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Review

# Hyphenation of capillary separations with nuclear magnetic resonance spectroscopy

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**Abstract**

The hyphenation of small-volume separations to information-rich detection offers the promise of unmatched analytical information on the components of complex mixtures. Nuclear magnetic resonance (NMR) spectroscopy provides information about molecular structure, although sensitivity remains an issue for on-line NMR detection. This is especially true when hyphenating NMR to capillary separations as the observation time and analyte mass are decreased to the point where reduced information is obtained from the eluting analytes. Because of these limitations, advances in instrumental performance have a large impact on the overall performance of a separation–NMR system. Instrumental aspects and the capabilities of cLC–NMR, CEC–NMR and CE–NMR are reviewed, and applications that have used this technology highlighted. Recent trends towards small volume capillary scale separations are emphasized, as is the recent success of capillary-isotachophoresis (cITP)–NMR.


*Keywords:* Reviews; Nuclear magnetic resonance spectroscopy; Hyphenated techniques; Microcoil; Detection, capillary techniques

## Contents



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## 1. Introduction

Separation and identification of chemical species is key to understanding the complex nature of chemical and biological systems. Small scale hyphenated separation techniques, such as capillary liquid chromatography (cLC), capillary electrophoresis (CE) and capillary electrochromatography (CEC), have revolutionized the ability to separate components in small samples. These techniques can provide faster analysis times, higher separation efficiencies and greater preconcentration abilities from less starting materials. Such features significantly benefit several areas, such as the pharmaceutical sciences, natural product chemistry, and biomedical research, that tend to have large numbers of mass-limited samples to analyze. What is capillary separation? Capillary liquid chromatography (cLC) has evolved from microcolumn chromatography and is now an established separation technique [1,2]. In general liquid chromatography (LC) performed with columns of less than 0.5 mm diameter is considered cLC [1]. The introduction of high resolution CE in the early 1980s by Jorgenson and Lukacs brought a new dimension to separation efficiency [3]. This technique was the method used to sequence the human genome and is gaining importance in a number of other areas. CEC is a technique that combines the separation efficiency of CE and the selectivity of LC [4–6]. In all cases, reducing the scale of the separation to microscale places unique constraints on sample handling and detection. This review focuses on the hyphenation of nuclear magnetic resonance (NMR) with capillary separations.

### 1.1. NMR spectroscopy

What information does NMR provide? NMR is a well-established and powerful technique for elucidating molecular structures, determining degradation products/impurities and studying molecular dynamics. As such, NMR is routinely used in pharmocokinetic studies [7], drug metabolism [8,9], organic synthesis [10] and natural product chemistry [11,12]. Methods based on NMR can also be employed to analyze mixtures without physical separation [13], and measure molecular properties [14] and bulk properties of the medium [15,16]. As a non-invasive technique, NMR is often used to study molecular interactions and is a vital tool to screen potential drug candidates [17], DNA/RNA binding to proteins, and other applications [18,19]. Therefore, NMR has become an essential analytical tool in academic and industrial environments. NMR is an inherently insensitive, but non-destructive technique, so that long observation times are used to increase the signal/noise ($S/N$) to acceptable levels. This becomes problematic when using on-line detection for capillary separations where peak durations tend to be short (several seconds long). The detection limits of NMR are several orders of magnitude poorer than other standard analytical techniques (Table 1) [20].

The sensitivity of NMR must be improved to

Table 1
Limits of detection (LOD) for common analytical detection methods used with capillary separations

| Method | LOD (mol) |
|---|---|
| Fluorescence | $10^{-18}$–$10^{-23}$ |
| Mass spectrometry | $10^{-13}$–$10^{-21}$ |
| Electrochemical | $10^{-15}$–$10^{-19}$ |
| Radiochemical | $10^{-14}$–$10^{-19}$ |
| UV–visible absorbance | $10^{-13}$–$10^{-16}$ |
| NMR | $10^{-9}$–$10^{-11}$ |

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adopt this technique as a more routine analytical tool and to use it as an online detector for separation techniques. Both theoretical and practical strategies have been developed to enhance NMR sensitivity. As a straightforward approach, higher field magnets have been introduced because the sensitivity increases with the 7/4th power of the magnetic field strength. Magnets, based on super conducting technology close to GHz field strengths, are now available at a fairly high cost. The search for higher field strengths with novel super conducting materials is a continuous effort [21]. Another approach involves polarization transfer from optically pumped nuclei such as $^{129}$Xe to improve NMR sensitivity. This method is known as the spin polarization induced nuclear Overhauser enhancement (SPINOE) technique [22]. A sensitivity improvement of 50-fold for $^{1}$H and 70-fold for $^{13}$C has been reported with SPINOE. However the applications are mostly limited to analytes dissolved in liquid Xe or non-aqueous media. Similarly, dynamic nuclear polarization (DNP) uses saturating electron spins coupled to nuclear spins to increase NMR signal intensity [23]. Several applications of NMR detection with DNP have appeared using chromatographic separations [24]. This method requires additional instrumentation to generate microwave power and most importantly the presence of electron radicals, but is certainly intriguing; hopefully, this will be further developed in the coming years. NMR probes constructed with super conducting materials [25] and cryogenically cooled radio frequency (RF) coils [20] can improve the sensitivity by minimizing receiver coil noise, although such technology has not yet received much attention for capillary separations. This review focuses on both standard and microcoil based NMR probes for detection in capillary separation.

### 1.2. The NMR probe

The RF coil or the probe is an important component of NMR instrumentation. It must be capable of delivering radiofrequency energy necessary to excite NMR active nuclei and of collecting signals from the sample. It has been shown theoretically and experimentally that maximum coupling between the probe and the sample is achieved by miniaturizing the NMR coil just to accommodate the sample [26]. The minimum amount of analyte that can be detected is related to the mass sensitivity of the probe, as defined by Eq. (1):

$$S_{\mathrm{m}} = \frac{S/N}{mol\, t^{1/2}} \quad (1)$$

where *mol* and *t* are the number of moles within the observe volume ($V_{\mathrm{obs}}$) and the acquisition time, respectively. The use of $S_{\mathrm{m}}$ instead of concentration sensitivity ($S_{\mathrm{c}}$) is more appropriate for mass limited samples as measured with NMR microcoils and to compare the performance of different probes. Descriptions of microcoils for high resolution NMR spectroscopy and their applications can be found elsewhere [20,26].

The ratio of the volume available for detection ($V_{\mathrm{obs}}$) to the total amount of volume ($V_{\mathrm{tot}}$) needed for analysis is known as the observe factor ($f_0$) [20]. To reach maximum sensitivity, $f_0$ should approach unity. In practice, preventing magnetic susceptibility boundaries near the sample being measured is done by extending the sample beyond the NMR observation region. For example, a standard 5-mm NMR experiment requires ~750-μl sample volume although the NMR active volume is ~250 μl.

Two arrangements of NMR coils, the solenoidal RF coil (Fig. 1A) and the saddle-type (Helmholtz) RF coil (Fig. 1B) have been employed as on-line NMR detectors for capillary separations. Maximum sensitivity for a solenoidal coil is achieved by keeping the coil axis to the static field, $B_0$, whereas in saddle coils, the maximum signal is gained with its axis parallel to $B_0$. Typically solenoidal mi-

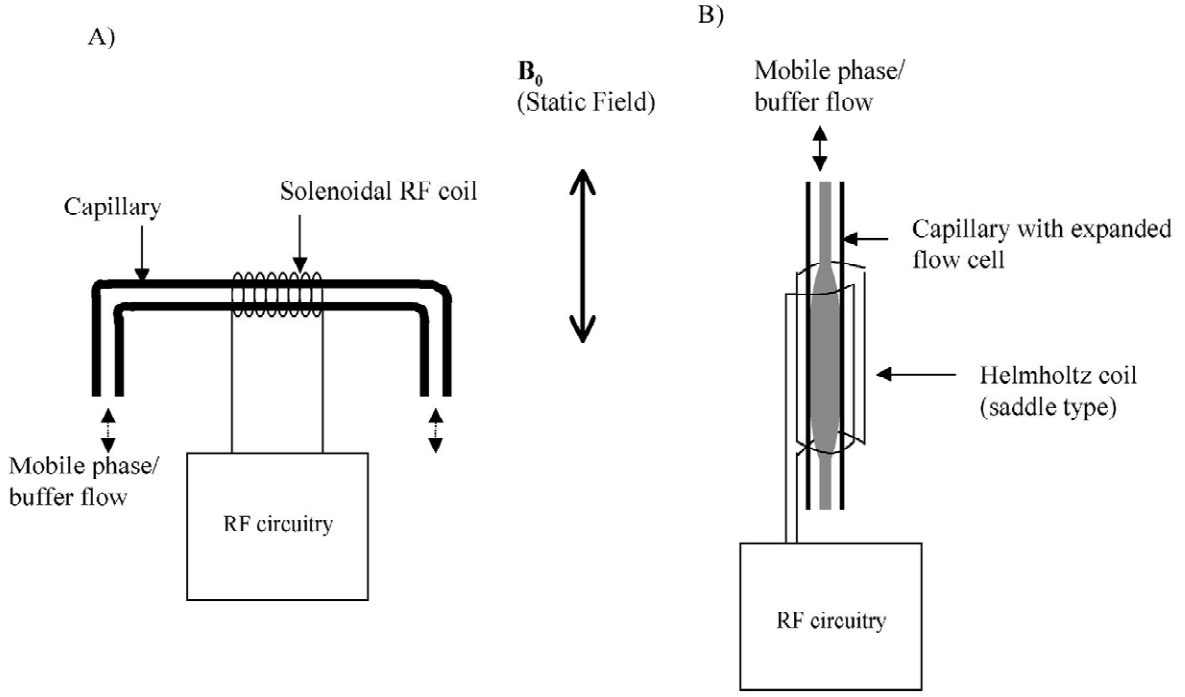


Fig. 1. (A) Solenoidal RF coil wrapped around a fused-silica capillary placed orthogonal to $B_0$. (B) Helmholtz coil with an expanded flow cell is placed parallel to $B_0$.

crocoils are designed to hold nanoliter to low microliter sample volumes. Commercial probes with flow configuration became available after the introduction of microcoils in 1995 [27]. As a major development, a saddle coil that houses 1.7-mm diameter sample tubes has been introduced by Varian [28–30]. Another significant contribution is the design of an inverse coil to accommodate a 3-mm diameter sample tube with a detection volume of 60 μl and total volume of 140 μl [31]. To date, the smallest reported volume for a saddle coil probe is 5 μl, with an active volume of 2.5 μl [32]. This probe shows a $S/N$ enhancement of 5 in comparison to the conventional 5-mm NMR probe. However no on-line capillary separation data have been reported with this probe yet.

The $S/N$ per unit volume for solenoidal coils with diameter ($d_c$) greater than 100 μm is approximated by Eq. (2) [33]:

$$S/N \propto \frac{\omega_0^{7/4}}{d_c} \quad (2)$$

where $\omega_0$ is the Lamor frequency. Because $S_m$ is proportional to $S/N$, smaller diameter RF coils provide higher mass sensitivity. For a given length and diameter, solenoidal RF coils are about two to three times as sensitive as saddle type coils [20,26].

Solenoidal microcoils can be constructed in-house by carefully wrapping a conductive wire around the capillary with the conductive wire acting as the detection cell. This allows easy coupling of the NMR spectrometer to cLC, CE or CEC. When using such an on-line detection cell, NMR active volumes are in the range of microliters to nanoliters and can easily be changed to the demands of a particular application by using the appropriate capillary. Different probe designs have been developed with several of them being designed especially for microscale separations [34–44].

### *1.3. Hyphenation of capillary separation–NMR*

In principle, complete structure elucidation of separating analytes during a chromatographic or electrophoretic process can be performed using NMR

as an on-line detector. This reduces off-line analysis of samples and may also allow the analysis of sensitive and unstable mixtures.

Watanabe and Niki introduced the first LC–NMR system in 1978 as a stopped-flow experiment [45]. In 1978 Bayer et al. [46] reported the first continuous-flow LC–NMR experiment. Nonetheless, detection sensitivity and other constraints imposed by this combination hindered the routine analytical application of LC–NMR for years. Over time, the development of higher field strength magnets, better solvent suppression techniques, more sensitive small diameter transmitter/receiver coils, on-column sample preconcentration and expanded flow cells have improved the sensitivity of NMR spectra recorded in LC separations. Since then LC–NMR has become a vital and an attractive analytical tool for stereochemical analysis of drugs in biological fluids [47], and has been used in combinatorial chemistry for separation and identification of libraries of small organic molecules [48] and peptides [49]. Separation and identification of terpenoid mixtures [50], and analysis of nitroaromatic compounds containing environmental samples [51] and plant natural products [52] have also been performed with LC–NMR.

The conventional LC column (150×4.6 mm I.D.) requires relatively large volumes of eluents. As a result, it is not often economically feasible to use more expensive deuterated solvents for LC–NMR measurements. However the intense signals generated from protonated solvents must be reduced to increase the dynamic range of analyte signals. NMR solvent suppression techniques remove large solvent signals; however, the spectral features of NMR signals in the proximity of solvent resonances are also affected. Consequently, important spectral information required to deduce structures, especially of minor analytes, is lost. The introduction of microcolumn LC and cLC dramatically reduced the mobile phase volume to a few milliliters or less, permitting deuterated solvents to be used as mobile phase at reasonable cost.

Johnson and He introduced a non-capillary based method on electrophoresis–NMR (E–NMR) to study electrophoretic mobilities and diffusion coefficients [53], although no separations were performed. The first coupling of CE–NMR was described by Sweedler and co-workers in 1994 [39,40]. A number of CE–NMR applications using solenoidal microcoils and saddle type microcoils have since been published [36,41,42,54]. CEC–NMR has been used to analyze biological fluids [41,42] and polymer mixtures [55] by the Tübingen groups. In 1998, Bayer, Albert and their groups introduced an integrated CEC–NMR system with CE and cLC [36]. To date, only a few researchers have reported using CE–and CEC–NMR, partially due to lack of commercial instrumentation. In this review article we will describe the developments and the applications of cLC–, CE–and CEC–NMR.

## 2. Capillary LC–NMR

Because of the small solvent volumes involved with cLC–NMR, most experiments are performed with deuterated mobile phase. This eliminates the need for solvent suppression and allows access to the total chemical shift range. Another advantage of microcolumn/capillary LC techniques, enhancing detection performance, is the higher concentration of analytes in an analyte peak for a given injected amount injected [1]. The chromatographic dilution ($D$) of a compound during the separation process is approximated by Eq. (3).

$$D = \frac{C_0}{C_{max}} = \frac{r^2 \varepsilon \pi (1+k)\sqrt{2LH\pi}}{V_{inj}} \quad (3)$$

where $C_0$ is the initial concentration of the analyte, $C_{max}$ is the final compound concentration at the peak maximum, $r$ is the column radius, $\varepsilon$ is the column porosity, $k$ is the retention factor, $L$ is the column length, $H$ is the column plate height and $V_{inj}$ the injected sample volume. Even with chromatographic preconcentration approaches, the column diameter dictates the peak volume of the eluting analyte, so that smaller columns produce high concentration eluting peaks. As the NMR detection cell also achieves higher mass sensitivity as it is scaled to smaller sizes, this indicates that smaller diameter cLC columns with size-matched NMR microcoils achieve the highest mass sensitivity performance. For example, the peak intensity and mass sensitivity should increase several hundred-fold for a reduction in column diameter of 4.6–0.3 mm. Table 2 shows

Table 2
Typical liquid chromatography separation parameters and resulting peak concentrations for 1 nmol analyte

| Column I.D. (mm) and mode of separation | Flow rate (μl/min) | Peak volume (μl) | Peak concentration (m*M*) |
|---|---|---|---|
| 4.6 Standard | 1000 | 200 | 0.005 |
| 1.0 Microbore | 30 | 10 | 0.1 |
| 0.32 Capillary | 3 | 1 | 1 |
| 0.18 Capillary | 1 | 0.3 | 3 |

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the variation of expected peak concentrations for a 1-nmol analyte injection with column diameter [56]. As evident from the table, the sensitivity increases significantly when approaching capillary scale. This suggests that capillary techniques are especially useful to analyze small sample volumes.

### 2.1. Capillary LC–NMR instrumentation

The basic instrumentation to perform cLC–NMR is shown in Fig. 2. Depending on the application, the LC outlet capillary can be directly connected to the NMR probe. Often online UV–Vis is used prior to the NMR detection cell; the absorbance detector can be used to trigger NMR acquisition or carry out stopped-flow NMR experiments.

How close can the column and the LC pumps be located to the NMR magnet? Obviously, magnetic materials perturb the required homogeneous field of the magnet, reducing the spectral quality that one can obtain. The column may be positioned closer to or inside the magnet bore if they are non-magnetic; not

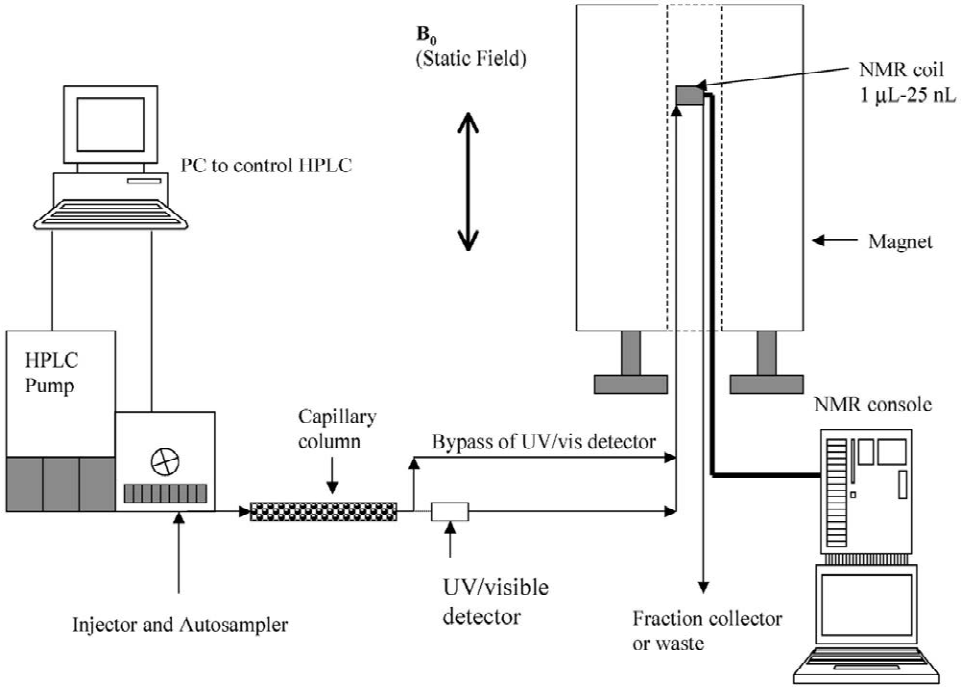


Fig. 2. Instrumental schematic for a typical cLC–NMR set-up showing the arrangement of the separation capillary, pumping system and NMR spectrometer.

surprisingly, steel-jacketed columns are problematic. To avoid any adverse effects of magnetic field on LC instrumentation, usually a long transfer line is required to couple the capillary column to the NMR probe. The internal diameter of transfer lines is generally kept between 50 and 100 μm to avoid extra column broadening. However with shielded magnets, the LC instrumentation can be placed close to the magnet (within 30 cm) with minimum NMR spectral line broadening. Therefore, the separation can be performed on a faster time scale. Commercial NMR probes specifically designed to interface with capillary LC systems have become popular tools in the pharmaceutical industry [57]. In addition, cLC–NMR instruments with more sophisticated control systems are also commercially available [58].

An important consideration in designing cLC–NMR instrumentation is the active volume of the NMR probe. A typical NMR microcoil is constructed by winding a Cu wire of a suitable gauge around a fused-silica capillary [27] or polyimide sleeve [59] which can house the sample capillary. A range of capillaries with internal diameters in the order of hundreds of micrometers can be used to construct a suitable NMR active volume. In general, the term microcoil denotes an active volume of less than several microliters. The typical length of solenoidal microcoils ranges from 0.3 to 3 mm. While saddle coils are not available in this size range, the saddle coil configuration allows direct insertion of a column or an outlet capillary into the NMR probe. Capillaries with internal diameters of 315 μm (12-mm probe, 900-nl active volume) and 180 μm (2-mm microprobe, 200-nl active volume) have been used to separate mixtures using cLC–NMR instrumentation [58]. As is obvious from probe and capillary dimensions, the poor filling factor decreases the NMR sensitivity. Two flow cells, cylindrical and expanded bubble cells [36] are used in cLC–NMR experiments with the saddle coil configuration to aid concentration sensitivity.

Chromatographic peak volumes for cLC processes range from ~1 μl to ~100 nl. In separation techniques, the detection volume should be limited to one-third the volume of the eluting peak or peak distortion can occur. However, to improve the NMR sensitivity larger active volumes can be used at the expense of chromatographic efficiency.

Besides chromatographic effects, the residence time of an analyte in the NMR flow cell affects NMR performance. The residence time, $\tau$, of an analyte in a continuous-flow experiment can be related to the NMR active volume ($V_{\text{Detection}}$) and the applied flow rate ($F$) [60]:

$$\tau = \frac{V_{\text{Detection}}}{F} \tag{4}$$

As an example, a fixed detection volume of 1 μl and a flow rate of 5 μl/min results in a 12-s residence time. Decreasing the flow rate by half increases the residence time to 24 s allowing a two-fold increase in NMR acquisitions and an ~40% increase in $S/N$. The effective relaxation rates ($T_{\text{n}}$) of flowing spins can also be related to residence time [60]:

$$1/T_{\text{n}} = \sum 1/T_i + 1/\tau \tag{5}$$

The spin-lattice relaxation time ($T_1$) and spin-spin relaxation time ($T_2$) are reduced in a flow system and described using Eqs. (6) and (7) [60]:

$$\frac{1}{T_{1\text{flow}}} = \frac{1}{T_{1\text{static}}} + \frac{1}{\tau} \tag{6}$$

$$\frac{1}{T_{2\text{flow}}} = \frac{1}{T_{2\text{static}}} + \frac{1}{\tau} \tag{7}$$

where $T_{1\text{flow}}$ and $T_{2\text{flow}}$ are spin-lattice and spin-spin relaxation times of spins in a flowing system, respectively. The $T_{1\text{static}}$ and $T_{2\text{static}}$ are relaxation times for spins in a stationary system. In NMR the highest $S/N$ is recorded by choosing a suitable pulse repetition time with respect to the spin-lattice relaxation time. In a static system this is generally in the range of three to five times $T_1$ [61]. In a flowing system, reduction of the effective $T_1$ permits the pulse repetition time to increase. Therefore, NMR parameters must be optimized to record spectra in continuous-flow LC–NMR experiments.

### 2.2. The modes of LC–NMR

#### 2.2.1. On-flow

In this method the NMR spectra are recorded while the sample is flowing through the NMR probe. Normally, results are presented in a two-dimensional display showing NMR spectra as a function of

retention time. This mode tends to preserve chromatographic resolution best; however, the number of scans recorded per retention time is limited preventing the on-flow technique from obtaining the highest $S/N$ and from performing information-rich multidimensional NMR experiments. Continuous-flow cLC–NMR has been reported with sample sizes as small as a few micrograms (nmol) with a 1.1-μl NMR observe volume [62].

The use of solvent gradients to achieve faster/better separation is a common liquid chromatographic technique. On-flow LC–NMR has a unique dependence on solvent composition. In a $D_2O/CD_3CN$ system, a change in $CD_3CN$ composition from 50 to 60% changes many resonance frequencies by ~15 Hz per percentage change in solvent. Therefore even shallow 1–2% gradients may cause the $S/N$ and the spectral linewidths to deteriorate [63]. Fig. 3 illustrates the adverse effects on NMR spectra of solvent gradients moving across the NMR observe volume [50]. The change in solvent composition during solvent gradients changes chemical shifts and degrades the magnetic field homogeneity across the NMR coil. All protons are effected differently; for example, the proton chemical shift of HOD is more affected by solvent composition than the protons of $CHD_2CN$. Both the chemical shift changes and line broadening make it difficult to perform on-flow gradient LC–NMR experiments. Therefore stopped-flow NMR measurement is a common practice with gradient LC–NMR experiments.

### 2.2.2. Direct stopped-flow

In stopped-flow experiments the flow is stopped when the desired analyte peak reaches the NMR coil and the spectra are acquired under static conditions. Typically another LC detector (e.g. UV–Vis) is used to detect and select when to stop the desired peak in the flow cell. The appropriate time delay after detecting the peak allows the analyte peak to reach the NMR coil, before the flow is stopped. This technique allows one- and two-dimensional NMR experiments as well as eliminating the negative effect of solvent gradients on NMR spectral properties. Under static conditions, the magnetic homogeneity can also be optimized to achieve high resolution. However, long NMR experiments often degrade chromatographic separation, especially when more than one peak is to be analyzed within a single run, requiring multiple stopped-flow periods. This is

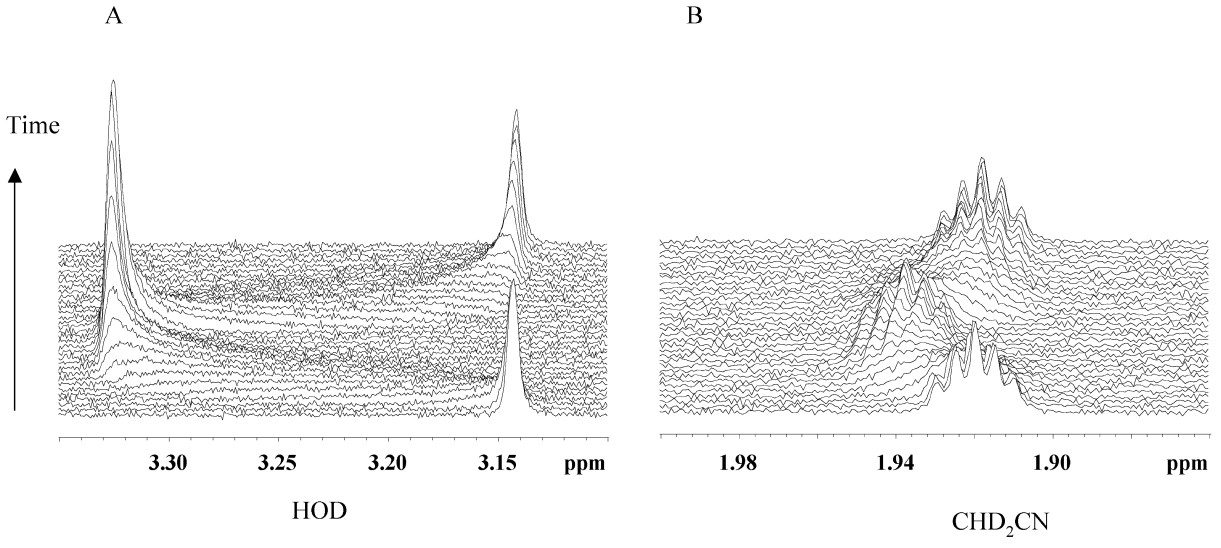


Fig. 3. Stacked plot of $^1$H NMR spectra as a function of time: (A) HOD peak, (B) residual acetonitrile (ACN) peak that shows the effect of a 3-μl injection of $D_2O$-$d_3$ACN (15%, 85%) into $D_2O$-$d_3$ACN (10%: 90%) mobile phase. The flow rate is 3 μl/min. The total NMR experimental time for acquiring each spectrum is 5 s (a relaxation delay of 3 s and an acquisition time of 1.99 s). (Reprinted with permission from Ref. [50]. Copyright 2001 Elsevier)

because diffusion of the analyte occurs during periods of stopped flow, broadening the peaks. However, the relatively smaller I.D. (50–100 μm) transfer line in cLC–NMR minimizes peak broadening [2,58].

### 2.2.3. Loop storage/loop transfer

Unlike the stopped-flow method, in the storage loop method, the separation is continued while extracting the desired peaks using a UV detector for timing. The selected eluant time periods are placed into storage loops for later interrogation by NMR. The individual peaks are then serially transferred to the NMR probe for measurement. This technique also avoids possible peak contamination, as illustrated in Fig. 4 [63]. The retention time difference for peaks 1 and 2 is 0.4 min; under the direct stopped-flow condition, ~20% of peak 1 is carried over to peak 2. However the loop storage/transfer removes the contamination of these two peaks. This method eliminates several of the problems of the stopped-flow method but requires more complex fluidics, and usually dilutes the peaks because of the switching required. This is especially true on the capillary scale where the dead-volume introduced by many capillary connections is especially important.

## 2.3. Applications of cLC–NMR

cLC–NMR is a powerful combination resulting in a method that provides structural information of compounds in complex mixtures; the NMR information complements the information available from cLC mass spectrometry. Examples of cLC–NMR include the analysis of kitols [38], mixtures of vitamins [35], cis/trans isocohumulones [36], amino acids [34,37] and terpenoids [50].

Bayer and co-workers demonstrated the first coupling of capillary LC to NMR [37]. In their work a binary mixture of dansylated amino acids (glutamate and phenylalanine) were separated in under 40 min. Separation was achieved using a $C_{18}$ (120×0.315

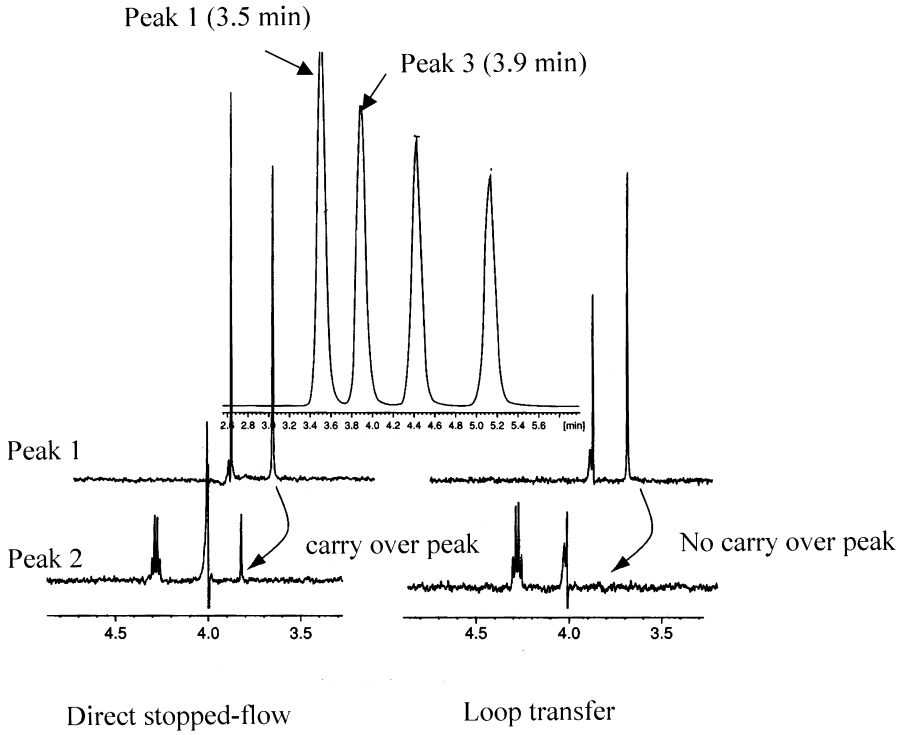


Fig. 4. NMR spectra of closely eluting peaks ($\Delta t = 0.4$ min) under two different working modes, stopped-flow and loop storage/transfer. (Reprinted with permission from Ref. [63]. Copyright 2002 John Wiley and Sons Ltd)

mm I.D.) column with solvent gradient ($D_2O$/deuterated ACN ($CD_3CN$). A 1.2-cm-long saddle type coil and an inset detection cell based on a 2.5-mm microprobe (600 MHz) were used in this study. On-line NMR spectra were recorded with a 900-nl NMR active volume probe for a mixture containing 1.7 nmol of dansyl glutamate and 2 nmol of dansylphenlyalanine. Despite the modest NMR probe filling factor (~1.6%), the LOD achieved with stopped-flow measurements is ~70 pmol in 20 min.

Analysis of submicrogram to microgram quantities of amino acids has been reported with cLC–NMR using a $C_{18}$ (150×0.5 mm I.D.) column and a 1.1-μl observe volume solenoidal probe (500 MHz) [62]. Fig. 5A shows a two-dimensional display of a cLC–NMR chromatograph recorded with online continuous-flow NMR spectra for a mixture of simple amino acids. The extracted one-dimensional NMR spectra shown in Fig. 5B demonstrate the capability to execute continuous-flow NMR with nanomole (less than 5 μg) amounts. It is important to note that each NMR spectrum represents just 12 coadded scans acquired in 12 s. The limit of detection reported in this study for Ala was ~750 ng. This clearly demonstrates the feasibility of using cLC–NMR with solenoidal microcoil-based NMR probe to analyze mass limited samples.

A particularly attractive feature of NMR is its capability to deduce molecular structures. Using this capability a previously unknown kitol isomer has been identified [38]. A capillary with 180-μm I.D. corresponding to 200-nl active volume is used inside a 2-mm microprobe. Fifty micrograms of analytes was injected for separation. The total correlated TOCSY spectrum shown in Fig. 6A and the one-dimensional proton spectra (Fig. 6B) allow the elucidation of the structure of the unknown kitol.

As an added advantage, hyphenated techniques permit the analysis of mixtures with unstable substances. A mixture of vitamin A derivatives which are susceptible to air and/or light has been analyzed with cLC–NMR [35]. In this study both continuous

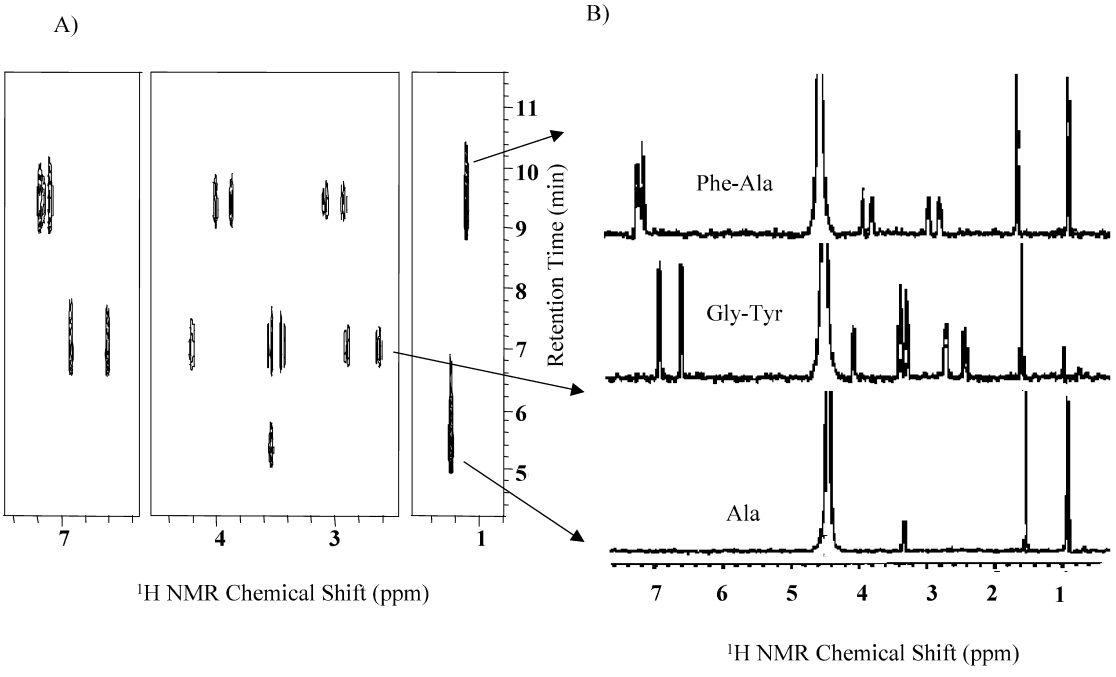


Fig. 5. (A) cLC–NMR chromatogram. Each NMR spectrum represents 12 coadded scans acquired in 12 s. Injected amounts: 2.3 μg (26 nmol) of Ala, 4.8 μg (20 nmol) of Gly–Tyr and 4.8 μg (21 nmol) of Phe–Ala. (B) Extracted $^1$H NMR spectra at each retention time. (Adapted with permission from Ref. [62]. Copyright 1999 American Chemical Society)

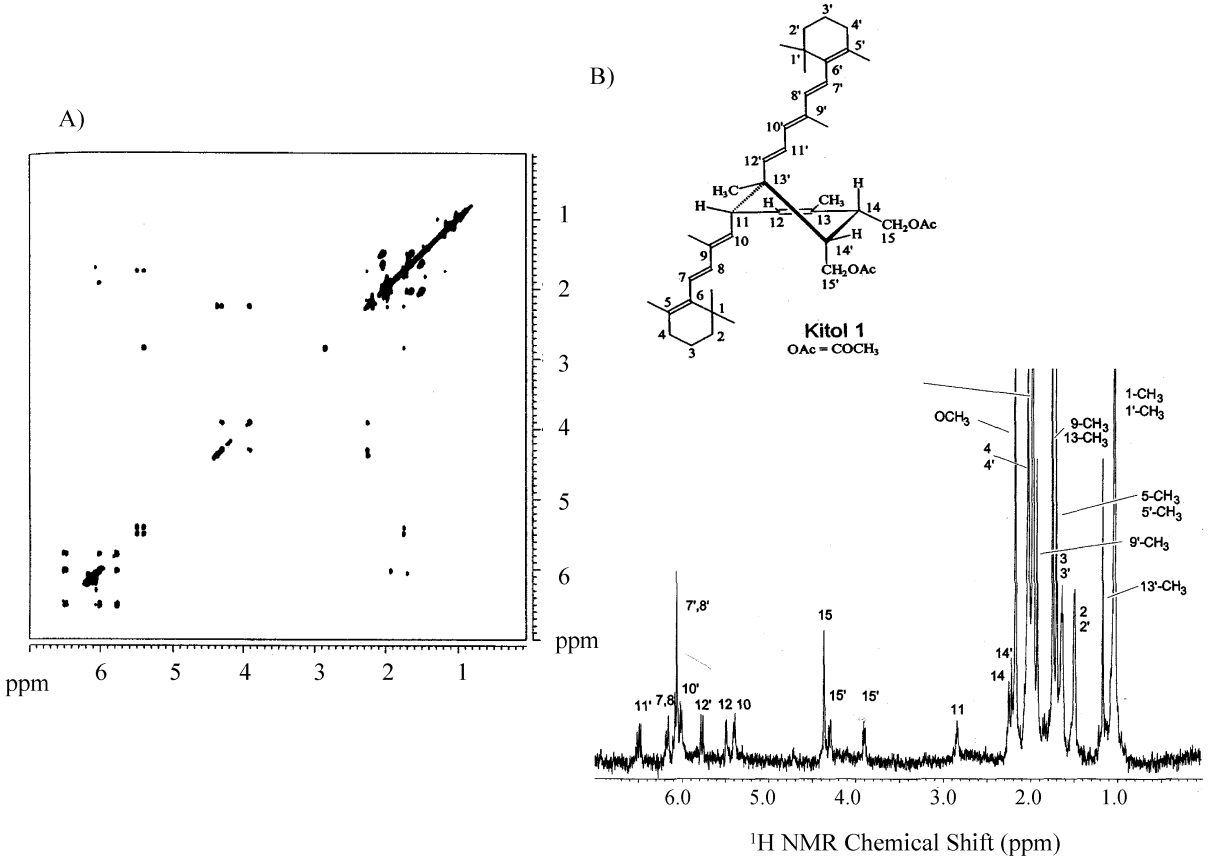


Fig. 6. (A) Stopped-flow TOCSY spectrum of an unknown kitol detected during a cLC–NMR experiment. The spectrum is recorded with 88 transients per increment with a total acquisition time of 15 h using 200-nl observe volume. (B) Stopped-flow $^{1}$H NMR spectrum of the same unknown kitol obtained with 128 scans in 5.3 min. (Reproduced with permission from Ref. [38]. Copyright 1997 American Chemical Society)

and stopped-flow experiments were performed with a column (180 μm I.D., ODS) to separate vitamin A acetate and vitamin A dimerization products.

In these studies, high resolution NMR spectra with 1–2-Hz linewidth have been reported with both solenoidal and saddle coils. The cLC–NMR with solenoidal microcoils confirmed the theoretical and experimental studies of higher mass sensitivity. The saddle coil configuration allows a wide range of observe volumes to be implemented by changing capillaries. In general mass limited samples are best analyzed with cLC coupled to solenoidal NMR microcoils, whereas high concentration sensitivity at the expense of separation efficiency can be achieved with saddle type NMR coils.

## 3. Capillary electrophoresis (CE)–NMR

The low-mass sample requirements with high separation efficiencies and fast separations are keys to the success of CE. Combined CE–NMR can offer the separation capability of CE and the superior detection of NMR, although the smaller volumes and shorter residence time of analytes makes NMR detection problematic in CE.

### *3.1. CE–NMR instrumentation and experimental aspects*

As shown in Fig. 7, the NMR microcoil probe can be coupled to the capillary CE system with no major

modification to the existing CE instrumentation. As in the case of cLC–NMR, magnetic objects must be kept several meters from the magnet depending on the magnetic field strength except with shielded magnets. The non-magnetic plastic buffer vials and Pt electrodes can even be positioned within the magnet bore to perform CE–NMR [43]. In prior CE–NMR studies, the solenoidal microcoil was either wrapped around the capillary or the capillary was housed inside the saddle coil. However, magnetic susceptibility effects degrade line shapes and $S/N$ if thinner-walled capillaries are used as the coil comes into closer contact with the sample. In this case, the capillary is the sample holder as well as the coil form. The number of turns of the solenoidal RF coil and the diameter of the capillary control the length of the NMR active volume. Larger observe volumes can be realized with expanded flow cells. Use of an expanded detection cell improves the sensitivity at the expense of separation efficiency.

### *3.2. Electrophoresis effects on NMR spectral properties*

As stated earlier the solenoidal coils with axis orthogonal to the static magnetic field ($B_0$) form the most mass sensitive RF coils (Fig. 1A). However, in this configuration, the electrophoretic current induces a second local magnetic field gradient that perturbs the magnetic field homogeneity (see inset in Fig. 8). The strength of this induced magnetic field ($B_i$) can be related to the permeability constant, $\mu_0$, the electrophoretic current, $i$, the radial distance from the center of the capillary, $r$, and the capillary I.D., $R$ [20]:

$$B_i = \frac{\mu_0 ir}{2\pi R^2} \tag{8}$$

The magnetic field inhomogeneity from this effect is difficult to restore by shimming procedures employed in NMR to restore magnetic field homo-

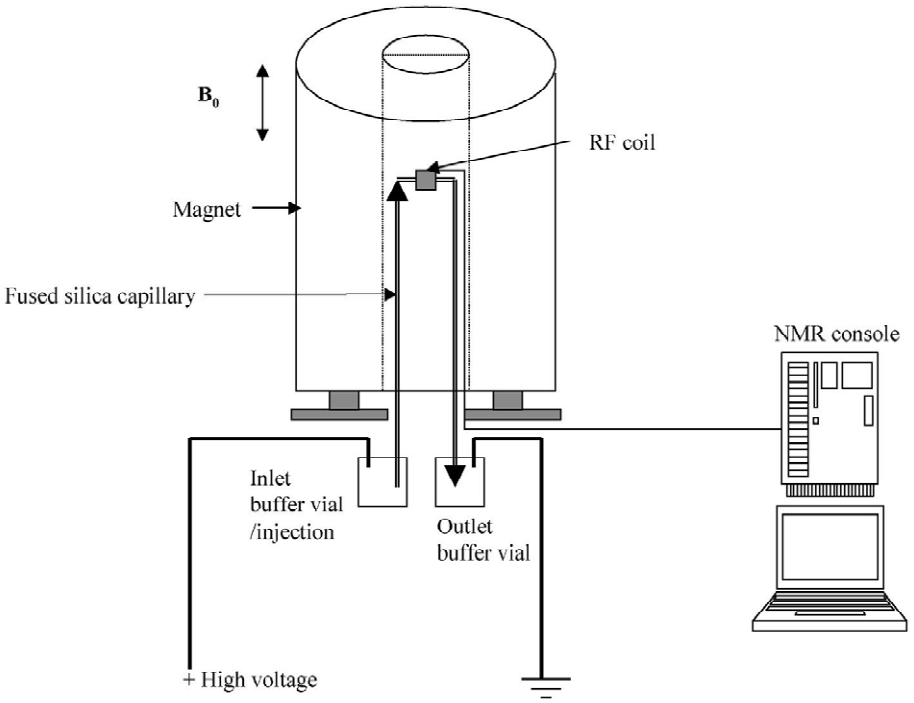


Fig. 7. Instrumental schematic used for CE–NMR showing the arrangement of the separation capillary and NMR detection probe.

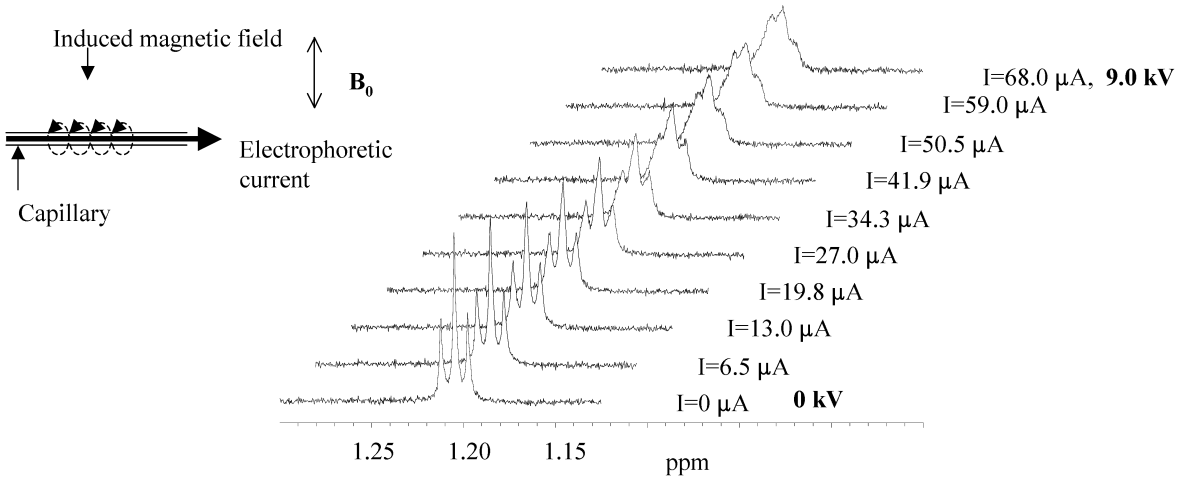


Fig. 8. Microcoil CE–NMR spectra of triethylamine methyl peak in 1 *M* borate buffer with an applied voltage of 0.0–9.0 kV increased by increments of 1.0 kV. The inset shows electrophoretic current induced magnetic field. (Reprinted with permission from Ref. [64]. Copyright 2002 American Chemical Society)

geneity. The effect of induced magnetic field gradient leads to broader NMR lines, loss of scalar coupling, and poor $S/N$. As shown in Fig. 8, the scalar coupling of the methyl triplet of triethylamine disappears with high field magnetic filed gradient created with increasing current [64]. A significant loss of $S/N$ and increase in NMR linewidth are also observed at higher voltages. A voltage increase from 0 to 8 kV causes a linewidth increase from 1.5 to 15 Hz with corresponding $S/N$ decreases from 147 to 19 [43].

A simple answer to the current induced thermal and magnetic field gradient effects is stopped-flow experiments. This technique also allows for the acquisition of time consuming but more informative two-dimensional NMR experiments. A unique technique which acquires NMR spectra under quiescent conditions has been described [43]. In this method, once the analyte reaches the NMR microcoil observe region, the voltage is periodically interrupted and 1-min high resolution NMR spectra with good $S/N$ are acquired for every 15 s of applied voltage. However, the termination of voltage may adversely affect the separation efficiency and reduce peak resolution due to diffusion over the longer period of the experiment.

Fig. 9 illustrates novel CE–NMR instrumentation with a dual microcoil probe to record continuous-flow NMR data under stopped-flow conditions [64]. The dual coil probe is fabricated by individually wrapping two coils around two separate outlet capillaries. The outlet capillaries are connected to the separation capillary through a capillary splitter. In this study, CE–NMR experiments were performed with one outlet capillary while keeping the second capillary at open circuit. A series of NMR spectra obtained by alternating electrophoretic flow between the two coils is shown in Fig. 10. The current induced effects observed in Fig. 8 on NMR spectra are eliminated, because with this instrumentation only the NMR spectra of the sample in the floating capillary are obtained.

The best NMR sensitivity for analytes using a saddle coil probe is achieved when the coil axis is parallel to $B_0$. In this case, the electrophoretic current-induced magnetic field does not contribute to magnetic field inhomogeneity along the $z$-axis. As shown in Fig. 11, high resolution NMR spectra have been reported with similar $S/N$ with varying electrophoretic currents [36]. The slight chemical shift change is attributed to Joule heating at higher voltages. The absence of current induced magnetic field permits on-flow CE–NMR experiments to be performed with saddle NMR probes.

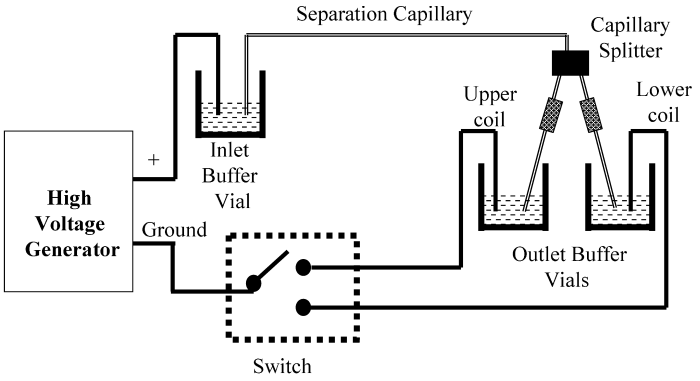


Fig. 9. Instrumental schematic of CE with dual-microcoil NMR detection showing the arrangement of the separation capillary, the two outlet capillaries and the two NMR detection coils. (Reprinted with permission from Ref. [64]. Copyright 2002 American Chemical Society)

The rate of migration of each analyte band in CE is characteristic of electrophoretic mobility, so that each moves past the detector at a different rate. These differential migration rates can influence the NMR signal intensity and linewidth of each migration species. The effective relaxation rates and thus the effect on NMR spectra have been described previously.

The high surface-area to volume ratio in capillary scale (I.D.≤100 μm) electrophoresis dissipates the

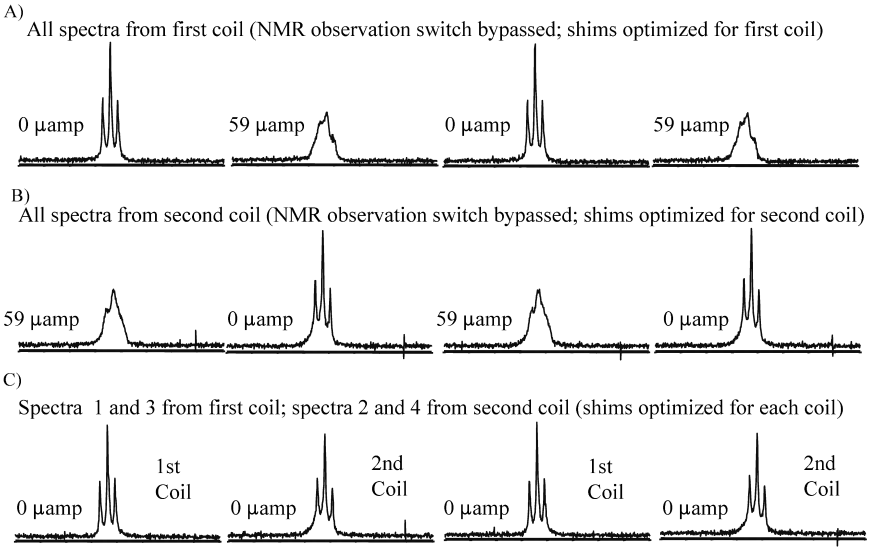


Fig. 10. Array of two-microcoil CE–NMR spectra of the methyl peak of triethylamine in 1 *M* borate buffer. Spectra acquired during alternation of electrophoresis flow between two outlet capillaries. (A) Spectra acquired from the upper coil, with the shim settings optimized for the upper coil and the NMR observation switch by-passed. (B) Spectra acquired with the lower coil, with the shim settings optimized for the lower coil and the NMR observation switch by-passed. (C) NMR spectra acquired from the microcoil on the outlet capillary without electrophoretic flow, with the shim settings optimized for the active coil and the NMR observation switch in-line. (Reproduced with permission from Ref. [64]. Copyright 2001 American Chemical Society)

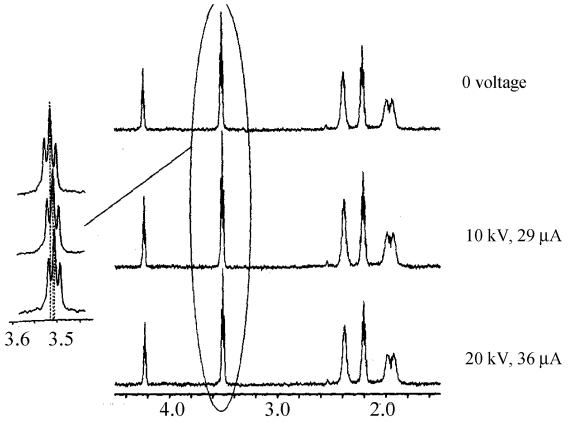


Fig. 11. Static 600-MHz $^{1}$H NMR spectra of lysine under CZE conditions: (1) without voltage, (2) with 10 kV (29 μA), (3) with 20 kV (29 μA). Inset shows slight change in chemical shift due to temperature. (Adapted with the permission from Ref. [36]. Copyright 1998 American Chemical Society)

Joule heat effectively, therefore allowing voltages as high as 30 kV. The high voltages enable faster separations. Because of the insensitivity of NMR compared to other capillary separation detectors, CE–NMR often requires capillaries with a diameter of 75–200 μm to enhance the NMR active volume specially when employing solenoidal coils. However, increases in internal diameter decrease the surface-area to volume ratio. For example a greater than 50% decrease in surface-area to volume ratio occurs when increasing from 75 to 200 μm [65]. The inability to dissipate heat effectively with larger capillaries may cause Joule heating and contribute to chemical shift changes of more solvent sensitive protons [66,67]. This complicates the interpretation of NMR data.

### 3.3. NMR observe volume

Analyte peaks in CE typically contain low nanoliter volumes. High resolution CE electropherograms and NMR spectra can be obtained using nanoliter volume NMR observe volumes; however the obtainable NMR sensitivity often precludes the use of such small NMR active volumes. CE–NMR experiments have been successfully performed with microcoils having observe volumes as small as 5 nl [40,43]. A high filling factor is one of the advantages of using microcoils directly wrapped on CE capillary. However, this excludes the ability to change the observe volume. These problems have been solved with sleeve probe technology in which the solenoidal coil is wrapped around a polyimide sleeve which can house different sizes of CE capillaries [59]. High-resolution spectra with linewidths of 1–2 Hz can be obtained with this new coil fabrication approach. With a saddle coil NMR probe as the online NMR detector, CE–NMR data can be recorded in a continuous mode. Because of the difficulty in fabrication, saddle coils tend to be larger than solenoids, although a 2.5-μl saddle coil probe for static samples has recently been described [32]. By using insets in the larger volume saddle coils, the effective flow cell volume of saddle coils reported for CE ranges from 250 to 400 nl [36,68].

### 3.4. Applications of CE–NMR

The first hyphenation of CE–NMR detected 800 m*M* arginine, had the CE inlet and outlet vials within the bore of the NMR magnet [39], and reported the separation of arginine, cysteine and glycine with detection limits of 50 ng under static modes [40]. In order to record NMR spectra in 16 s, a relatively high injection volume (20 nl) has been used, reducing the separation efficiency. For example the efficiency reported for cysteine is ~5000.

The periodic stopped-flow technique excludes the electrophoretic current induced effects associated with the horizontally-positioned solenoidal coil, and was used to analyze a mixture of arginine and triethylamine (TEA) [40]. Improved concentration LODs for arginine with similar mass sensitivities (57 ng; 330 pmol) and TEA (9 ng; 88 pmol) have been reached with field amplified stacking. Because of the sample stacking and stopped-flow measurement the sensitivity was increased by 2- to 4-fold without compromising the separation efficiency. Separation efficiencies of 50 000 were reported for arginine with periodic stopped-flow.

Continuous-flow CE–NMR with a saddle coil has been used to separate a mixture of lysine and histidine in phosphate buffer [36]. In this report a detection limit of 336 ng (2.3 nmol) has been recorded for lysine. CE–NMR using a saddle coil probe has been used to analyze drug metabolites. The ability to separate and identify drug metabolites

and bio-transformed products in biological fluids is a considerable challenge. CE–NMR analyses of the major metabolites of paracetamol in human urine has been demonstrated [41,42]. In this study CE–NMR successfully analyzed two major metabolites, paracetamol glucuronide and paracetamol sulfate conjugates, as well as endogenous material (hippurate). Fig. 12 illustrates the separation of paracetamol glucuronide and paracetamol sulfate and endogenous material hippurate. The experiment was performed with a 400-nl NMR detection cell at 600 MHz, and used an injected volume of 8 nl for an 80-μm I.D. column (effective length 1 m). Comparison of chemical shifts confirmed the identification of these compounds. The estimated amount that could be detected in this study with a $S/N$ of 3 was ~10 ng.

Often the separation process can be improved by optimizing the buffer pH. Continuous-flow CE–NMR has been used to analyze two commonly found soft drink ingredients, caffeine and aspartame at two pH values [44]. This separation using a glycine buffer at pH 10 was achieved in less than 70 min.

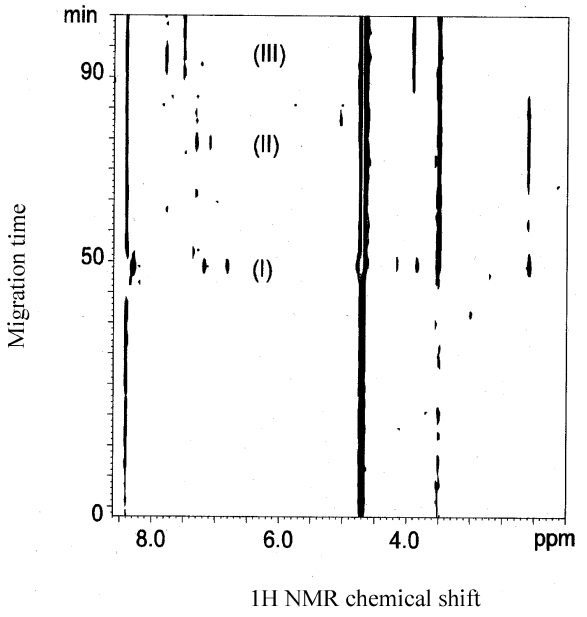


Fig. 12. CE–NMR electropherogram for the separation of paracetamol and its metabolites from a human urine sample: (I) paracetamol glucuronide; (II) paracetamol sulfate conjugate; (III) endogenous hippurate. Data have been acquired with 64 FIDs per increment. (From Ref. [41]; reproduced with permission from The Royal Society of Chemistry)

However, some NMR signals overlap the glycine resonances. Changing the buffer to formate (pH 5) removes the spectral overlap but reversed the order of migration and increased migration time. However at pH 5, both caffeine and aspartame migrate closer to each other. The experimental conditions often are a compromise between separation efficiency and NMR spectral quality. Injections of larger analyte volumes and concentrations greater than the buffer concentration to improve NMR sensitivity usually degrade the separation efficiency of CE.

As described in the section above, online concentration methods can enhance the sensitivity by concentrating the sample during the separation process [43]. Capillary isotachophoresis can concentrate either positively or negatively charged analytes [69]. During the cITP process the analytes are stacked between the leading electrolyte and the trailing electrolyte according to their electrophoretic mobilities. Using cITP, concentration sensitivity enhancements of two orders of magnitude have been reported for on-line nanoliter-volume NMR detection [59]. Fig. 13A shows a reference proton NMR spectrum of 5 m$M$ tetraethylammonium bromide (TEAB) acquired with a solenoidal coil of 30-nl observe volume. Fig. 13B illustrates the cITP–NMR spectrum of 8 μl TEAB with initial concentration of 200 μ$M$. The $S/N$ recorded for TEAB under the cITP condition is twice as high as TEAB without preconcentration. The overall concentration enhancement is ~1000. Furthermore, the sample observation efficiency has also been increased from 0.5 to 50%. The cITP stacking enables the recording of high $S/N$ NMR in a shorter period of time. For example, the spectrum shown in Fig. 13B (inset) represents a stopped-flow COSY spectrum acquired in 22 min.

Wolters et al. further extended the application of cITP–NMR for trace impurity analysis [70]. The application of cITP–NMR is attractive for separating and identifying trace drug metabolites and synthetic organic products. In this study, 200 μ$M$ (1.9 nmol) atenolol, a beta blocker used for treatment of cardiovascular disease, was successfully isolated in the presence of 200 m$M$ sucrose. At the pH of analysis, atenolol is positively charged and therefore is effectively separated from sucrose. As shown in Fig. 14, the intensities of atenolol signals are well below the intensity of $^{13}$C satellite signals of anomeric protons

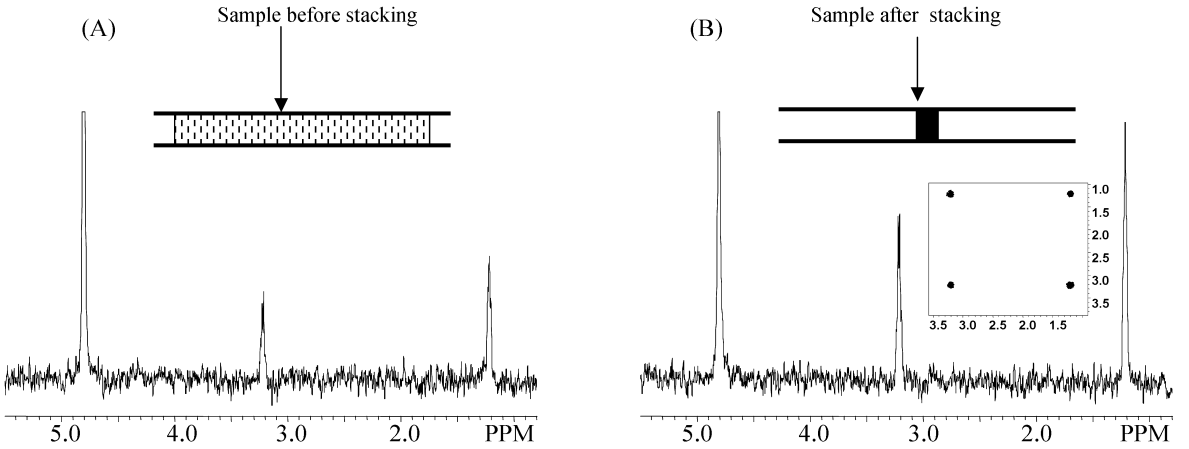


Fig. 13. $^{1}$H NMR spectra of TEAB. (A) Capillary filled with 5 m*M* TEAB without sample stacking (*S*/*N* of peak at 1.2 ppm: 13). (B) 8 μl of 200 μ*M* TEAB injected; spectrum after sample stacking by cITP (*S*/*N* of peak at 1.2 ppm: 30). The inset shows a COSY spectrum. One-dimensional spectral acquisition parameters: acquisition time 1.24 s, 55° pulse, delay time 0 s, eight acquisitions, total experiment time 10 s. (Reproduced with permission from Ref. [59]. Copyright 2001 American Chemical Society)

of sucrose. This clearly demonstrates the difficulty of obtaining good *S*/*N* data for trace amounts in a standard NMR experiment even when there is no spectral overlap. Fig. 14B illustrates a $^{1}$H NMR reference spectrum of 25 m*M* atenolol directly injected to the same coil. The on-flow cITP–NMR spectrum of atenolol (200-μ*M* injected concentration) which has been obtained and processed under the same parameters is shown in Fig. 14C. Comparison of the *S*/*N* of these two spectra shows that the concentration of the focused atenolol band is ~40 m*M*. Capillary scale ITP significantly improves the concentration sensitivity of the most mass sensitive NMR microcoils by allowing microliter samples to be concentrated and measured using 10–100-nl NMR observe volumes. As demonstrated in this study, the cITP–NMR system has successfully analyzed trace materials at ~0.1% in the presence of excess uncharged species.

The narrow (few millimeters) length of the focused analyte bands makes it difficult to trap individual peaks. To solve this problem, new instrumentation with two solenoidal microcoils in a single probe has been designed [70]. The first (scout) coil is used to detect the analyte, while the second coil is used for data acquisition (Fig. 14D). The low electrophoretic mobility in cITP keeps the electrophoretic current at $<10$ μA. Therefore the cITP process permits on-flow NMR data with solenoidal microcoil RF coils.

### *3.5. Diagnostic capabilities of CE–NMR*

Besides compound identification, NMR can be used to prove the separation process. Using NMR, a number of electrophoretic parameters have been monitored. The presence of protonated species in the buffer allows NMR to provide insight into the separation. NMR has followed injection performance, sample plug profile [43], and intra capillary temperature measurements [66]. Such findings will increase our understanding of the dynamic processes occurring during separation.

## 4. Capillary electrochromatography (CEC)–NMR

CEC is a hybrid separation technique that combines the separation efficiency of CE and the selectivity of LC. In addition CEC also provides more sample loading capacity in comparison to CE. With these features CEC has become an attractive analytical tool to separate complex mixtures. The increased loading capacity is an important advantage to improve NMR sensitivity.

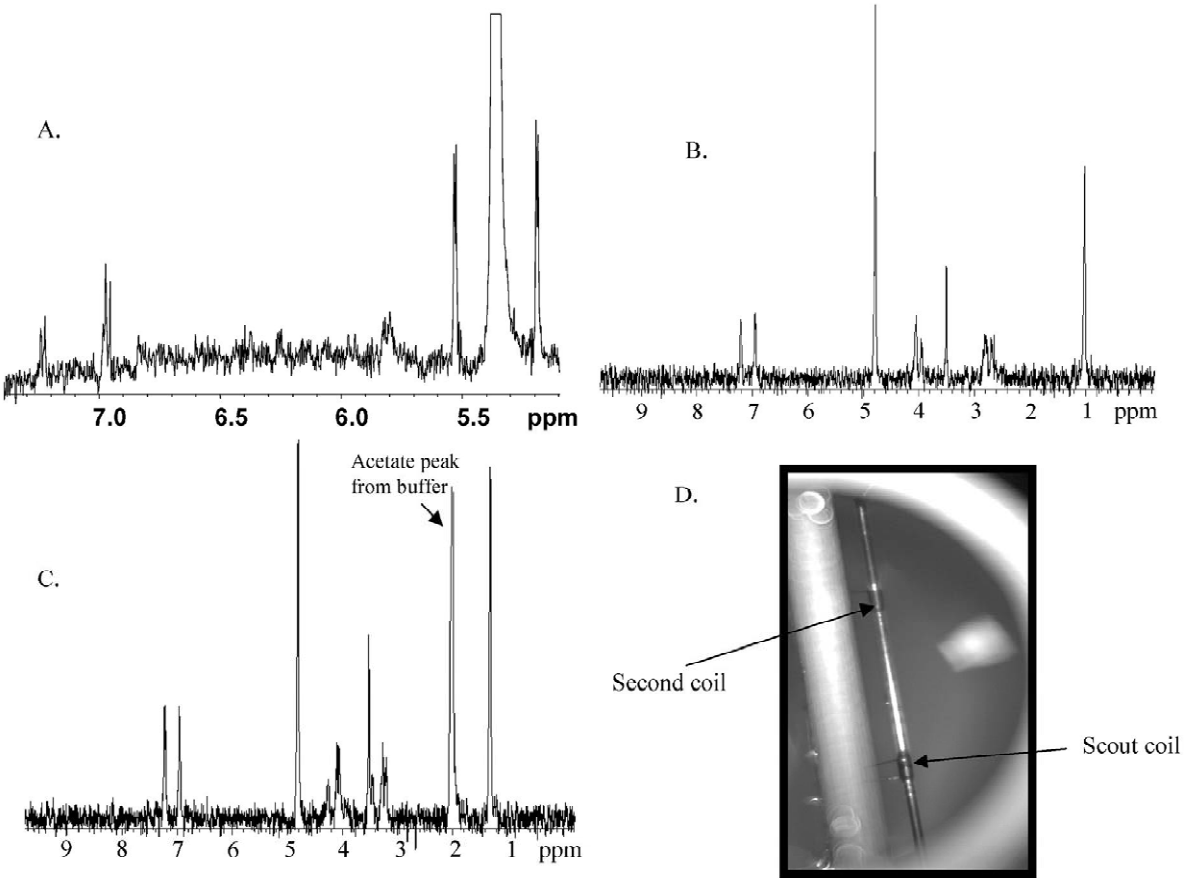


Fig. 14. (A) $^{1}$H NMR spectrum of the trace impurity sample (200 μ*M* atenolol and 200 m*M* sucrose in 50% TE/$D_2O$) from 5-mm probe. The expanded and vertically increased area is shown. (B) Static NMR spectrum obtained with direct injection of 25 m*M* atenolol to the NMR microcoil. The *S*/*N* of atenolol methyl peak is 21. (C) On-flow cITP–NMR spectrum of atenolol at peak maximum during analysis of the trace impurity sample (200 μ*M* atenolol and 200 m*M* sucrose in 50% TE/$D_2O$). No sucrose peaks are observed. The *S*/*N* atenolol methyl peak is 34. Microcoil $^{1}$H NMR spectra shown in (B) and (C) were recorded and processed with the same NMR spectral parameters. (D) Photomicrograph of the two-coil probe, with each wrapped around the same polyimide sleeve. (Adapted with permission from Ref. [70]. Copyright 1998 American Chemical Society)

### *4.1. CEC–NMR instrumentation and experimental aspects*

Fig. 15 illustrates a typical instrumentation schematic used for CEC–NMR measurements. In CEC, the separation is performed using capillary LC columns. The separation is performed with electro-osmotic flow instead of pressure flow. However, low pressures (>30 bar) are also used to prevent air bubble formation at frits and/or achieve faster separation [55]. Integrated instrumentation to perform cLC–, CE–and CEC–NMR has been designed and shown to be a promising tool to analyze complex mixtures [36]. The instrumentation for CEC–NMR is a hybrid between CE–NMR and cLC–NMR and so few additional details are provided here.

### *4.2. Applications of CEC–NMR*

A continuous-flow CEC–NMR experiment has analyzed drug metabolites in human urine [41,42]. The two-dimensional electropherogram in Fig. 16A demonstrates successful separation of three com-

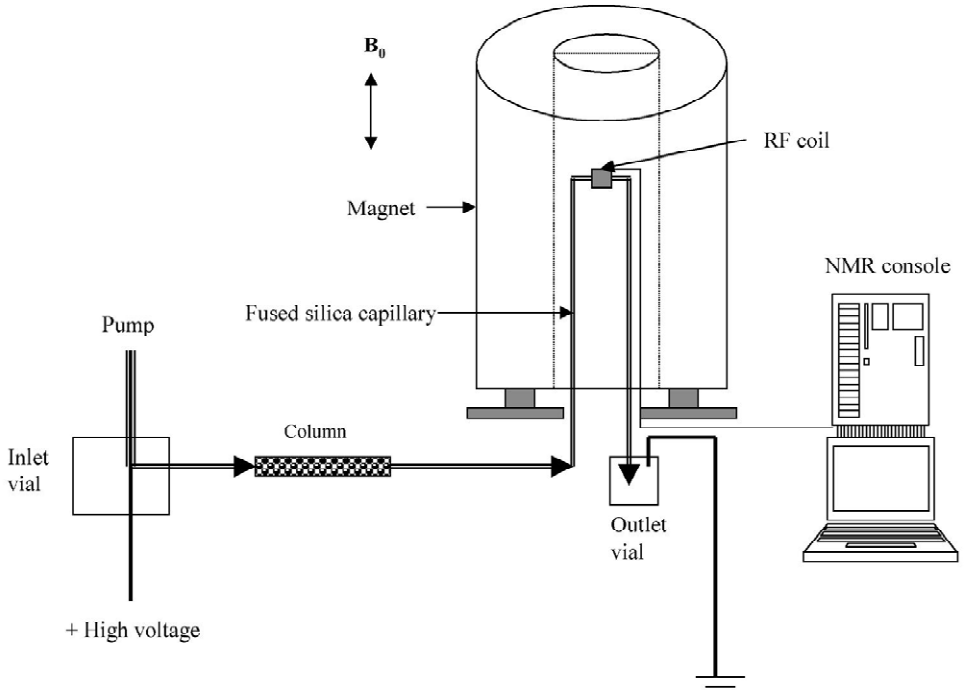


Fig. 15. A typical instrumental schematic for CEC–NMR showing the arrangement of the separation system and NMR detection cell.

ponents in a human urine sample. Taking advantage of the high loading capacity of CEC (a 500-nl sample injection) NMR spectra with good $S/N$ have been recorded. CE–NMR analysis of the same mixture under identical conditions (Fig. 12) yielded poorer NMR spectra. Time-slices of the proton NMR spectra at each migration time are used to identify the three peaks as paracetamol glucuronide, paracetamol sulfate and hippurate (Fig. 16B). The stopped-flow TOCSY further confirms the presence of paracetamol glucuronide [42].

The power of gradient elution has been realized with CEC–NMR while successfully analyzing an analgesic mixture containing caffeine, acetaminophen and acetylsalicylic acid [44]. In this report poorly separated peaks with a solvent peak eluting close to acetaminophen are observed under isocratic conditions (2 m$M$ borate, 80% of $D_2O$ and 20% of $CD_3CN$). The large sample injection volumes used to improve NMR sensitivity appear to have caused the separation efficiency to deteriorate. By using a solvent gradient of 0–30% $CD_3CN$ in 25 min, an improved separation has been achieved in a shorter time period.

In addition to the electrophoretic flow, pressure can also be applied during CEC to decrease the separation time [55]. Pressurized capillary electrochromatography (pCEC) coupled to NMR separated and identified a mixture of unsaturated fatty acid esters. The total analysis time was reduced by a factor of 10 compared to non-pressurized CEC. The plate heights increase with increasing pressure in pCEC; however, the plate height recorded even at 100 bar is still below that recorded with cLC. To compare the same analysis, cLC–NMR operating at 16 bar separates the mixture in 110 min, whereas pCEC–NMR (16 bar and 20 kV) analyzed the mixture in 13 min. However, better resolution was achieved with cLC. Further pressure increases during pCEC do not shorten the migration time significantly but cause the separation efficiency to deteriorate. The reduced separation time as in pCEC provides extra

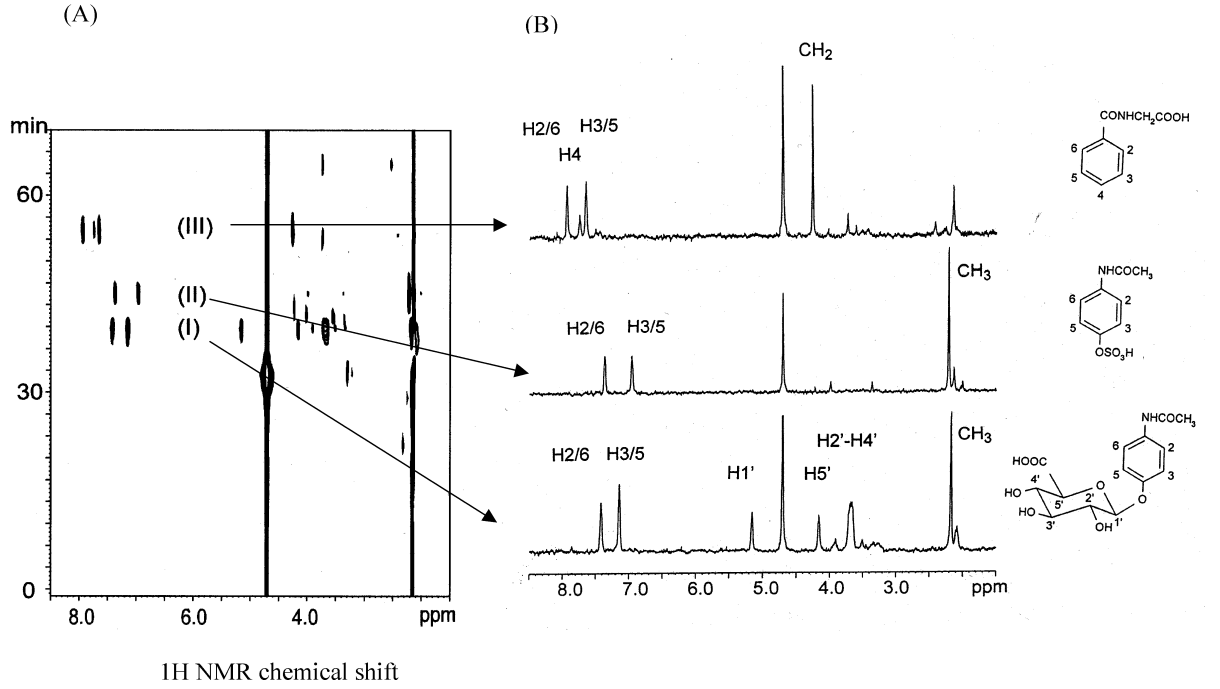


Fig. 16. (A) Contour plot of a two-dimensional electropherogram recorded on-flow with CEC–NMR for separation of human urine extract. (B) Single rows extracted from (I, II and II) the continuous flow CEC–NMR from Fig. 16A: (I) paracetamol glucuronide; (II) paracetamol sulfate conjugate; and (III) endogenous hippurate. (From Ref. [41]; reproduced with permission from The Royal Society of Chemistry)

time to obtain more chemical information with multidimensional NMR experiments.

### *4.3. Diagnostic capabilities of CEC–NMR*

As in CE–NMR, CEC–NMR allows one to follow the separation process, although far fewer reports of this use have appeared. The frit used to contain the packing material creates a region of increased electrical resistance, which leads to localized temperature rises. A recent study using CEC–NMR has revealed the temperature events before, after and within the frit [67]. A better understanding of CEC process will assist efforts to improve its separation capability and the reproducibility of CEC–NMR.

## 5. Conclusions and future of capillary separation–NMR

Hyphenated capillary separation techniques, cLC–NMR, CE–NMR and CEC–NMR, are capable of analyzing complex mixtures while providing structural details of analytes. The improved efficiency at the capillary level benefits the enhanced NMR sensitivity. Capillary techniques are especially useful for analyzing mass limited samples as required in pharmaceutical analysis, biomedical research and environmental analysis. Sample amounts as small as picomoles have been analyzed with capillary–NMR techniques. The cLC–NMR instrument is becoming an accepted analytical tool in the pharmaceutical industry. Although it is not commercially available, integrated cLC–, CE–and CEC–NMR instrumentation provides data with complementary separations.

NMR sensitivity must be increased to improve the utility of NMR as the detector for these capillary separations. The miniature size (~1 mm in length) permits more than one solenoidal coil to be used within the homogeneous region of the static magnetic field [71,72]. Multiple detectors on a single column or capillary can record the NMR spectra of a single migration band at multiple points. A multiple coil system can also be developed so that separated

peaks are parked and probed with the NMR spectrometer while continuing the separation. Such approaches increase the overall flexibility of the hyphenated system. Acquiring data from multiple samples simultaneously, greatly increases NMR throughput. The multiple coil approach will be ideal to perform two-dimensional separation using two separation techniques (cLC and CE).

Higher field strength magnets will continue to become available, increasing the performance of NMR. Applications of CE–NMR and CEC–NMR to analyze peptides, proteins and complex mixtures have not been reported extensively. For example, the separation and identification of molecular classes can be performed with these techniques. Such approaches can minimize long analytical procedures involved in metabonomics and proteomics. Carbohydrate analysis will greatly benefit from CE and CEC with NMR as an on-line detector because NMR does not require the presence of a chromophore. Also exciting is the trend to hyphenate multiple techniques such as LC with NMR, MS and even infrared spectroscopy [73]. We expect this trend to accelerate, with such hyphenated combinations occurring on the capillary scale in the not too distant future. We also expect probes with the ability to analyze additional nuclei such as $^{13}C$, $^{31}P$ and $^{15}N$ to be implemented in the near future, further increasing the analytical utility of this combination of capillary scale separation and NMR detection.

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