too many solvent signals increase the probability of superimposition of the analyte signals and thus make a comprehensive and reliable interpretation, based on the integrals, multiplicities and coupling patterns, impossible.

In the stopped-flow measurement method, the chromatographic separation is stopped when the maximum of the peak under investigation reaches the active detection volume of the flow probe.

The advantage of this method is that by increasing the acquisition time for a spectrum, one greatly increases the SNR. Thus, even minor components in a mixture can be investigated by NMR spectroscopy. Another great advantage of this measuring method is that it offers the possibility to record also 2D (correlation) NMR spectra.

In more than a few cases, complete structure elucidation can only be performed by means of 2D NMR spectroscopy. There are several instrumental possibilities for conducting stopped-flow measurements. The simplest method is to break off the solvent flow to the probe by a switching valve, placed after the separating column to avoid the occurrence of unnecessary dead volumes. However, care must be taken to ensure that there is no peak diffusion. Therefore, the outlet should also be closed at the same time using a switching valve. After the spectra have been recorded, the separation process can be continued for the investigation of the next peaks. To avoid peak diffusion, which can occur after long flow interruptions, the peaks to be examined can be stored temporarily in loops. Loops are capillaries which have the same volume as the probes. The separation of the analytes is performed before the NMR spectroscopic analysis. After the temporary storage of the peaks in the loops, they are transferred in sequence to the probe for detection [22].

Another possibility is to use a SPE–NMR unit (Fig. 3). In this method a SPE column is utilized to increase the concentration of analytes by multiple trapping. Afterwards

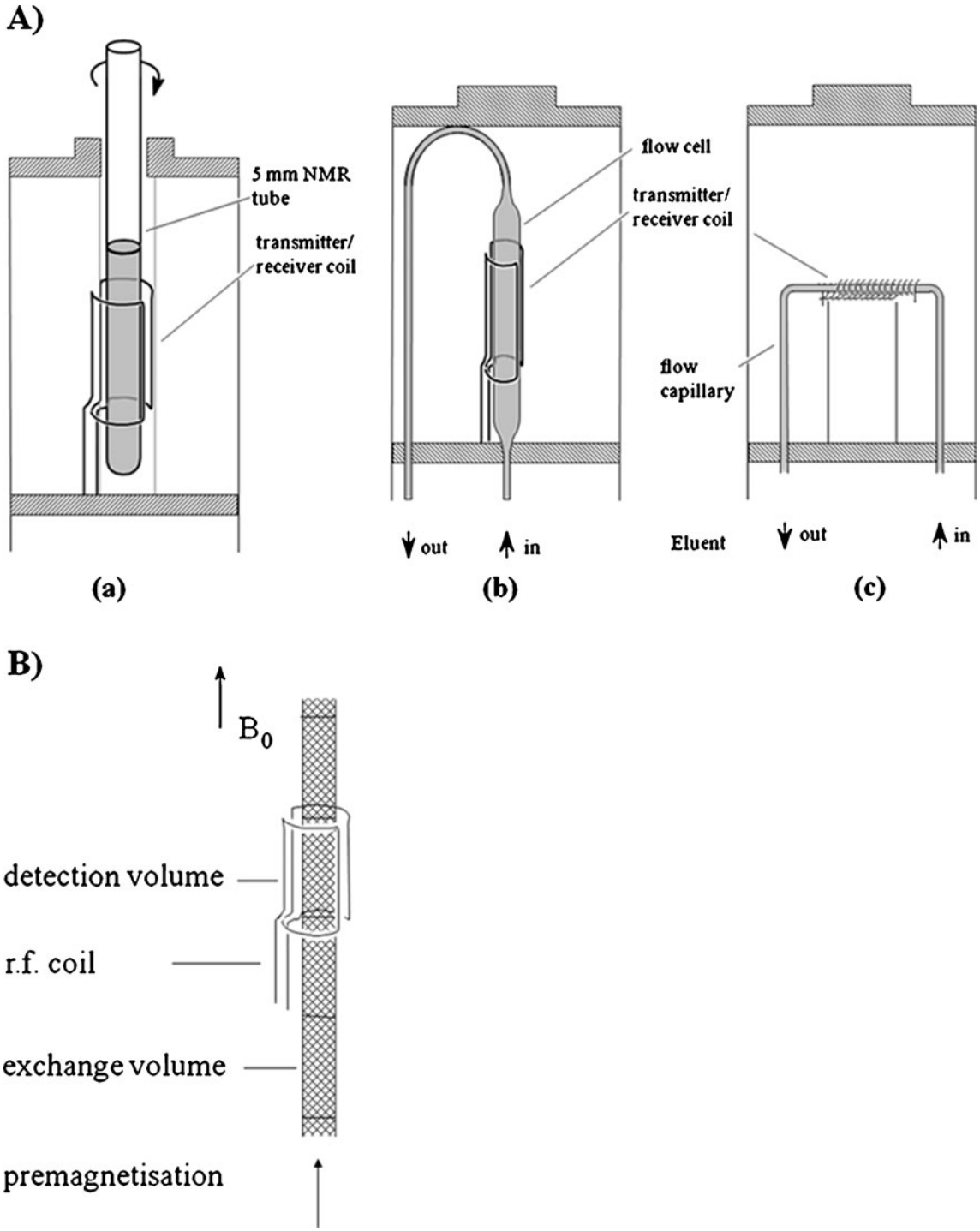

Fig. 1 **A** Commonly used 5-mm nuclear magnetic resonance (*NMR*) probe (*a*), conventional NMR flow probe (*b*), and capillary flow probe with solenoidal microcoil (*c*). **B** A flow probe in a magnetic field. *r.f.* radio frequency. (Adapted from [20])

the analytes can be eluted completely from the SPE precolumn and transferred into the detection cell [27–30]. The immense advantage of the capillary HPLC-NMR spectroscopy coupling is the minimum solvent consumption. Thus, for the online coupling fully deuterated solvents can be applied. By using deuterated solvents, one significantly reduces the intensity of the solvent signals. As a consequence, solvent signal suppression is often not necessary. For structure elucidation of unknown compounds ^{13}C NMR spectroscopy is routinely used in addition to ^{1}H NMR spectroscopy. An important 2D technique is the inverse ^{1}H-^{13}C correlation for the determination of direct correlations between adjacent protons and carbon atoms. Owing to the low natural

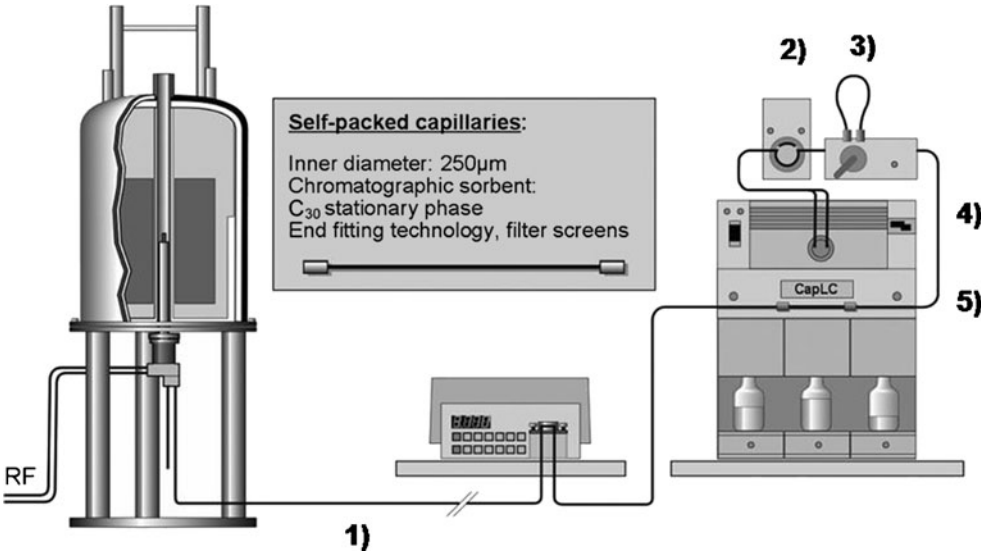

Fig. 2 Experimental setup for online capillary high-performance liquid chromatography (HPLC)–NMR spectroscopy. *1* transfer capillary (50-µm inner diameter), *2* NMR peak parking valve for stopped-flow measurements, *3* injection valve, *4* HPLC pump, *5* HPLC capillary column. (Adapted from [32])

abundance and low magnetogyric ratio of the ^{13}C isotope, a time of several hours is required to record the heteronuclear 2D NMR spectrum of an injected sample amount in the low microgram range in a detection cell with an active volume of 1.5 µL.

For small sample amounts, consisting of very soluble compounds, the capillary HPLC-NMR spectroscopy coupling with a detection volume of 1.5 µL is the method of choice [23]. Through advances in both chromatographic separation and NMR detection, substance amounts in the nanogram range can now be analyzed easily [5, 21].

An online-detected NMR separation of dansylated amino acids in a 315-µm-ID fused-silica capillary packed to a length of 12 cm with C_{18} stationary phase was demonstrated by Behnke et al. [31]. They obtained NMR spectra with a resolution on the order of 3 Hz, using a 50-nL detection cell

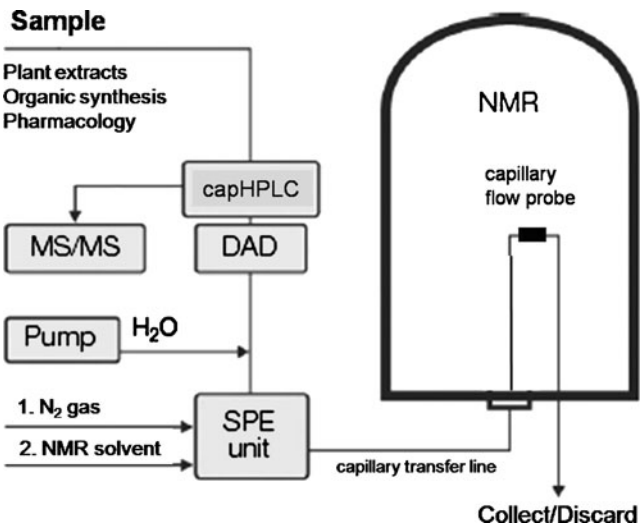

Fig. 3 Experimental setup for online capillary HPLC– solid-phase extraction (*SPE*)–NMR spectroscopy. *DAD* diode-array detector, *capHPLC* capillary HPLC system, *MS/MS* tandem mass spectrometer

to measure 1.1 nmol of dansylated γ-aminobutyric acid under static conditions in a 75-µm-ID capillary. After isolation from biological matrices by the matrix solid phase dispersion method, separation and structural identification of extremely air and UV sensitive bixin stereoisomers was also successfully performed by performing shape-selective C_{30} capillary HPLC–NMR spectroscopy in the stopped-flow mode [32]. Good-quality stopped-flow $^1H,^1H$ correlation spectroscopy (COSY) NMR spectra of 9′-(*Z*)-bixin (Fig. 4a) and all-(*E*)-bixin (Fig. 4b) were obtained.

In 2004, Krucker et al. [33] showed the considerable potential of capillary separations coupled with NMR spectroscopy for the investigation of restricted amounts of sample. They employed a 250-µm-ID fused-silica capillary, self-packed with C_{30} phases, for the separation of tocopherol homologues. Despite the small amount of the tocopherol homologues (vitamin E) available (1.33 µg of each tocopherol), they were able to monitor the capillary HPLC separation by ^1H NMR spectroscopy under continuous-flow conditions, which allowed an immediate peak identification (Fig. 5). Hentschel et al. [34] studied the regulatory phosphorylation sites in a tryptic fragment containing amino acids 485-496 (ALGADDSYYTAR). They synthesized, separated (Fig. 6a), and detected (Fig. 6b) four possible peptides with phosphorylation at none, one, or both of the Y-492 and Y-493 tyrosines by ^1H NMR spectroscopy at 600 MHz using a capillary HPLC-NMR microprobe. Thus, an unambiguous discrimination of the peptides via the effect of chemical shifts of phosphorylation on the aromatic tyrosine protons was possible. With the capillary probe and the detection volume of 1.5 µL, they were able to perform structure elucidation with the very small amounts available for the various peptides. For the syringe injection, they used 15 µL of the analyte. They recorded the capillary HPLC-NMR spectra in the stopped-flow mode from less than 400 ng of each peptide, using 1D and 2D techniques [$^1H,^1H$-

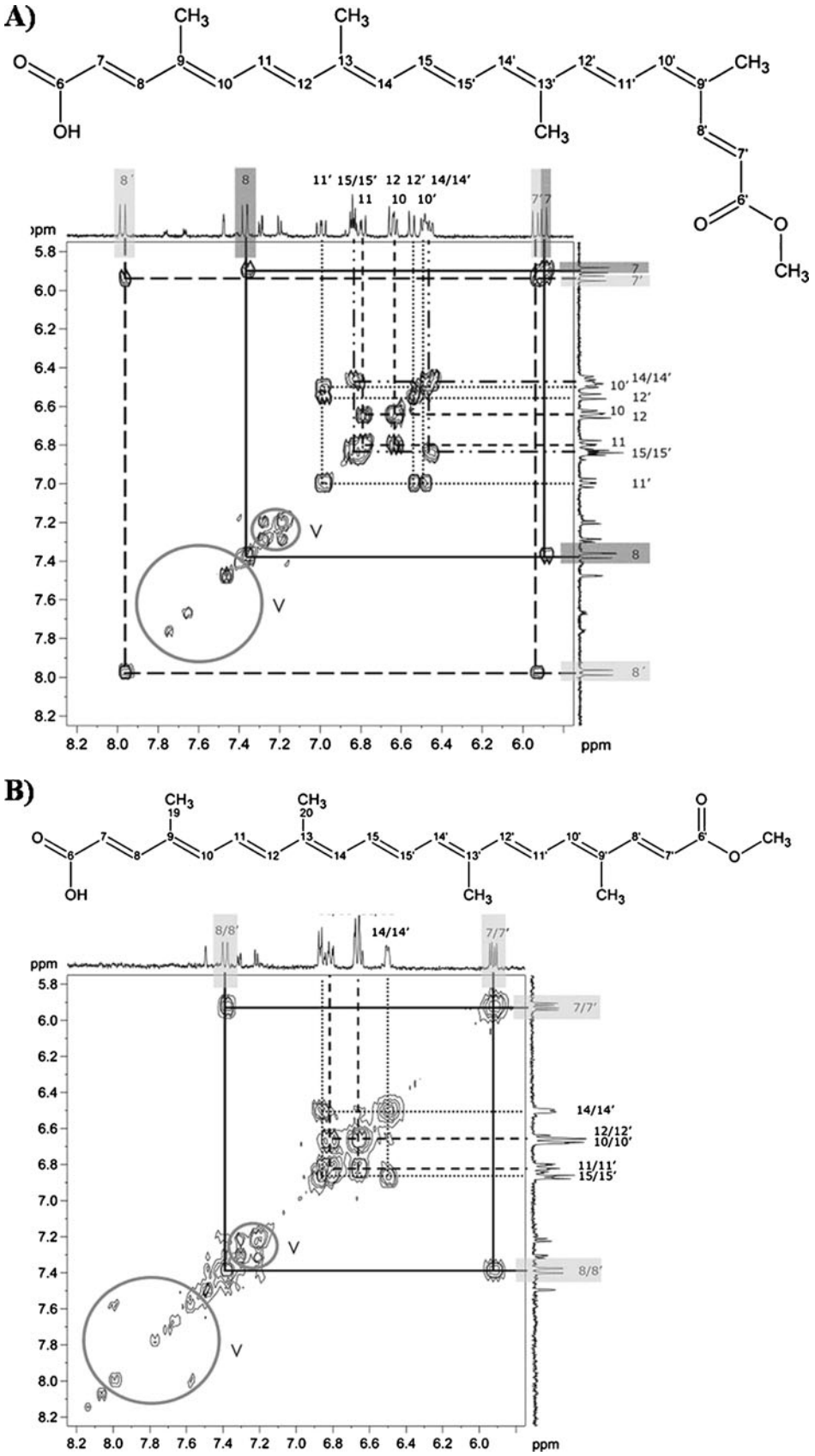

Fig. 4 **A** Stopped-flow $^{1}H,^{1}H$ correlation spectroscopy (COSY) NMR spectrum (600 MHz) of 9′-(*Z*)-bixin. **B** Stopped-flow $^{1}H,^{1}H$-COSY NMR spectrum (600 MHz) of all-(*E*)-bixin. (Adapted from [32])

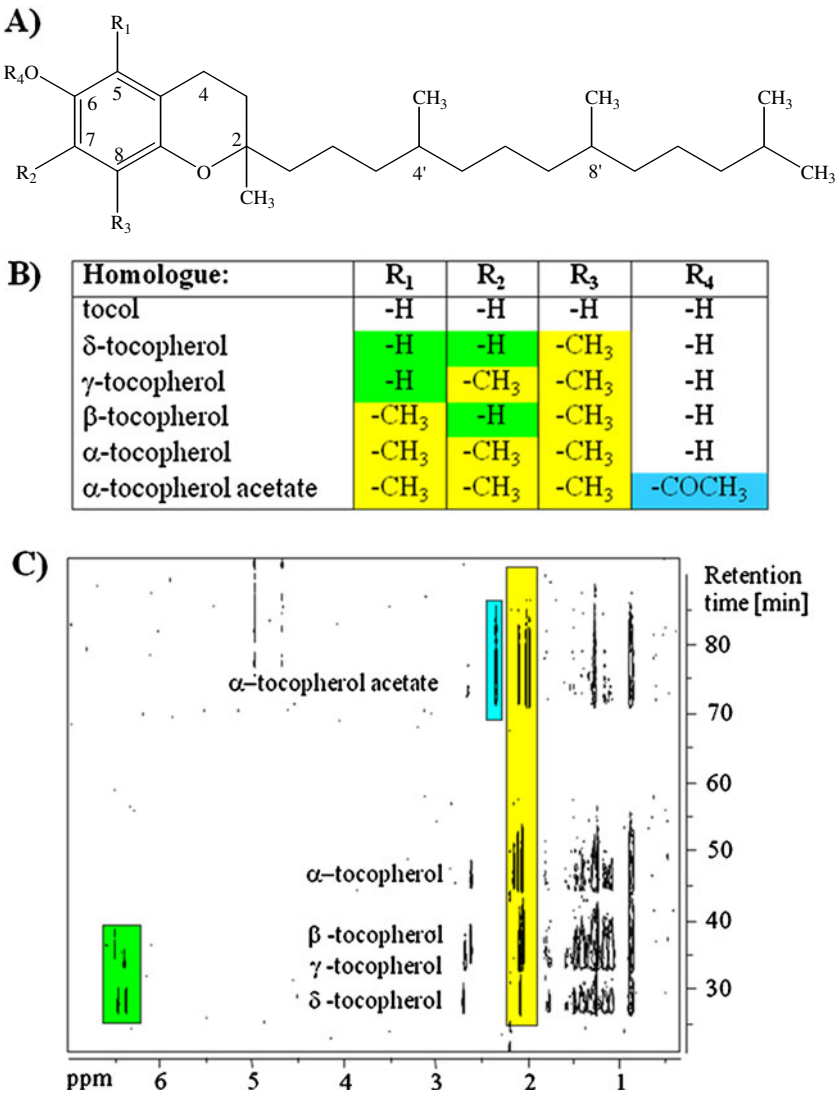

Fig. 5 **A** General structure of tocopherols. **B** Tocopherol homologues investigated. **C** Continuous-flow ^{1}H NMR contour plot of the separation of the tocopherol homologues. Capillary liquid chromatography conditions were as follows: 90:10 (v/v) MeOH-d_4/D_2O, flow rate 5 μL/min. Continuous-flow NMR conditions were as follows: pulse program lc2pnps, 16,384 transients with 4,096 complex data points and a spectral width of 9,615 Hz, relaxation delay 1 s, 256 rows with an acquisition time of 36 s per row. (Adapted from [33])

COSY-90, ^{1}H,^{13}C heteronuclear single quantum coherence (HSQC), and ^{1}H,^{13}C heteronuclear multiple bond coherence (HMBC)].

CE-NMR spectroscopy

In recent decades, CE and related techniques have proven to be important separation methods, especially when it comes to problems where other (micro-) separation methods provide unsatisfactory results or no results at all [19, 35]. In contrast to a variety of highly sensitive techniques for detection (e.g., laser-induced fluorescence, refractive index, MS, and UV/vis spectroscopy), the lack of sensitivity of NMR spectroscopy made its utilization as a coupled detection technique in CE impossible for years. Only in recent years has it been possible to combine the detailed structural and molecular dynamic information obtainable by NMR spectroscopy with the high separation power of CE [36]. The online coupling of capillary isotachophoresis (cITP) and NMR has been described as the most promising variant of the coupling of CE with NMR spectroscopy [37]. By means of cITP, charged species can be separated and concentrated because of their electrophoretic mobilities. In this method a high voltage is applied on a capillary, which is equipped with a two-buffer system, consisting of a leading electrolyte and a trailing electrolyte. Under very favorable conditions, it is possible to concentrate the charged analytes by 2–3 orders of magnitude during the cITP separation. If coupled with NMR spectroscopy, cITP allows precise focusing of the maxima of the analyte peaks examined in the active detection volume of the flow capillary [38, 39]. The focusing of the sample facilitates the application of probes with active volumes in the nanoliter range. This fact leads to an even higher mass sensitivity and makes the cITP-NMR instrumentation the most mass-sensitive NMR-spectroscopy-coupled technique. The potential of cITP-NMR spectroscopy for the online separation and analysis of bioactive compounds and drugs

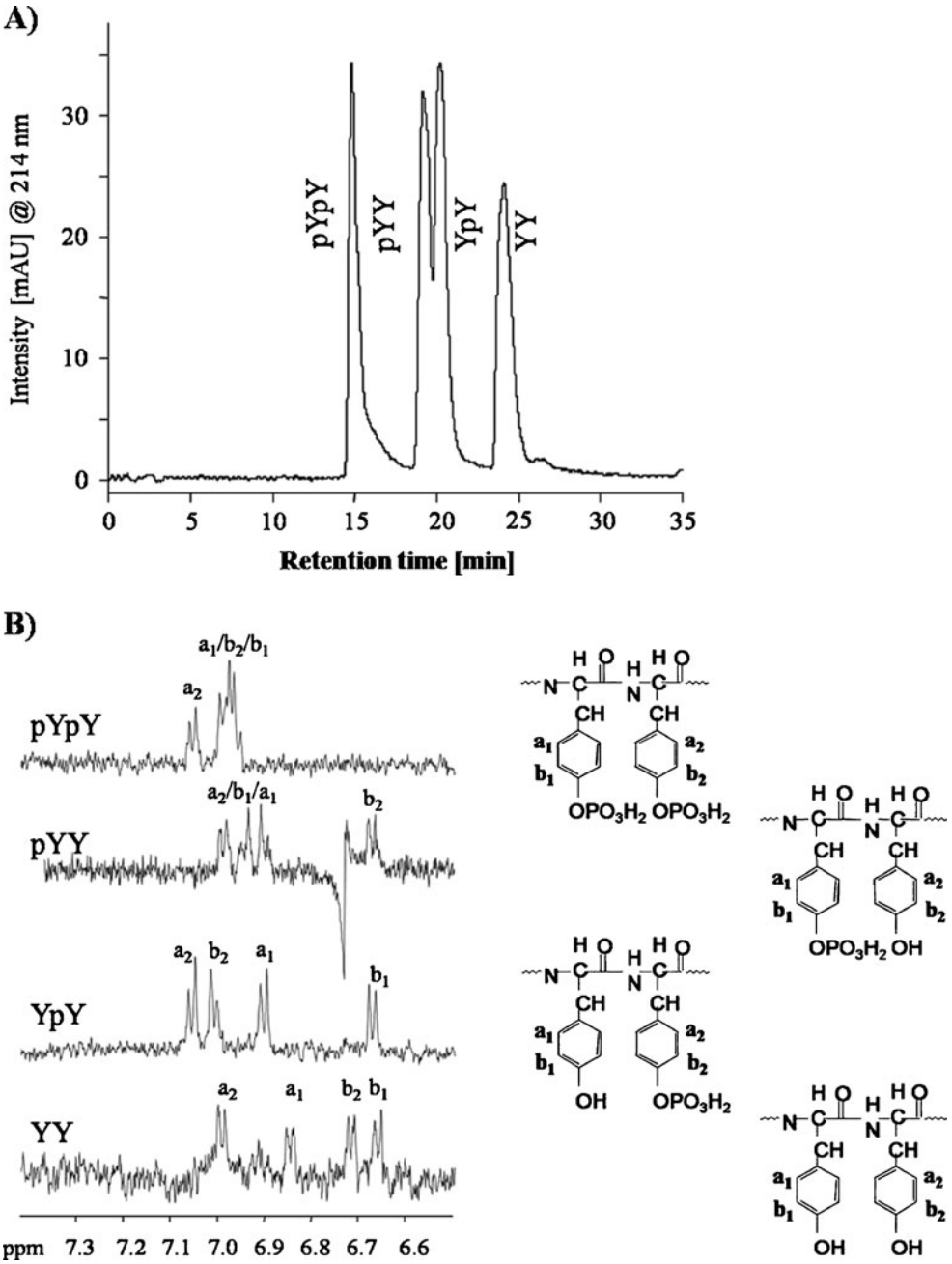

Fig. 6 **A** Capillary HPLC separation of four different synthetic peptides corresponding to the tryptic fragment 485–496 of the human protein tyrosine kinase ZAP-70. The conditions were as follows: laboratory-made ProntoSil C_{18}SH 200-Å, 3-µm capillary (15 cm×250 µm), flow rate 5 µL/min, MeCN-d_3/D_2O (+ 0.1% trifluoroacetic acid), gradient elution [6:94 →30:70 (v/v) in 45 min], 200-nL loop equivalent to 400 ng of each peptide. **B** Signal assignment of the stopped-flow ^{1}H NMR spectra of the peptides. (Adapted from [34])

in the nanomolar and micromolar range has already been demonstrated by very active groups [37–42].

In contrast to capillary HPLC–NMR spectroscopy, which is more and more being established as a standard analytical method, the development of CE–NMR spectroscopy coupling is still in its early stages. Most of the CE–NMR spectroscopy results obtained are based on homemade probes, which are equipped with handmade capillaries with very small active volumes [39, 43]. However, the results seem to be promising and suggest that the CE-NMR spectroscopy coupling will be developed further and find use in solving special problems in the various fields of instrumental analysis.

Generally, there are two problems regarding the CE–solenoidal-type microcoil NMR coupling: firstly, the electrophoretic current induces magnetic field effects [41, 44] and, secondly, the low concentration sensitivity of NMR spectroscopy compared with other capillary detection techniques.

The resulting magnetic field gradient effects can be minimized by the application of stopped-flow techniques (albeit at the cost of longer CE analysis times), or by the placement of the microcoil probe at the magic angle, or by combining both methods [44]. However, stopped-flow measurements cause signal-band dispersions, whereas the placement of microcoil probes at the magic angle lowers the intensities of NMR signals.

The magnetic field generated by the electrophoretic current has no influence on NMR coils positioned parallel to the static magnetic field [45]. If a loss of mass sensitivity can be tolerated, the coupling of the CE system with a conventional Helmholtz-coil-prepared NMR flow cell is more favorable. Owing to its low NMR active volume, the concentration sensitivity of the microcoil NMR probe is several orders of magnitude lower than that of an analytical flow probe. The online concentration of the samples during the CE-NMR experiments increases the effective concentration sensitivity of the NMR detection [44]. Even though the production of nanocoil probes is no longer a problem, their modification to obtain higher efficiency and the injection of the required sample volumes in the nanoliter range are still challenging tasks. However, a concentration of microliter sample volumes to nanoliter sample volumes can compensate the low concentration sensitivity of solenoidal-type nanocoil flow probes [46]. Using this very mass sensitive coupled technique in the continuous-flow mode, Schewitz et al. [47] investigated nanoliter amounts of a crude adenosine dinucleotide sample synthesized by solid-phase chemistry. They showed that the combination of the CE separation technique and NMR spectroscopy (Fig. 7) proved well suited for the identification of the main components and for the detection of by-products of the synthesis of the nucleotide. In the stopped-flow mode they were also able to acquire 2D CE-NMR spectra. The separation and identification of metabolites (Fig. 8a) of the drug paracetamol in an extract of human urine have also been performed successfully by the application of both capillary zone electrophoresis (CZE) (Fig. 8b) and capillary electrochromatography (CEC) on nanoliter sample volumes [48]. The utilization of the continuous-flow CZE-NMR (Fig. 8c) and stopped-flow CEC-NMR (Fig. 9) techniques allowed the detection of the major metabolites, the glucuronide and sulfate conjugates of the drug and the endogenous material hippurate.

In 2007, Korir and Larive demonstrated [41] the potential of cITP-NMR spectroscopy to facilitate the evaluation of sample purity and the identification of novel oligosaccharides by interpretation of the ^{1}H NMR spectrum measured for 1–3 μg (Fig. 10a) of the analyte. Although important information could be obtained from the ^{1}H NMR spectra, the definitive assignment of all the tetrasaccharide resonances was not possible owing to resonance overlap and the absence of measurable coupling constants (Fig. 10). The resonance at 5.98 ppm arises from the characteristic H-4 proton of the unsaturated uronic acid residue produced by enzymatic digestion. They noticed that other unknown impurities also produce signals in the polysaccharide resonance region. The ^{1}H NMR spectrum of the heparin-derived tetrasaccharide obtained by using a capillary NMR probe is shown in Fig. 10b. Other than the characteristic H-4 proton (5.98 ppm) of the uronic acid at the nonreducing end of the oligosaccharide, only three anomeric proton resonances were well resolved, at 5.56, 5.50, and 5.44 ppm. In this spectrum the signals around the HOD resonance, including the peaks at approximately 4.6 ppm, are weak because the HOD resonance was suppressed by selective saturation. Since Korir and Larive did not apply water suppression in the cITP-NMR experiment (Fig. 10a), the peak at approximately 4.6 ppm could be clearly detected in the coadded cITP-NMR spectrum of the oligosaccharide. They stated that the absence of a resonance in the 4.90–5.30-ppm region suggests that the internal uronic acid residue in the heparin-derived tetrasaccharide is the D-glucuronic acid form (GlcA). This is supported by the presence of a characteristic D-glucuronate H-4 triplet at 3.38 ppm shown in the inset in Fig. 10b [49]. With use of on-flow cITP–^{1}H NMR spectroscopy, (*R*)-alprenolol and

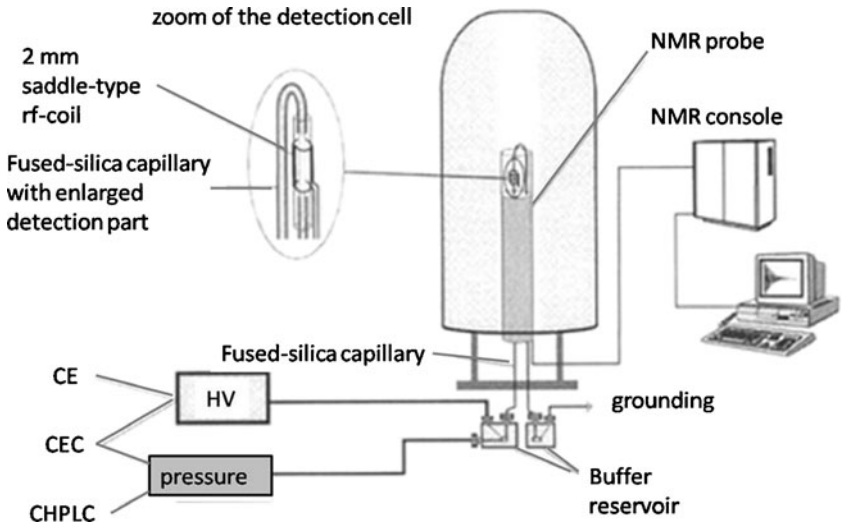

Fig. 7 Instrumentation for coupling capillary electrophoresis with NMR spectroscopy. *CE* capillary electrophoresis, *CEC* capillary electrochromatography, *CHPLC* capillary HPLC, *HV* high voltage. (Adapted from [47])

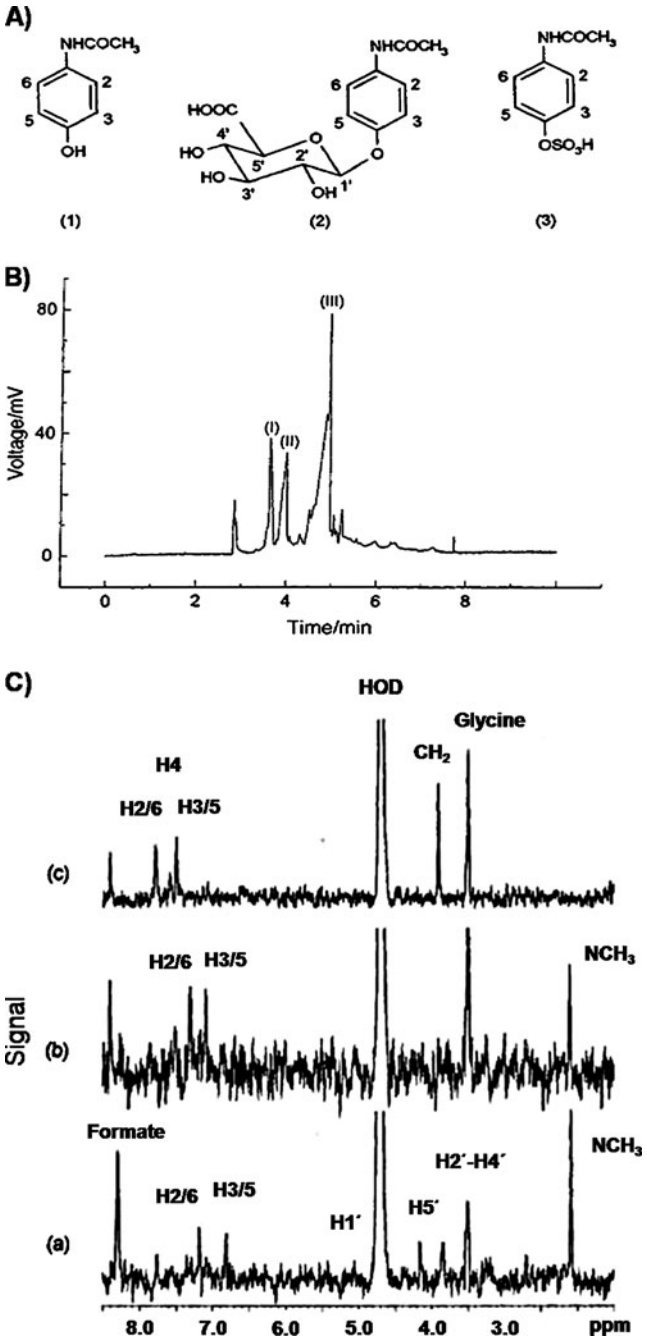

Fig. 8 **A** Structural formula of paracetamol (*1*), paracetamol glucuronide (*2*), and paracetamol sulfate (*3*). **B** Capillary zone electrophoresis (CZE) separation of the paracetamol metabolites in human urine extract using UV detection at 254 nm. Two major peaks not normally found in control urine were detected at 3.6 and 4 min (*I* and *II*). Also, a third component gave a sharp peak at 4.9 min. **C** Continuous-flow CZE-^{1}H NMR spectra of urine extract: *a* paracetamol glucuronide, *b* paracetamol sulfate, and *c* the endogenous material hippurate. (Adapted from [48])

(*S*)-alprenolol were separated and detected by using a mixture of 2 nmol racemic alprenolol in acetate buffer with α-cyclodextrin and sulfated β-cyclodextrin at pD 6 (Fig. 11). In this chiral separation of nanomole analyte

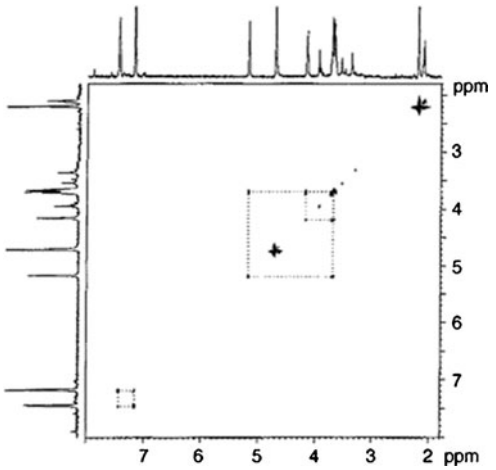

Fig. 9 Stopped-flow capillary electrochromatography–^{1}H,^{1}H total COSY NMR spectrum of paracetamol glucuronide. The 1D NMR spectrum is also plotted vertically and horizontally for convenience. (Adapted from [48])

amounts, Jayawickrama and Sweedler [42] observed that the concentration enhancement achieved for the *R* and *S* isomers was 224-fold and 200-fold, respectively. Their work extends the application of cITP–NMR spectroscopy to separate racemic mixtures as well as to identify

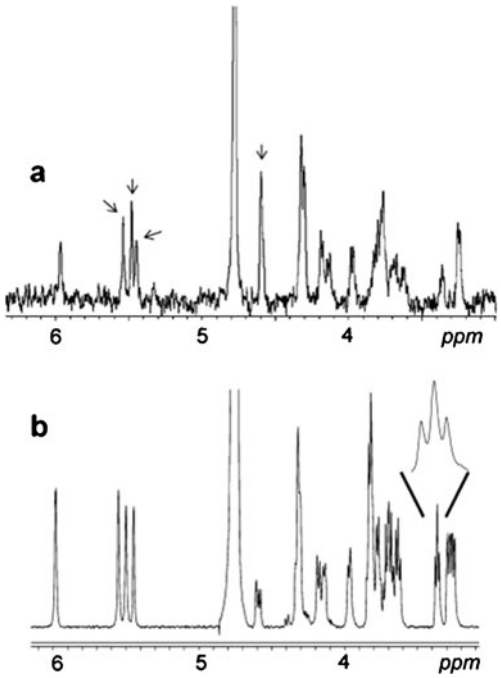

Fig. 10 **a** ^{1}H NMR spectrum obtained by postacquisition coaddition of the capillary isotachophoresis (cITP)–NMR spectra of 2.5 μg of the heparin oligosaccharide. There are four anomeric protons, indicated *by arrows*, suggesting that the unknown oligosaccharide is a tetrasaccharide. **b** ^{1}H NMR spectrum of the heparin tetrasaccharide acquired using the capillary NMR probe with 30 μg of the oligosaccharide in the probe flow cell. The *inset* is an enlarged portion of the spectrum showing the characteristic D-glucuronate H-4 triplet at 3.38 ppm. (Adapted from [41])

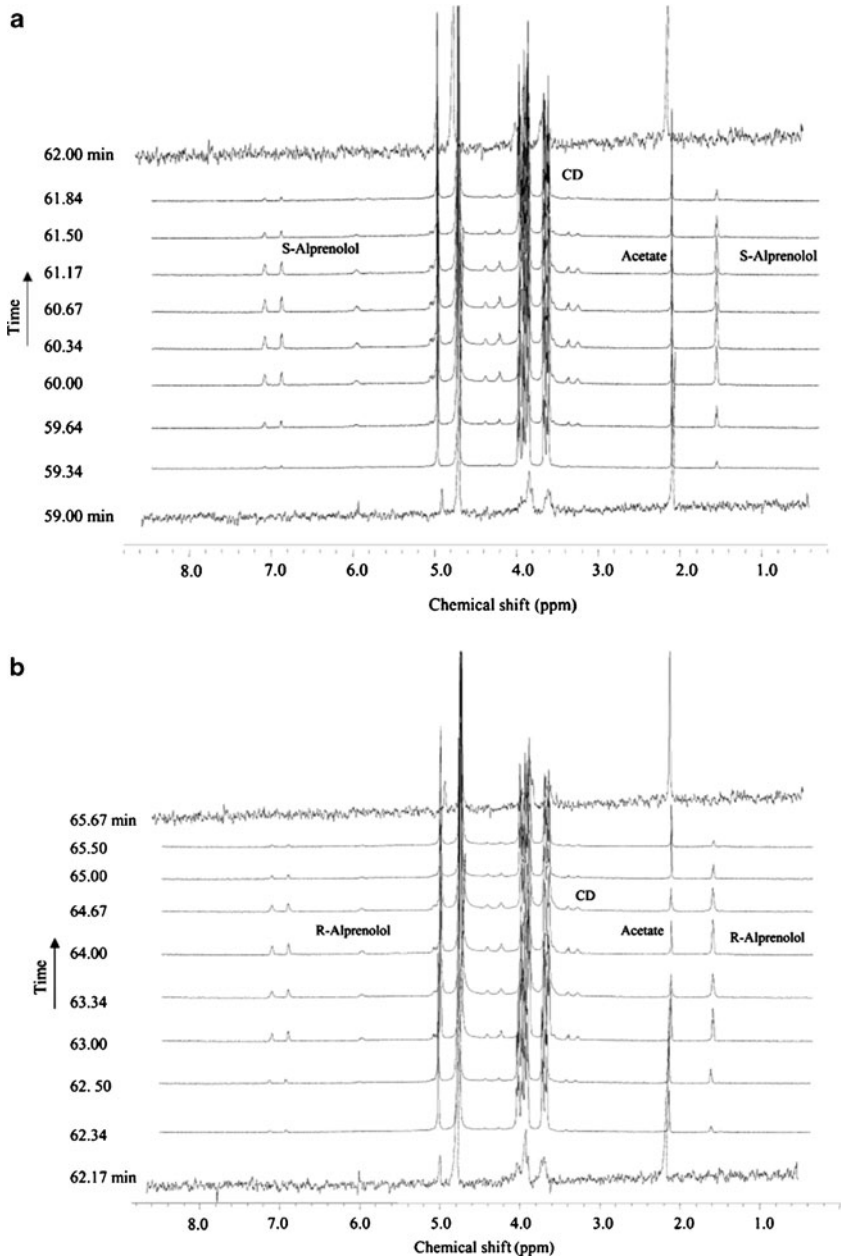

Fig. 11 Microcoil ^{1}H NMR spectra. **a** On-flow cITP–NMR spectra of (*S*)-alprenolol as a function of run time. The spectra at the *bottom* (59.0 min) and the *top* (62.0 min) mark the boundaries of the sample plug and are of reduced quality because of the susceptibility mismatch that occurs at sharp boundaries. The estimated concentration at the peak maximum is approximately 25 mmol/L. **b** On-flow cITP–NMR spectra of (*R*)-alprenolol as a function of run time. The spectra at the *bottom* (62.2 min) and the *top* (65.7 min) mark the boundaries of the sample band. The estimated concentration at the peak maximum is approximately 28 mmol/L. *CD* cyclodextrin. (Adapted from [42])

interacting sites between analytes and chiral selector molecules. They successfully demonstrated that a slight difference in the scalar coupling pattern of the two isomers is indicative of the extent of interaction with cyclodextrin. One drawback of this method is that the chiral separation of alprenolol isomers requires a significant concentration of cyclodextrin. The proton signals from these selectors within the analyte plug mask a significant portion of the chemical shift range available to observe analyte protons. Therefore, they suggested the application of deuterated chiral selector molecules for cITP–NMR analyses. Jayawickrama and Sweedler emphasized that the use of cITP–NMR spectroscopy together with a thermometric and conductivity detector [50] could enhance the performance of cITP by identifying times to stop the flow for stopped-flow NMR measurements. The NMR-invisible molecules can be characterized with such detectors and so they complement NMR detection during cITP. The results obtained from this study illustrate that NMR spectroscopy is also useful to probe the pH [51, 52], temperature [53], and flow dynamics occurring during a separation [54, 55], which are not observable with most other detectors.

Capillary GC-NMR spectroscopy

The coupling of GC with MS has become one of the most important analytical techniques [56–58]. The coupling of GC with FTIR spectroscopy has also been utilized for

several years for the identification of volatile compounds [59, 60]. However, little is known about the coupling of GC with NMR spectroscopy, although the coupling is very attractive, since the separation power of GC can be combined with the comprehensive and unprecedented structural information provided by NMR spectroscopy.

Capillary GC–NMR spectroscopy (Fig. 12) is a new coupling method for the structure elucidation of organic molecules and is thus an interesting and useful addition to the established GC-MS technique for the analysis of stereoisomers. A detailed stereochemical analysis is only possible by NMR spectroscopy. A reliable detection of enantiomers can be performed only on the basis of two exactly identical NMR spectra. By use of capillary GC-NMR spectroscopy, the separation and online detection of a solvent mixture has already been achieved [16]. Although NMR spectroscopy suffers from lower sensitivity compared with capillary GC, it is possible to perform continuous-flow ^{1}H NMR measurements that allow an intermediate structure determination of the analytes. Separations of stereoisomers and enantiomers and their online-flow NMR detection in the gas phase were also conducted successfully [61, 62]. In contrast to mass-spectrometric detection, it is now possible to distinguish between different stereoisomers and enantiomers and to obtain stereochemical information of the molecules. Stopped-flow GC-NMR measurements, which are required for the analysis of low-concentration selectands, have also been performed successfully [63].

Owing to the experimental difficulties in handling samples and the low SNR of spectra at atmospheric pressure [64], in contrast to liquids [20], gases have been studied much less by NMR spectroscopy. High-pressure NMR spectroscopy has been used successfully in various fields for a long time, such as supercritical fluid chromatography-NMR spectroscopy [65], online monitoring of chemical reactions and studies of equilibrium systems [66]. GC-NMR spectroscopy is performed with the usual carrier gases (helium, nitrogen, argon), which offer the advantage that the NMR spectra show no great background solvent signals. Buddrus and Herzog [67] reported the first GC-NMR studies in 1981. They performed the experiments with a JEOL FX-100 NMR spectrometer, equipped with a 260-μL flow probe and a 2-mm (ID) packed GC column (Chromosorb with 20% squalane). Two years later, the same group reported results regarding NMR spectra of gaseous organic compounds with higher boiling points [68] obtained with the use of a 4-mm (ID) packed column (Carbowax 4000), equipped with a heated interface, discharging into an air-heated (conventional) 5-mm NMR tube and the application of a temperature gradient (150-200 °C). In 2007, Grynbaum et al. [16] reported the first coupled continuous-flow capillary GC–solenoidal microcoil ^{1}H NMR spectroscopy experiments, resulting in reasonable SNR for sample masses of 1-2 μg. They described the use of a custom-built double-resonant solenoidal NMR microprobe with an active volume of 2 μL for the ^{1}H NMR detection of several compounds at 400 MHz with full coupling to the capillary GC system, equipped with an SE-54 column (50 m, 250-μm ID, 0.25-μm film thickness). They performed the separation of a mixture consisting of the volatile components diethyl ether, dichloromethane, and tetrahydrofuran (3.3 μL each) at a constant column oven temperature of 60 °C (Fig. 13). In 2008, Kühnle et al. [61] demonstrated that the separation and identification of volatile *cis*/*trans* stereoisomers is also possible by employing a coupled capillary GC-NMR spectroscopy system (Fig. 14). After optimizing the chromatographic and spectroscopic conditions with respect to the ^{1}H NMR detection of the mass-limited samples in the gaseous state, they developed

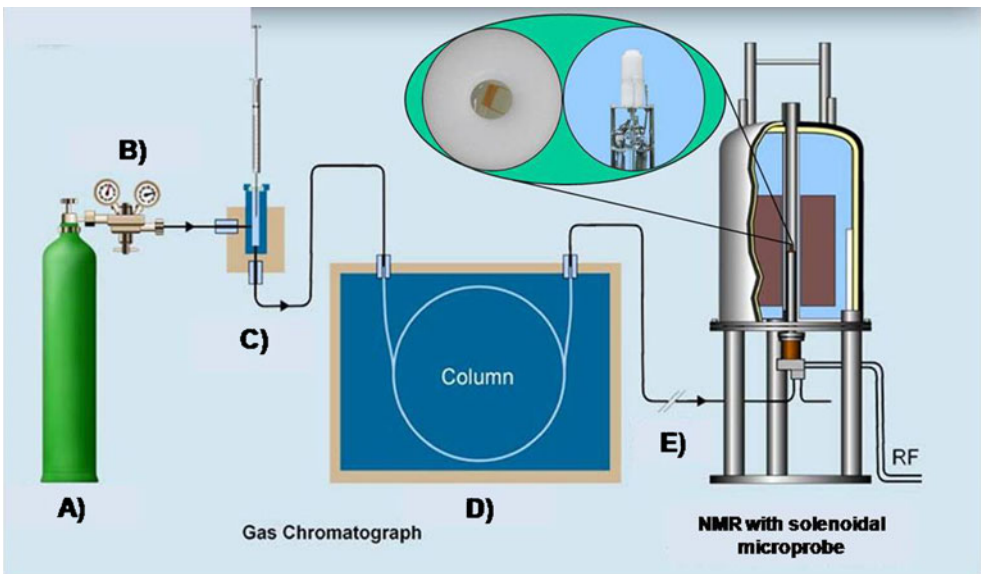

Fig. 12 Experimental setup for gas chromatography (GC)–NMR spectroscopy. *A* carrier gas, *B* flow control, *C* sample injector, *D* column oven, *E* transfer capillary. (Adapted from [16])

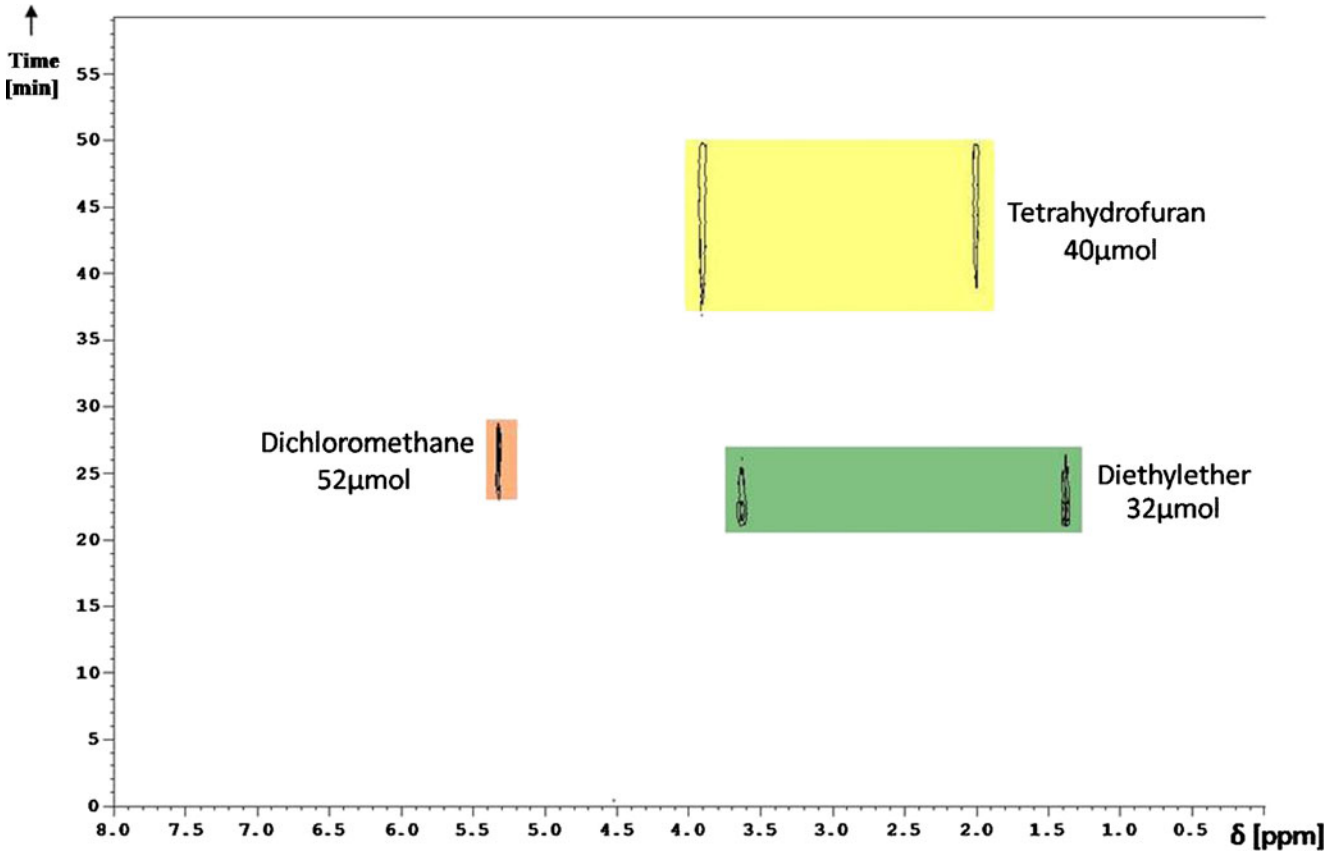

Fig. 13 Contour plot of the GC-NMR separation of diethyl ether, hexane, and tetrahydrofuran. (Adapted from [16])

a special spectrum-processing technique to obtain unambiguously interpretable spectra. They successfully revealed the structures of the stereoisomers by processing the stopped-flow ^{1}H NMR spectra of the gas chromatographic peaks investigated.

In 2010, Kühnle et al. [62] described the first online coupling of enantioselective capillary GC with ^{1}H NMR spectroscopy for the unfunctionalized chiral alkane 2,4-dimethylhexane resolved on octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin at 60 °C. Accomplishing this experiment, they showed that unfunctionalized enantiomers, displaying identical NMR spectra, can be identified or characterized indirectly on the basis of their different retention times.

Micromixer-equipped NMR microprobes

Over the past several years, much effort has been put into shrinking the analytical techniques to the microscale. Owing to the successes achieved in the field of microanalytical techniques, the developments are now continuing in the direction of nanotechnology. These miniaturized devices are so desirable because they offer, additionally to their space-saving size, more significant benefits such as mobility, speed, and cost savings. Microscale and nanoscale chemistry greatly reduce chemical consumption and thus are far more environmentally friendly than the conventional technologies.

These miniaturized devices also include micromixers (also known as microreactors) that integrate (multiple) reactor functions on a single chip or capillary cell of only a few millimeters to a few centimeters in size and which are capable of handling extremely small fluid volumes down to picoliters (Fig. 15a) [69]. Consequently, attempts have been made to use such tiny reactor systems together with microanalytical techniques. From the micromixer-prepared capillary probes, completely new possibilities have arisen for real-time reaction monitoring in NMR spectroscopy [70]. The NMR system contains a micro-flow cell (having a volume in the nanoliter to microliter range) with an integrated microreactor (Fig. 15b) [71, 72]. In this cell different substances or reactants can be mixed very quickly and completely (within a few milliseconds) owing to the narrow channels and accurately controllable injection flow rates in the measuring head, and can be detected immediately after mixing [8]. Thus, qualitative and quantitative in situ studies of very fast chemical reactions are also possible by means of high-resolution NMR spectroscopy. Inves-

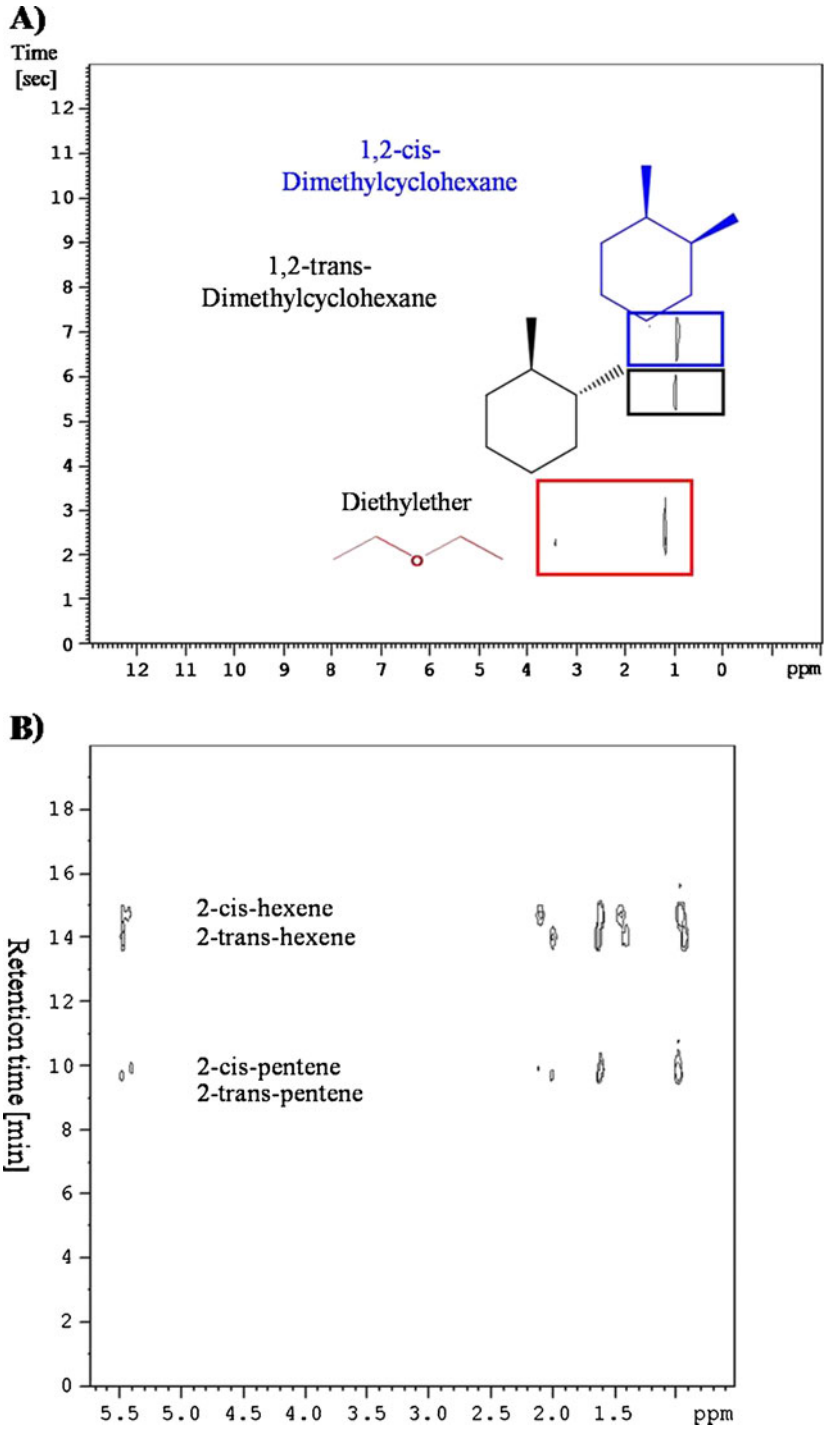

Fig. 14 **A** Contour plot of the GC-NMR separation of 1,2-*trans*-dimethylcylohexane and 1,2-*cis*-dimethylcylohexane. **B** Contour plot of the GC-NMR separation of 2-*trans*-hexene, 2-*cis*-hexene, 2-*trans*-pentene, and 2-*cis*-pentene. (Adapted from [61])

tigations of the kinetics of very fast reaction processes and short-lived intermediates are just two of several possible applications of the micromixer-prepared NMR capillary probes [73]. The temperature of the microreactor is controlled by the temperature-control system of the NMR spectrometer.

In recent years, interest in the use of miniaturized systems for the study of chemical reactions grew notably [74]. The advantages that microreactors offer are a faster and better heat exchange, a fast and complete mixing of the components, and extremely high surface-to-volume ratios in microchannel reactors for heterogeneous catalysis. Another advantage is that progress in the field of micro-electromechanical systems has enabled implementation of functional elements, such as active mixers, heaters, and other components, for real-time in situ reaction monitoring

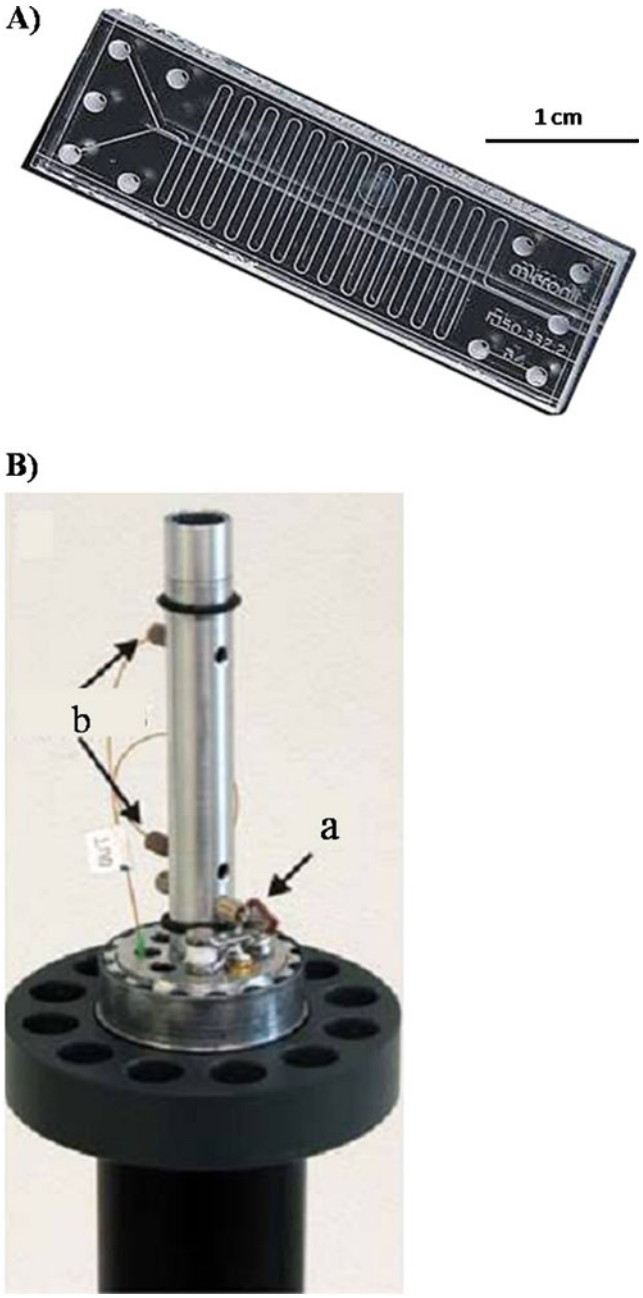

Fig. 15 **A** Microfluidic microreactor chip: 700-μm bottom glass with a 0.150 mm×0.150×mm×332 mm channel and a 3.7-μL internal volume. **B** Top part of the probe. The chip is aligned vertically in the aluminum tube, and connected electrically (*a*) and fluidically (*b*). (**B** Adapted from [73])

[75–78]. Investigations of protein conformational changes have already been successfully performed using a micro-electromechanical micromixer coupled to a microcoil NMR probe [79]. Wensink et al. [8] reported on the integration of a planar metal-film microcoil (already mentioned in "Introduction") coupled to an NMR microcoil flow probe for the excitation and detection of NMR signals. With this arrangement, they studied in real time the formation of imine by the reaction of benzaldehyde and aniline (Fig. 16). Starting from the experimental results obtained by investigations on the microfluidic chip system, developed for NMR-based continuous-flow reaction monitoring, van Bentum et al. [9] concluded that the stripline RF coil structure (Fig. 17) can be a valuable addition to the NMR instrumentation, as it combines high sensitivity with low susceptibility broadening and high power handling capabilities. In their work they also stated that the sensitivity of such a planar structure can be superior to that of a conventional wrapped microcoil. Utilizing the stripline RF coil "Micronit" microreactor chip, which they integrated in the probe, they also performed reaction-monitoring experiments on 600-nL sample volumes. They investigated in real time the reaction of toluene diisocyanate with pure ethanol

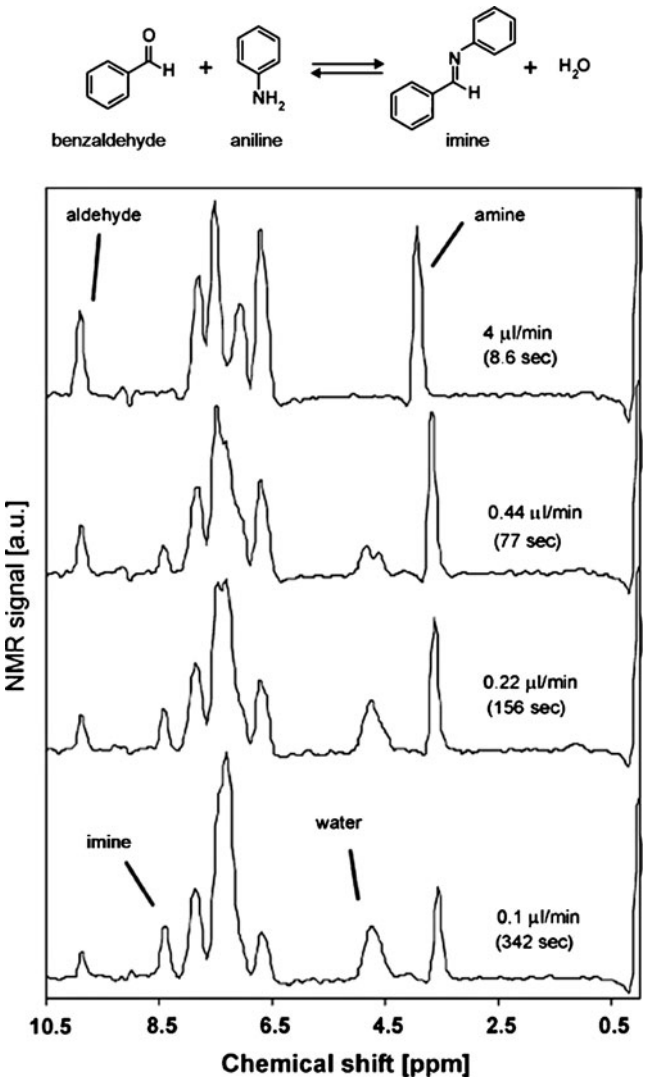

Fig. 16 ^{1}H NMR monitoring of the increase of the imine peak and decrease of the aldehyde signal with increasing residence time. (Adapted from [8])

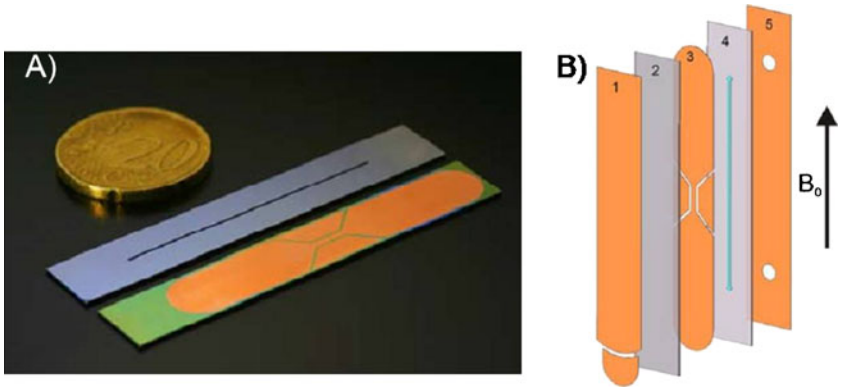

Fig. 17 **A** The two halves of the microfluidic chip (stripline chip) fabricated on silicon substrates before bonding. **B** Exploded view of a $\lambda/2$ stripline-based NMR chip. *Layer 1* is a grounded copper plane. The small separate plane represents the capacitive coupling to the external electronics. *Layer 2* is a substrate. *Layer 3* is the central line feeding the RF current. A constriction is defined by removal of narrow copper lines. The remaining trapezoid-shaped copper planes are electrically isolated. *Layer 4* is a substrate containing a microfluidic channel. Access holes are in the rear side. *Layer 5* is a grounded copper plane with microfluidic access holes. (Adapted from [73])

to form carbamate in the microreactor chip by NMR spectroscopy (Fig. 18). By varying the relative flow rates, they studied the carbamate formation as a function of ethanol stoichiometry.

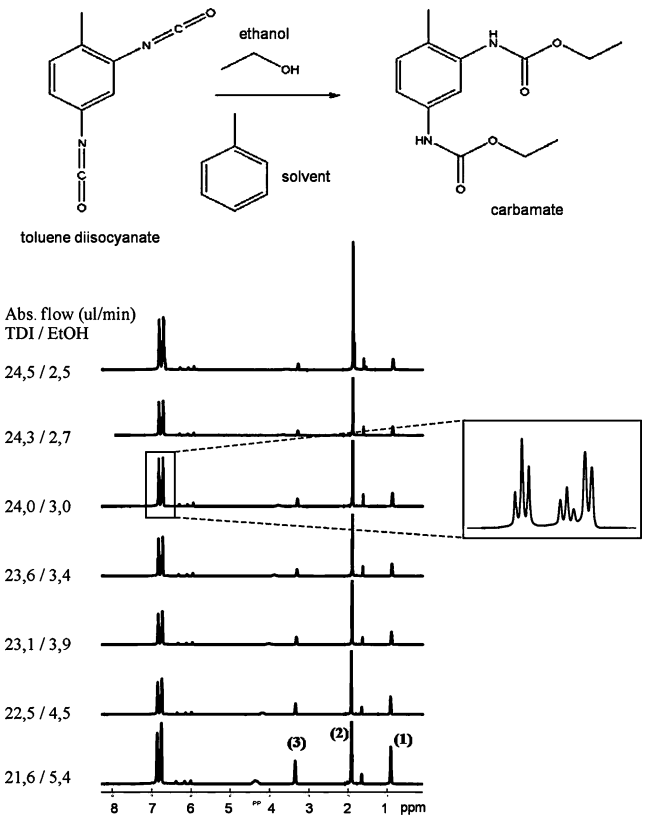

Fig. 18 ^{1}H NMR monitoring of carbamate formation from 0.5 mol/L toluene diisocyanate (*TDI*) and pure ethanol. If the relative flow rate of ethanol is increased, the product peaks rise. The zoomed spectrum segment shows *J*-coupling of aromatic peaks. *(1)*, *(2)*, and *(3)* indicate the signals of the carbamate, TDI, and ethanol, respectively.(Adapted from [92]).

(Multiple) microcoil flow probe arrangements

Automatic methods for high-throughput NMR analysis with two-, four- or eight-coil multiplex NMR probe arrangements have already been successfully developed [15, 80–82]. A two-cross-coil multituned probe head is shown in Fig. 19. Flow probes equipped with solenoid microcoils have been designed and optimized for the detection of small sample volumes and mass-limited samples. Up to eight samples can be injected simultaneously into an eight-coil multiplex probe head (Fig. 20) using an automated sampler and afterwards can be analyzed by selective excitation experiments. Under these conditions, the analysis time for 1D ^{1}H NMR studies is only a few seconds per sample. Current approaches for high-throughput NMR analysis include the use of automatic sample changers or flow probes in combination with automated liquid handlers. Sample changers have a relatively high failure rate, primarily due to troubles regarding the rotation of the NMR tubes or the finding of the ^{2}H spin lock

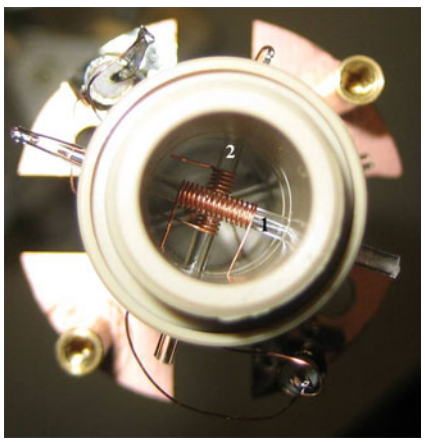

Fig. 19 Cross-coil arrangement: two-coil multituned probe head

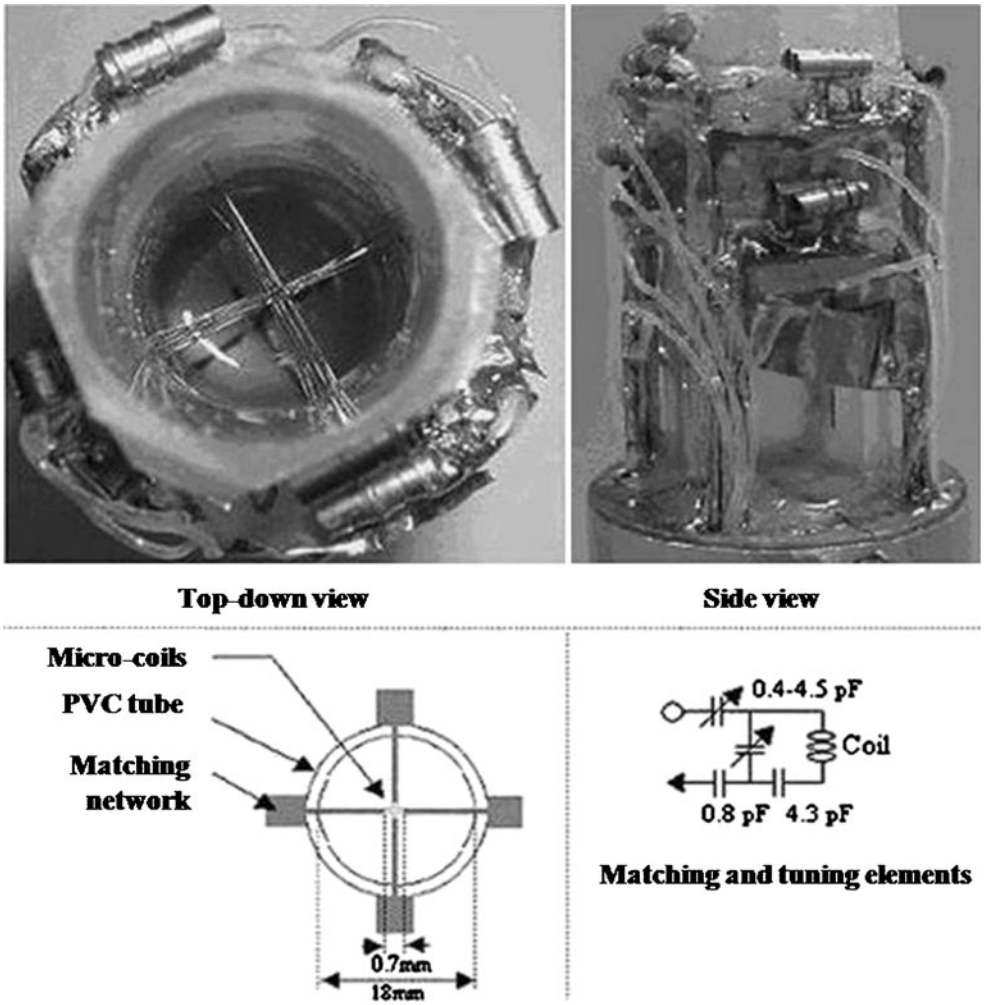

Fig. 20 *Top*: Photograph of the eight-coil probe head showing the configuration of the microcoils with a cross-coil arrangement and matching networks, and side view of the probe head. *Bottom*: The coil arrangement from top to bottom, and the circuit diagram for each matching network. (Adapted from [82])

and the shattering of NMR glass tubes [83]. The commercially purchasable automation systems for flow probes were found to be far more reliable [84, 85]. Conventional probe systems are equipped with flow cells, which are aligned parallel to the magnetic field. These flow-through cells are typically equipped with saddle-shaped Helmholtz coils and have sample volumes between 100 and 500 μL and active volumes between 40 and 400 μL (Fig. 1b). These systems include autosamplers, which rinse the measuring cell and inject the samples consecutively from well plates into the flow cell. The total recycling time for these systems is about 3 min per sample for 1D ^{1}H NMR analysis [84]. By the use of cryogenic flow probes, the concentration sensitivity of the flow probes has been improved by a factor of 4 [86]. Carryover of samples due to sample residues on the inner wall of the transfer capillaries, the tendency of clogging of the capillary induced by poorly soluble samples, and the unnecessary dilution of the samples by residual wash solvents are disadvantages of these robotic flow systems [84]. The development of microcoil NMR probes is another approach for the effective use of NMR flow cell systems. As already described in this work, the microcoil flow probe has been used together with several separation techniques, such as HPLC, CE, and GC. A high mass sensitivity and the need for only small sample volumes (nanoliters to a few microliters) are the main advantages of the microcoil probes [25, 26]. With use of a commercial microcoil flow probe with an active volume of 1.5 μL, the structure elucidation of a considerable number of bioactive compounds has been performed [87]. Owing to the high mass sensitivity of the microcoil probe, often only 1-15 μg of the pure compound dissolved in about 2 μL solvent is needed for 1D ^{1}H NMR, ^{13}C distortionless enhancement by polarization transfer (DEPT), ^{1}H,^{1}H COSY, ^{1}H,^{13}C HSQC, and ^{1}H,^{13}C HMBC experiments. In Fig. 21 the 1D ^{1}H NMR and ^{13}C DEPT NMR spectra of 2.3 μg isolated β-sitosterol (from *Trigonella foenum graecum* seeds) (Fig. 21, spectrum A) and 10 μg synthetic β-sitosterol (Fig. 21, spectra B, D), each dissolved in 1.5 μL solvent, are illustrated. The ^{1}H NMR spectrum of 15 μg isolated oxypeucedanin (from *Radix imperatoriae*) is shown in Fig. 22. Recently 2D ^{1}H,^{15}N COSY measurements of proteins have also been performed successfully by Peti et al. [88].

Even if the microcoil flow probe method is not operated fully automated but is used manually, the high throughput

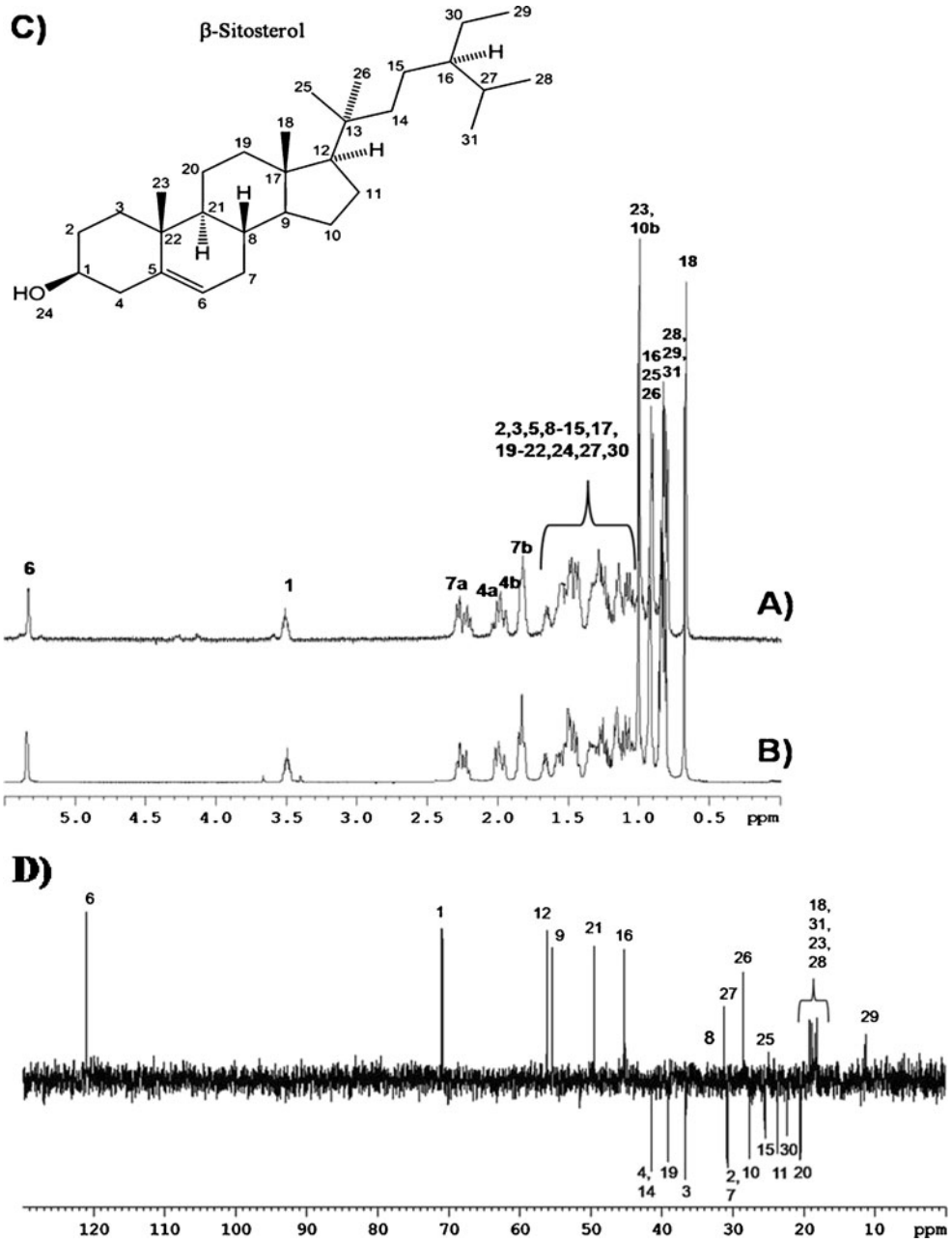

Fig. 21 The 600-MHz microcoil ^{1}H NMR spectra of 2.3 μg of the isolated β-sitosterol (*A*) and 10 μg of the β-sitosterol reference material (*B*) (both recorded in 1.5 μL $CDCl_3$). Structural formula of β-sitosterol with the assigned ^{1}H and ^{13}C NMR signals (*C*). The 150-MHz microcoil ^{13}C distortionless enhancement by polarization transfer NMR spectrum of the β-sitosterol reference material (10 μg in 1.5 μL $CDCl_3$) (*D*)

rate is as high as for other commercial fully automated analytical methods. On average it takes about 3 min to introduce the sample into the probe in addition to the time for washing and removing the sample. An alternative method to improve the throughput which can be used to enhance the efficiency of both the online and off-line flow-through systems is the application of a homemade probe head, equipped with more than one RF coil for parallel detection [15, 80–82]. Since solenoidal microcoils are oriented perpendicular to the magnetic field and are smaller than most saddle-shaped Helmholtz coils, more than one of them can be placed in the homogeneous region of the magnet. Parallel analyses have been performed for many years with other types of analytical instrumentation, but recently have been developed for NMR spectroscopy. In this regard, in recent years several approaches have been made, such as the use of isolated circuits, application of rapid selective sample excitations, and development of imaging methods [80, 89–91]. Macnaughtan et al. [81] demonstrated an improved injection system with a high level of automation, consisting of a multiplex NMR probe and a Gilson 215 multiprobe liquid handler (Fig. 23). Since four samples can be injected simultaneously by use of a multiplex probe automation system, the time for retrieving and delivering the samples has been reduced significantly. For this reason and owing to the time savings in the

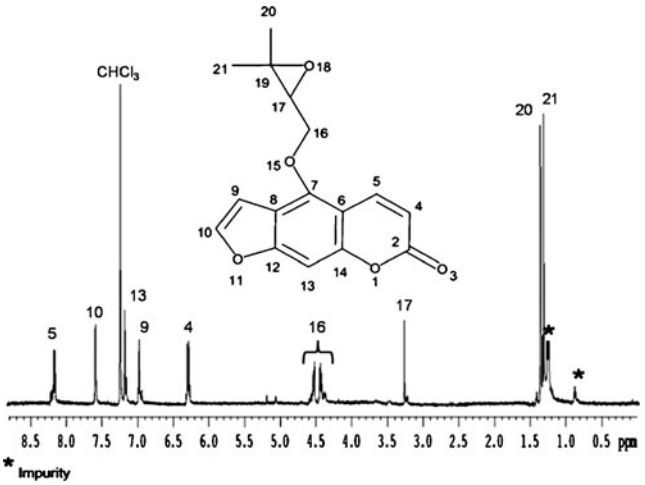

Fig. 22 The 600-MHz microcoil ^{1}H NMR spectrum of 15 μg (52 nmol) oxypeucedanin in 1.5 μL $CDCl_3$. The parameters were as follows: 32 transients, acquisition time 2.75 s, 1.0-s relaxation delay, 32,768 time domain points, sweep width 8.90 ppm (5,340 Hz), total acquisition time 2 min 20 s. (Adapted from [25])

sequential detection of even small sample amounts, compared with current NMR automation methods, the total recovery time for a sample can be greatly reduced.

The use of multiplex probe arrangements contributes to a reduction of the spectrometer dead time because multiple samples are delivered at the same time and the flushing of the capillaries and the retrieval and expulsion of the samples occur simultaneously. In addition, the samples are analyzed rapidly and successively, resulting in reduced relaxation delay and short analysis time. The routine instrumentation currently has the ability to analyze samples at an overall rate of a few seconds per sample to generate 1D ^{1}H NMR spectra with the presaturation-selective excitation pulse sequence. Even more important is that only 16 s is required to introduce new samples into the multiplex probe, leading to a more effective use of the NMR spectrometer [81]. In this fully automated process, the samples are obtained from well plates, frequencies of water are identified automatically, and each spectrum is stored immediately after recording by the controlling software.

As indicated above, with further developments an additional reduction in sample volumes by a factor of 4, an increase in sensitivity of twofold to threefold, and a doubling of the throughput should be realized soon. Owing to the combination of automated sample handling systems and multiplex NMR microcoil flow probes, mass-limited samples can be analyzed in a short time by NMR spectroscopy; therefore, the way for efficient high-throughput NMR analysis of a large number of samples has been cleared.

Wang et al. [82] recently described several advances in the design of multiple microcoil probes. They managed to increase the number of microcoils to eight. These coils were integrated into a commercial spectrometer, which had four separate receiver channels. The probe was designed to be run at 600 MHz, and to increase the sensitivity over that of previous probes, operating at 300 and 250 MHz.

The low sensitivity of NMR spectroscopy requires the use of NMR probes with the highest mass sensitivity for the CE-NMR spectroscopy coupling, such as those based on solenoidal microcoils. Wolters et al. [46]demonstrated a new method to avoid the microcoil NMR spectral degradation during continuous-flow CE by using a unique multiple solenoid microcoil NMR probe. They divided the electrophoretic flow from a single separation capillary into multiple outlets, each equipped with an NMR detection coil. While the CE electrophoretic flow passes through one outlet, high-resolution NMR spectra are

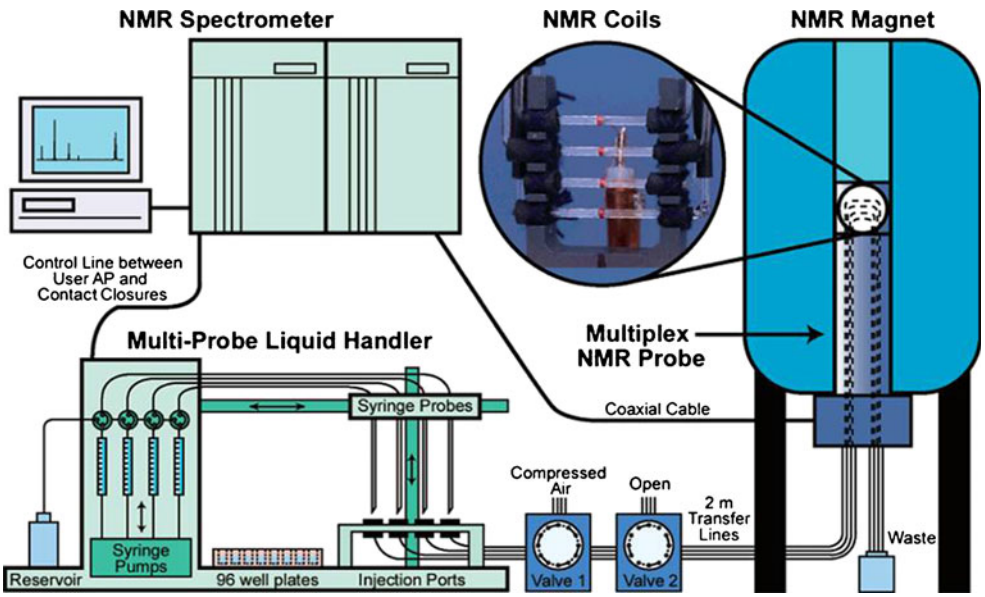

Fig. 23 Automation system consisting of a multiprobe liquid handler, two 12-port valves, the four-coil parallel-arranged multiplex NMR capillary probes (as shown in the photograph), and the NMR spectrometer. (Adapted from [81]). The liquid handler and spectrometer communicate through the connected electrical contact ports on the liquid handler and the user AP (analogue port) on the spectrometer

obtained from the coil at the other outlet in the stopped-flow mode (Fig. 24). Thus, the alternate processing of the electrophoretic flow and the NMR measurements enables a continuous-flow CE separation with stopped-flow mode detection. As a new approach for improving the performance of the multiple microcoil probes, the magnetic field homogeneity for each active coil was set automatically via the shim coils of the magnet. To demonstrate that electrophoretic current-induced line broadening in CE-NMR spectroscopy can be avoided, the multiple CE–microcoil–NMR spectroscopy coupling was used to analyze 3 μmol of an amine mixture. As a result, line broadening in the range between 1 and 2 Hz was obtained.

Summary and future directions

Although NMR spectroscopy will never achieve the sensitivity of MS in the picogram to femtogram range, it is an indispensable complementary method to MS. Highly selective enrichment and separation techniques in combination with MS and NMR spectroscopy will be used in the future in a very efficient way to solve complex separation and identification problems.

Thanks to the enormous progress in the development of microcoil-equipped flow capillaries, up to 16 probes will soon be stackable in the probe head along the magnetic field axis. Therefore, a maximum distance of about 1.5 mm between the probes is required and "cross talk" between adjacent coils must be minimized, which would be feasible by a better isolation of the inductors and the preference of a cross-coil arrangement instead of a parallel-coil system. The problem of RF "cross talk" between the different microcoils has already been solved by very active groups. The practical application of the parallel HPLC-NMR spectroscopy coupled analysis is still in its early stages. For this purpose an appropriate device configuration regarding the NMR instrumentation is necessary.

The simultaneous capillary HPLC-NMR spectroscopy detection with 16 measuring microcoils will be feasible in the coming years. A future-oriented capillary HPLC–multiplex NMR spectroscopy instrumental setup is proposed in Fig. 25.

Besides the practical viability of a parallel detection, the microcoil flow probe technique opens up the possibility for the coupling of capillary HPLC, capillary GC, and CE with microprobe NMR spectroscopy. The coupling of supercritical fluid chromatography with microcoil NMR spectroscopy is also an analytical tool which would be worthwhile to realize. A possible experimental setup is shown in Fig. 26.

The long spin–lattice relaxation times (T_1) in the gaseous and supercritical state can be reduced by the introduction of paramagnetic relaxation agents in the separation column before the NMR detection. This technique has already been used successfully for the acquisition of flow ^{13}C NMR spectra and brought about a drastic increase in sensitivity per unit time [20]. The application potential of this method needs to be explored. Thus, it is clear in which direction the development of the microseparation-microprobe NMR coupled techniques will continue: ^{13}C NMR spectroscopy will increasingly be used in the online coupling. In addition to the HPLC-DAD–electrospray ionization–MS and HPLC-DAD–atmospheric pressure chemical ionization MS coupled techniques, capillary HPLC-^{1}H NMR and HPLC-^{13}C NMR will be used in the future for the structure determination of unknown substances, increasingly in the multiple microcoil flow probe mode. Microreactors (micromixers) integrated in highly sensitive NMR microprobes will allow comprehensive real-time investigations of reaction kinetics of sample volumes in the lower nanoliter range under variable reaction conditions (e.g., at different temperatures and pressures). It will be possible to make qualitative and quantitative statements on both reaction products and intermediate products. The examination of structural changes of proteins, enzymes, and of course, smaller molecules cause by certain influences

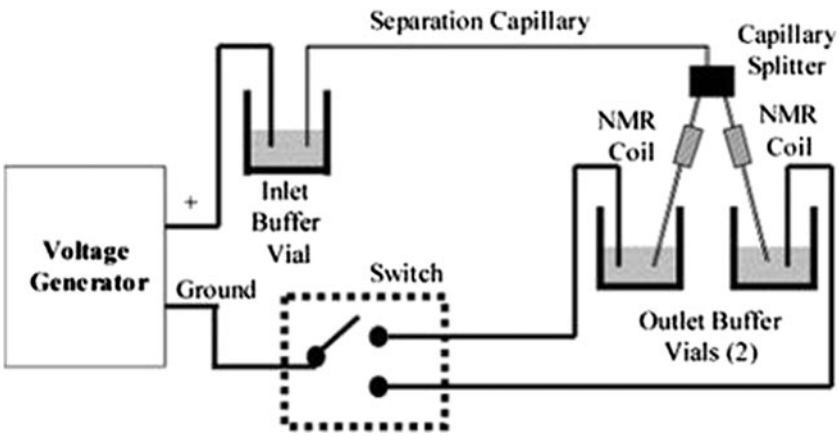

Fig. 24 The capillary electrophoresis–NMR system highlighting the arrangement of the separation capillary, the two outlet capillaries, and the two NMR detection coils. (Adapted from [46])

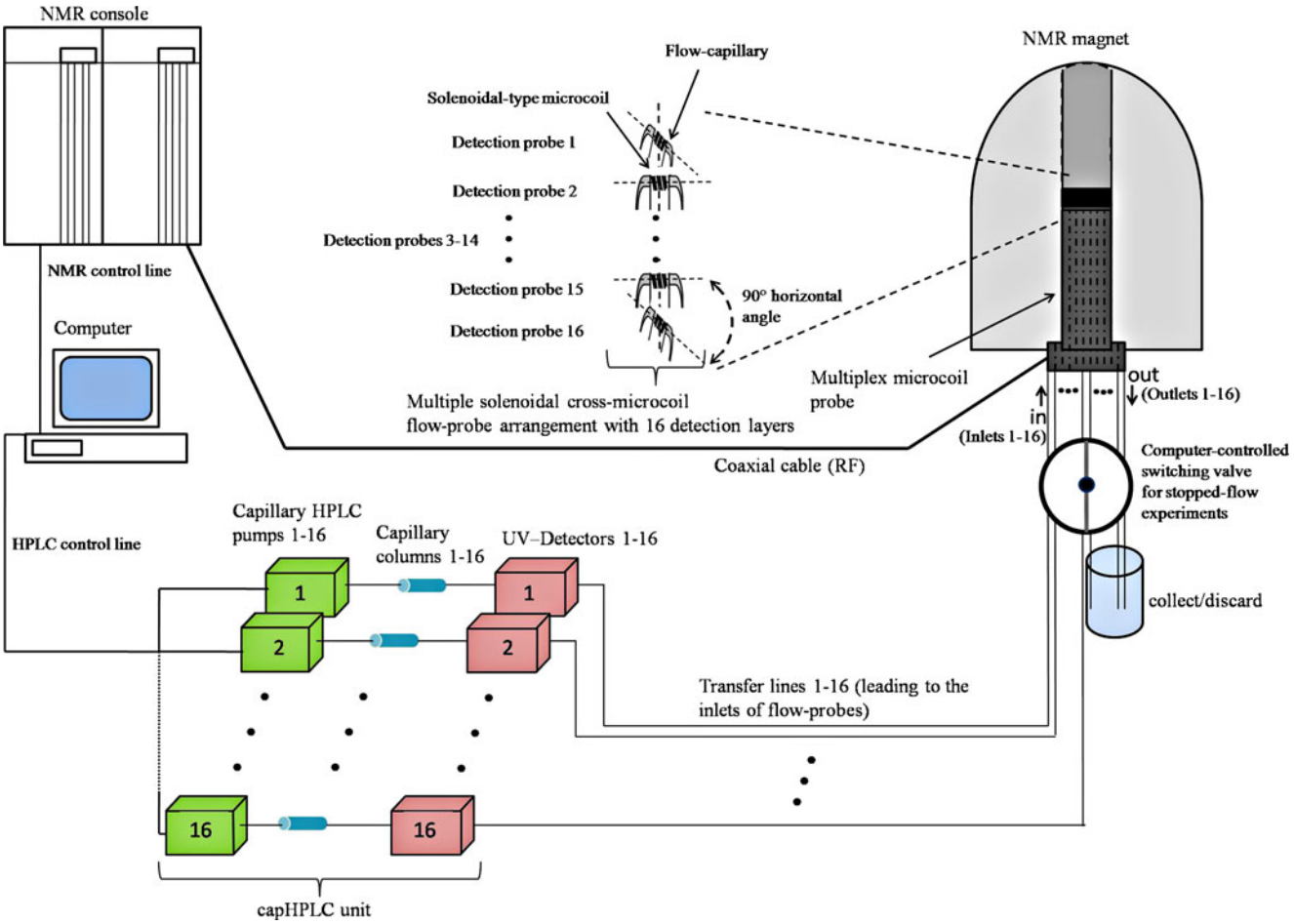

Fig. 25 The proposed instrumental setup of the automated multicapillary HPLC–16-micro-cross-coil multiplex probe head system

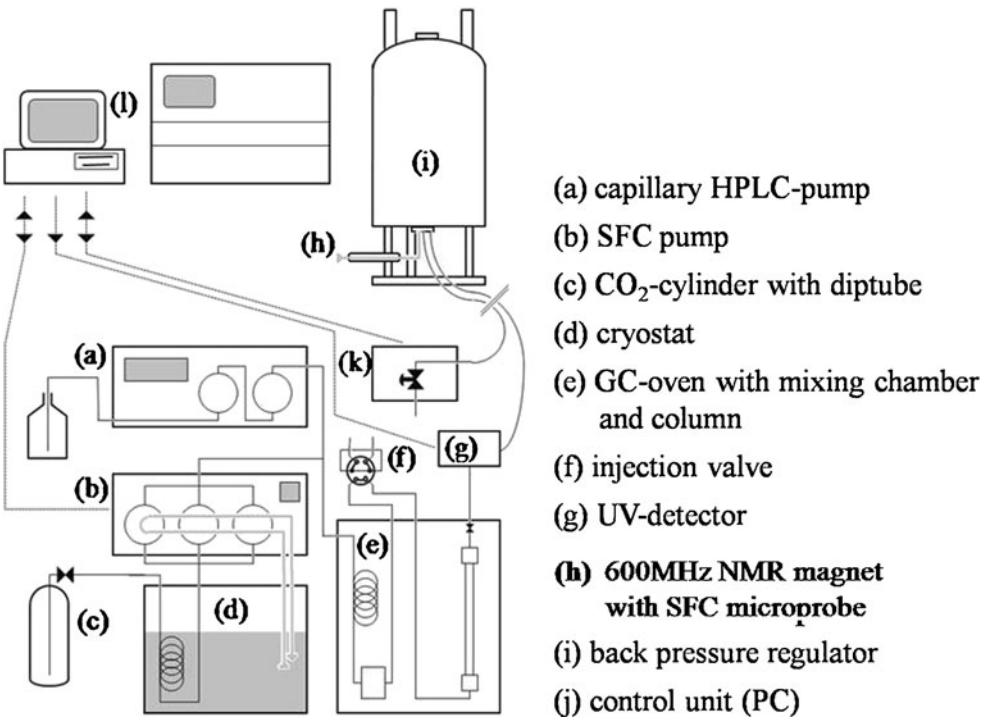

Fig. 26 The proposed experimental setup of supercritical fluid chromatography (*SFC*)–microcoil NMR spectroscopy instrumentation. (Adapted from [20])

will be accomplishable almost without exception. In the intermediate term and long term, simultaneous real-time microreaction monitoring of several reaction systems, with different educts, temperatures, pressures, catalysts, pH values, solvents, molar ratios of educts, etc., in multiplex NMR microprobes will be practicable.

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