Hyphenated NMR techniques

Julie R. KESTING, Kenneth T. JOHANSEN and Jerzy W. JAROSZEWSKI
Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark

Abstract. This chapter describes principles and applications of on-line coupling (hyphenation) of NMR spectroscopy with liquid-chromatographic and other separation methods as well as with other forms of spectroscopy. This results in highly integrated and automated systems for structure determination of components of complex mixtures at microgram and nanogram levels. Hyphenated NMR techniques provide accelerated and detailed insight into components of complex mixtures of biological origin without initial separation of the constituents.

Keywords. Hyphenated techniques, flow NMR probes, NMR sensitivity, cryogenic probes, chromatography, solid-phase extraction, capillary HPLC, electro-driven separations, gas chromatography

Introduction

In many areas of chemistry and biology there is a need for identification of components of complex mixtures, often available in very small amounts. The aim of hyphenation of a separation technique with a detection method is to gain maximal on-line information about individual constituents of a mixture as rapidly as possible. Hyphenated chromatographic techniques commonly employ separation by liquid chromatography (LC) or gas chromatography (GC) with detection by a photo-diode array (PDA) detector or a mass spectrometer (MS). Although PDA is an almost standard part of any HPLC installation, the structural information gained from this detector is very limited, which is also true for some of the more specialized detectors available such as fluorescence detectors (FLD), evaporative light scattering detectors (ELSD), and others. MS detection is very valuable because of its sensitivity. When employed in the electron-impact ionization mode, normally available only in conjunction with GC, it provides compound fingerprints useful for computer-based recognition, and also for structure determination of unknown compounds based on fragmentation patterns. When hyphenated with an LC separation, the common MS ionization techniques are electro-spray ionization (ESI) or atmospheric-pressure chemical ionization (APCI). These techniques provide very important information about molecular weights of compounds separated by LC, especially when implemented in high-resolution mode or aided by identification of isotope peaks. Apart from that, however, the structural information obtainable form LC-MS is very limited in comparison to that obtainable by NMR spectroscopy, even if additional information provided by tandem MS methods is included.

1 Corresponding Author.
Thus, only NMR spectroscopy, with its sophisticated, modern repertoire of 2D experiments provides truly comprehensive structural information needed for a definitive identification of organic compounds, especially in the case of new compounds. In this review, principles and applications of hyphenated chromatographic techniques involving NMR as a detection method are discussed. NMR has been coupled to a variety of LC techniques, including standard high-performance liquid chromatography (HPLC) but also capillary separations (CapHPLC), supercritical fluid chromatography, ion-exchange liquid chromatography, centrifugal partition chromatography, gel permeation chromatography, and electro-driven separations, as well as to GC. Being a dominant LC technique, HPLC remains to be the main separation technique used in hyphenation mode together with NMR, and hence HPLC-NMR is the primary focus of this review [1-6].

1. General considerations

The combination of separation methods and NMR spectroscopy was introduced in the late 1970s when the use of NMR in organic chemistry had already become widespread [7-11]. The initial hyphenated NMR experiments involved iron magnets but the advantage of superconducting coils was soon recognized [12, 13]. However, due to sensitivity issues it took almost two decades before improvements in probe design and general NMR technology enabled hyphenated NMR to produce data that are competitive in comparison to classical NMR experiments with compounds purified by chromatography in a separate workflow. A typical HPLC-NMR setup is based on a conventional HPLC system with binary, ternary, or quaternary pumps, RP-columns and a UV or PDA detector. The chromatograph is coupled on-line with the NMR spectrometer, creating a demand for appropriate flow-probe design. This equipment will usually be supplemented with a parallel MS detector to provide additional chromatographic traces (total ion current, extracted ion chromatogram, etc.) and complementary structural data (molecular ions and their fragments).

When hyphenating $^1$H NMR to HPLC, the solvent used in the chromatographic step is one of the major limitations. Since most of the solvents routinely used in HPLC contain hydrogen, the mobile phase will give rise to NMR signals $10^3$-$10^6$ stronger than those of the compounds present in the chromatographic elution bands. This requires application of solvent peak suppressing pulse sequences, such as the pulse sequence corresponding to one increment of the NOESY sequence with presaturation during relaxation delay and mixing time, typically used in 1D NMR, or the pulsed field-gradient-based WET sequence (water suppression enhanced through T$_1$ effects) used for 1D and 2D spectra [14]. These sequences are often applied with $^{13}$C-decoupling in order to collapse $^{13}$C-satellites of the solvent signals. Although this solves the dynamic range problem, the solvent peak suppression introduces another problem, since resonances under and in the vicinity of the solvent peak are likely to be suppressed as well, complicating structure elucidation. The strong solvent signal can also be eliminated by use of deuterated solvents for chromatography, but this is normally limited to deuterated water because of high cost of organic deuterated solvents. Use of non-deuterated organic additives such as amines or chiral auxiliaries is likely to compromise spectra of the analyte.

Hyphenation of NMR with LC methods requires optimization of the chromatographic step to maximize concentration of the analyte in the detection cell.
This calls for high column loading and narrow elution bands in order to maximize analyte concentration and to match the volume of the NMR detection cell as closely as possible. Broad and unsymmetrical chromatographic peaks will diminish the analyte concentration in the eluate and either compromise the NMR experiments altogether or greatly prolong the time necessary for data acquisition, especially in the case of 2D NMR spectra.

2. Sensitivity

In the field of hyphenated NMR, sensitivity is one of the major concerns due to small sample amounts. The S/N ratio can be increased through accumulation of transients. This is however, a time-consuming process as the S/N ratio is proportional to the square root of the number of transients. The noise generated in an NMR experiment arises from three main sources: thermal noise from the receiver coil in the probe, thermal noise from the preamplifier, and from the sample itself. Mathematically, the S/N ratio can be described as follows [15, 16]:

\[
\frac{S}{N} \propto \frac{N \times \gamma_e \sqrt{\gamma_d} \times B_0 \times B_c}{\sqrt{4k_B \times \Delta f \times [R_c(T_c + T_a) - R_e(T_e + T_a)]}}
\]

As seen from Eq. (1), NMR signal strength depends on factors related to the sample such as number of nuclei \(N\), the magnetogyratic ratio of the excited nuclei \(\gamma_e\), the magnetogyratic ratio of the detected nuclei \(\gamma_d\), the applied magnetic field \(B_0\), and on the coil design \(B_1/I_{coil}\), described by the ratio between \(B_1\) field of the radiofrequency pulse and coil current \(I_{coil}\). The noise generated depends on the resistance and temperature of the detection coil \(R_c\) and \(T_c\), respectively, and of the sample in the coil \(R_s\) and \(T_s\), as well as on the temperature of the amplifier \(T_a\). Furthermore, Eq. (1) contains the receiver bandwidth in Hz, \(\Delta f\), and Boltzmann's constant \(k_B\). The thermal noise is generated by the receiver coil due to stochastic motion of electrons in the conducting medium, described by \(R_c(T_c + T_a)\). Therefore, the noise decreases at lower temperatures. The cryogenic probe technology where the coil and preamplifier are cooled by helium gas, takes advantage of this dependence. For samples of low conductivity, a cryogenically cooled probe with low \(R_e\) and \(T_e\), reduces the noise by a factor of two. If the preamplifier is cooled as well (low \(T_a\)), noise is reduced by another factor of two [17]. The influence of cryogenic cooling, magnet strength and probe design on advances in NMR sensitivity is illustrated in Figure 1. The launch of a 1000 MHz NMR spectrometer in 2009 with a cryogenic probe operating at this field is a recent breakthrough in the quest for improvement of NMR sensitivity.

Since hyphenated NMR systems operate with small samples, advantage is taken of the fact that mass sensitivity of NMR coils is roughly inversely proportional to the coil diameter [15, 18, 19]. Thus, flow probes with sensitive volume down to 20 µL and capillary probes with sensitive volumes down to a fraction of µL are routinely used.
Figure 1. S/N ratio measured with 0.1% ethylbenzene in CDCl₃ for ¹H-observe probes as a function of time. The black dots represent the sensitivity of conventional probes at particular fields. The triangles mark the launch of cryogenically cooled probes at a given field. The dashed line indicates the increase in sensitivity of conventional 500 MHz probes over two decades (reproduced from ref. [17] with permission of the copyright owner).

3. NMR flow probes

Solution-state NMR data are traditionally acquired using tubes oriented along the B₀ field, and placed in Helmholtz-type radiofrequency coils that generate pulses of transverse B₁ field. This vertical design is most often preserved in NMR flow cells (Figure 2), which usually use tubes of 2-4 mm in diameter (corresponding to 30-120 µL sensitive volume, which is approx. half of the total cell volume) connected to inlet and outlet capillaries. Vertical flow cells can be placed inside a 5 mm cryogenic probe for increased sensitivity.

Figure 2. Vertical NMR flow probe with Helmholtz (saddle) coil (left) and horizontal capillary NMR cell with solenoid wrapped directly on the capillary (right).

Smaller cells are characterized by increased mass sensitivity and help avoid peak mixing and broadening, but diminish, for a given flow rate, the residence time τ (ratio
between cell volume and flow rate) of the sample in the cell. This not only diminishes the time available for acquisition of on-flow data, but results in line broadening due to increase of effective $T_2$ relaxation time according to Eq. (2). As seen from Eq. (2), a decrease in $\tau$ will decrease both $T_1$ and $T_2$.

$$\frac{1}{T_{\text{effective}}} = \frac{1}{T_n} + \frac{1}{\tau} \tag{2}$$

The initial LC-NMR experiments used a modified traditional probe where the mobile phase from the LC was transferred to an NMR tube via a capillary and subsequently led away through another capillary [7]. This allowed the sample to be spun but the setup introduced an increased risk of peak mixing. The bubble cell, which was introduced soon afterwards, employed the characteristics of a conventional flow cell [8]. In most HPLC-NMR studies, flow cells have been used, but in some cases this option has been abandoned in favor of eluting directly into NMR tubes, thereby maintaining high sensitivity [20, 21]. This introduces an added versatility in terms of conducting further experiments at a later stage if necessary, as well as the possibility of running several samples in parallel on existing NMR instrumentation. The collection can either be done manually, in a semi-hyphenated setup, or fully automated.

During the 1990s, solenoidal microcoil capillary NMR probes (Cap-probes) with sub-microliter sensitive volumes have been developed [22]. Cap-NMR probes are compatible with hyphenation with capillary electrophoresis (CE-NMR) and capillary LC-NMR with advantages such as high sensitivity and low solvent consumption, allowing use of deuterated solvents in the chromatographic step [23, 24]. Moreover, a CapNMR probe was also used in GC-NMR with a limit-of-detection of 200 µg for 2D experiments [25].

Supercritical fluid chromatography (SFC) is also an important separation method, but in order to fit the requirements of this technique, special SFC-NMR probes had to be developed. Due to the phase characteristics of the CO$_2$ used as mobile phase, NMR detection has to be conducted at temperatures above 305 K and pressures higher than 73 bar [26]. The first NMR probe hyphenated to SFC was announced by Allen et al. in 1988 [27]. This pioneering design combined a premagnetization volume for equilibrating the Boltzmann distribution together with a pressure-stable flow cell. More recent designs are derived from the flow cell used in HPLC-NMR probes where the inner glass tube of the original HPLC-NMR probe is substituted with a titanium flow cell and polymeric tubing is replaced by titanium [28].

4. HPLC-NMR

HPLC separations and NMR experiments have usually widely different time scales; while the former can often be performed in minutes, acquisition of comprehensive sets of 2D data from several peaks in a chromatogram may require many hours or even days. For these reasons, HPLC-NMR experiments are performed in a number of different formats (continuous-flow, stopped-flow and loop-storage modes as well as hyphenation with solid-phase extraction, HPLC-SPE-NMR), discussed below. A schematic diagram of a modern hyphenation system, accommodating various modes of operation, is shown in Figure 3. Such system can operate in HPLC-SPE-NMR mode as
well as in direct hyphenation modes. As already mentioned, NMR hyphenation systems typically include a parallel mass spectrometer in addition to the serially connected PDA detector [20, 21, 29-40], of particular advantage in case of non-UV-absorbing compounds. If an integrated mass spectrometer is not available, advantage is taken from parallel HPLC-NMR and HPLC-MS runs using separated systems [23, 30, 41-51]. The HPLC-SPE-NMR system may also include an additional UV or PDA detector placed after the SPE unit for detection of analyte break-through during SPE trapping [21, 52].

Figure 3. Schematic description of a hyphenated NMR system consisting of a liquid chromatograph module (HPLC), flow splitter, photodiode-array detector (PDA), NMR spectrometer, and a parallel mass spectrometer (MS). Alternative solvent lines for continuous-flow, stopped-flow, loop-storage and SPE operation modes are illustrated with solid arrows. MP = chromatographic mobile phase.

4.1. Continuous-flow HPLC-NMR

As the analyte resides only for a short period of time in the NMR flow cell during continuous-flow experiments, the limit-of-detection is no less than 10 µg (compounds with MW 300-500) and the technique is limited to quick 1D experiments. Nevertheless, it is still used as a means of getting a preliminary overview of major constituents in a sample [53-62]. Continuous-flow HPLC-NMR experiments have been widely used in studies of natural products [53-60, 62-66]. The majority of studies used gradient based reversed-phase C18-column separations with CH3CN:D2O as the mobile phase. Some of the studies employed large (up to 125 mm × 8.0 mm) columns enabling loading of up to 10 mg of extract and with flow rates down to 0.1 mL/min to extend the analyte residence time in the 60 µL flow cell used [60, 64-66]. Other studies employed continuous-flow HPLC-NMR experiments using isocratic elution with CH3CN:D2O at 0.7-0.8 mL/min, 250 mm × 4.6 mm columns, and 120 µL flow cells operated at 600 MHz [54, 57, 58]. Continuous-flow HPLC-NMR has been used for analysis of beverages [61, 67] and in connection with high-loading capacity C30 columns [68, 69].

Attempts have been made to acquire homo- and heteronuclear 2D NMR data with only one or a few transients as described by Frydman and co-workers [70-72] and Kupče and Freeman [73-78]. The rapid 2D NMR technique presented by Frydman and co-workers involve heterogenic initial excitation as opposed to the homogeneous excitation usually employed in 2D experiments and utilize position-dependent evolution of spins. The method has been employed for acquisition of 2D data in a continuous-flow mode on a home-built LC-NMR system at 500 MHz with a 100-µL
In this study, a mixture of 30 mg of each of three low-molecular compounds was analyzed giving an average concentration in the flow cell of 15-20 mM in the active cell volume.

Another rapid acquisition technique uses multichannel excitation and detection of NMR signals in the frequency domain according to Hadamard matrix [73]. The Hadamard-based fast 2D acquisition method distinctively differs from the conventional 2D NMR experiments by detection in the frequency domain. Traditional 2D experiments explore frequencies indirectly by allowing an evolution period before data collection in the time domain. All possible frequencies are sampled although most of these only carry noise. Hadamard-based 2D NMR experiments require prior knowledge of NMR frequencies in the sample from 1D experiments. An encoding scheme based on Hadamard matrices is used for simultaneous multichannel excitation of signal-bearing regions, creating NMR signals in the receiver. These signals can then be disentangled by referencing the chosen encoding scheme. The Hadamard approach was employed in a conventional continuous-flow setup analyzing a urine sample with acquisition of TOCSY spectra of the major components (4.9-6.1 mM concentrations of amino acids) with a 60 µL active volume flow cell at 500 MHz [78]. The sensitivity of the Hadamard method is higher than that of the method based on spatial encoding, but while the latter can be used on completely unknown samples, the Hadamard method requires prior knowledge of the analyte spectrum to define the encoding Hadamard frequency matrix.

4.2. Stopped-flow HPLC-NMR

In stopped-flow HPLC-NMR, the mobile phase flow is paused when a particular peak of interest reaches the flow cell in order to perform the necessary NMR experiments. Peak selection and positioning within the NMR probe is commonly guided by UV absorption, although MS signal can also be used. In addition to increased analysis time in the NMR experiment itself, the stopped-flow experiments allows for individual careful optimization of field homogeneity and acquisition parameters. Since the entire analyte band rarely resides fully within the flow cell and due to probe design with thin tubing on either side of the cell, it is possible to perform very time-consuming experiments with hardly any loss of the analyte due to diffusion out of the cell. When all the necessary experiments have been conducted, the flow is resumed until the next peak of interest reaches the NMR cell. Although the stopped-flow technique enables collection of a lot of data on-line, the price to be paid is a risk of diffusion-mediated band broadening in the chromatographic column. This diffusion can be manifested both as a decrease in concentration and as collapsing peaks. To circumvent these effects, one can acquire NMR data from different chromatographic peaks using successive injections, provided that a sufficient amount of the sample is available. Numerous stopped-flow HPLC-NMR experiments have been reported in the literature. These are usually preceded by continuous-flow experiments and the chromatographic conditions are usually similar, with CH$_3$CN:D$_2$O as the most often used eluent. Again, natural products are the most popular area of applications of the technique [53, 54, 56-58, 63, 64, 66, 68, 69, 79-86]. Stopped-flow HPLC-NMR was also used to obtain TOCSY and NOESY spectra of components of an artificial protein mixture (cytochrome c and lysozyme) separated on a 50 x 4.6 mm cation-exchange column, but the amounts of the proteins used were rather large [87]. Other examples include analysis of bile and urine samples from rats and dogs dosed with an anti-tumor agent [88] and study of human
amniotic fluid [89], in both cases using 500 MHz spectrometers and 60 or 120 µL sensitive volume flow cells, respectively.

An extension of the stopped-flow mode is the time-slice experiment, where the flow is stopped at regular intervals. The major advantage is that it effectively eliminates the dependency of a detector to position the sample in the flow cell. Time-slice stopped-flow HPLC-NMR experiments have been used in several studies [61, 88, 89].

4.3. Loop-storage HPLC-NMR

Loop-storage HPLC-NMR could be characterized as intermediate between on-line and off-line hyphenation. By sealing and storing chromatographic elution bands of interest in capillary loops for later NMR analysis, the need of stopping the flow and thus the risk of diffusion-mediated band broadening on the column is eliminated. Another advantage is that it is possible to thoroughly wash the NMR flow cell between analyses to avoid cross-contamination. A possible drawback is the risk of degrading compounds from long-term storage of the solubilised compounds in capillary loops. Loop-storage HPLC-NMR experiments have been employed using a variety of sample types [67, 90-93].

5. HPLC-SPE-NMR

HPLC-SPE-NMR is the newest approach in the field of hyphenation of HPLC and NMR. In direct HPLC-NMR techniques, e.g., continuous-flow, stopped-flow and loop-storage HPLC-NMR, the NMR data are acquired using analytes dissolved in the mobile phase from the chromatographic separation. This still requires suppression of the large solvent peaks. Moreover, chromatography sets the upper limit for the amount of material available for the NMR experiments, e.g., the NMR experiment is performed using a solution of the analyte at a concentration deliverable in the chromatographic elution band following a single injection to the column. These limitations are circumvented by the HPLC-SPE-NMR technology.

5.1. Principles of HPLC-SPE-NMR

A dramatic improvement of the direct HPLC-NMR hyphenated techniques was the introduction of an automated solid-phase extraction step (SPE) between the HPLC module and the NMR spectrometer. Several studies set stage for the present-day commercial systems. In 1986, Wilcox and Phelan reported a procedure where an automated fraction collector was connected to a reversed-phase HPLC column, the fractions were diluted with water, analytes were trapped on C18 SPE cartridges, and finally the separated compounds were eluted with deuterated solvents for data acquisition using a 250 MHz NMR spectrometer [94]. All individual processes were performed off-line, but the procedure itself can be regarded as a forerunner of modern HPLC-SPE-NMR experiments. Analyte concentration on a trapping column was exploited in 1998 by Griffiths and Horton [95]. They demonstrated that the post-chromatographic column trapping led to an increased S/N ratio in the NMR spectra, further improved by use of a deuterated solvent for back-flushing of the trapping
column, which, however, was not dried prior to desorption of the trapped analyte. Nyberg et al. employed in 2001 post-column off-line SPE analyte trapping followed by elution with a deuterated solvent into an on-line NMR flow probe [96, 97].

Modern HPLC-SPE-NMR systems are operated in a fully automated mode by hyphenation software. After chromatographic separation typically on a reversed-phase HPLC column using non-deuterated solvents, the peaks of interest are directed to small (typically 2 x 10 mm) SPE cartridges with a sorbent capable of retaining the analyte. Stop and start signals for this SPE trapping can be provided in a variety of ways including UV absorption thresholds at multiple wavelengths, MS signal (total ion current level or extracted ion chromatogram level), or chromatographic retention time. Alternatively, the trapping can be triggered manually. SPE trapping in a time-sliced manner is also possible and can be used to ensure that sections of chromatograms with no apparent peaks or with many weak peaks do not contain NMR-detectable but non-UV-absorbing or poorly ionizable species [33, 37]. A make-up flow of water is added post-column to the mobile phase for lowering the eluotropic strength, hence facilitating the SPE process. Subsequently, the SPE cartridges containing analytes from individual HPLC peaks are dried using pressurized nitrogen gas, after which the compounds are eluted with a small volume of deuterated solvent for NMR analysis (Figure 4).

![TRAPPING](image)

![ELUTION](image)

**Figure 4.** Principles of HPLC-SPE-NMR. Compounds eluted from the chromatographic column are directed to individual SPE cartridges and subsequently eluted into the NMR flow probe. Alternatively, the SPE eluate can be collected in NMR microtubes.

**5.2. Advantages of HPLC-SPE-NMR**

The principal advantage of HPLC-SPE-NMR, in addition to replacement of a non-deuterated mobile phase with a deuterated NMR solvent, is analyte focusing. Thus,
analytes are typically eluted from HPLC columns with internal diameter of 4.6 mm in at least 300 µL of mobile phase, often considerably more. On the other hand, the bed volume of a 2 x 10 mm SPE cartridge is 31 µL, and the adsorbed analyte can be stripped off the SPE stationary phase with a few tens of µL of a solvent with high eluting power. A typical flow probe used in HPLC-SPE-NMR has a total volume of 60 µL and sensitive volume of 30 µL, and thus the total amount of analyte eluted from the column will be placed in the detection cell. This corresponds to increase of concentration of the analyte by a factor of five. In practice, this analyte focusing effect may be considerably higher in case of broadened or tailing peaks. This contrasts the situation in direct HPLC-NMR experiments, where the elution bands are typically larger than the NMR cell volume (usually 120 or 240 µL), especially when large HPLC columns are used, and thus only a fraction of material eluted from the HPLC column contributes to the NMR signal, which is acquired using analyte concentration determined by the chromatography. The chromatographic peak broadening, which can be a problem in direct HPLC-NMR [30, 98], is circumvented. Increased analyte focusing can be achieved using 1 x 10 mm SPE columns (bed volume 8 µL), which, however, requires even smaller NMR cells [49].

As the solvent from the chromatographic separation is removed from the SPE cartridges in the drying process, the need for solvent suppression schemes during
acquisition of NMR spectra is diminished, which gives better spectra with less suppression artefacts. The use of pure deuterated solvents instead of mixed HPLC solvents gives standardized chemical shift data. As MS is still done using non-deuterated mobile phase, MS data will not be further complicated, as no artifact peaks due to deuteration will arise [99, 100].

Another great advantage of introducing solid-phase-extraction is that the chromatographic separation can be repeated a number of times, allowing for multiple trapings of an analyte on the same cartridge. This will increase the amount of analyte available for NMR analysis, thereby increasing the S/N ratio in the NMR spectra linearly [42, 101]. An example of the effect of multiple trapping is shown in Figure 5.

The advantage of analysis of plant extracts by HPLC-SPE-NMR as compared to the traditional approach was illustrated in a study of Phyllantus reticulatus [48]. In the traditional procedure, 1.32 kg of plant material, 40 L of organic solvents, and three months of work were required, resulting in the isolation of eight known compounds. In HPLC-SPE-NMR analysis, a fraction of the plant extract was investigated using a 250 × 4 mm C18 column, polymeric resin cartridges, and transferring trapped peaks to a 400 MHz spectrometer equipped with a 120 μL inverse probe. One mg of sample (corresponding to 0.3 g of dried leaves) and one month of work was used to characterize six known compounds, only two of which were identified following the traditional approach. This study demonstrates the superiority of HPLC-SPE-NMR in terms of workload and results.

5.3. Limitations of HPLC-SPE-NMR

Although HPLC-SPE-NMR circumvents a lot of problems associated with the use of direct HPLC-NMR hyphenation, a number of limitations exist. To achieve a maximal S/N ratio, SPE trapping of the analyte should be quantitative and the analyte should be eluted from the SPE cartridge in a sharp band (preferably with the eluting solvent front). In reality, both trapping and elution are new chromatographic steps. Although the make-up flow of water lowers the elutropic strength of the chromatographic solvent, the resulting mixture may still cause analyte breakthrough. The fact that the analyte is initially delivered in the HPLC mobile phase limits the number of variables that can be altered as compared to conventional cleanup procedure by SPE [102]. Since HPLC-SPE-NMR is most often used for the analysis of complex mixtures containing compounds not only of varying polarities, but also with initially unknown structure, it is difficult to optimize the procedure for individual components. This leads to compromises with regard to choice of make-up flow, stationary phase for SPE, and eluting solvents. This may negatively affect the amount of analyte that actually reaches the NMR probe.

An example of the difficulties with regard to trapping is a study of isomeric tropane esters from Schizanthus grahamii, for which trapping efficiency was estimated to be around 50% [52]. To test efficiency of the SPE step, Sandvoss et al. chromatographed an equimolar mixture of phthalic acid, benzoic acid, propiophenone, valerophenone and octanophenone on a 150 × 4.6 mm C18 column using a CH3CN gradient in H2O; the most and the least polar compound eluted at approx. 32% and 92% of CH3CN respectively. Peaks were trapped on 2 × 10 mm polystyrene-type resin (Resin GP) cartridges following three-fold dilution of the mobile phase with water, and the trapped compounds were eluted with CD3CN for NMR. The results showed a significant variation between the amounts of material transferred for each of the
compounds in the mixture and in the efficiency of multiple trapping, which was negligible for the most polar compound [5]. On the other hand, Clarkson et al. investigated trapping of a range of natural products eluted from a C18 column with CH$_3$CN percentages varying from 5% to 85%, on eight different SPE stationary phases ranging from very polar (silica) to non-polar (C18 bonded phases and polymeric resins) [103]. The results indicated that prediction of SPE retention properties on the basis of HPLC elution orders is difficult, but resin-based sorbents are efficient for the greatest range of polarities, with 83-99% of material retained for compounds eluting with 20-85% of CH$_3$CN. The retention was demonstrated in several cases to be critically dependent on the exact amounts of CH$_3$CN in the mobile phase, with few percent points changing the retention from very high to almost negligible [103]. Another study confirmed the high versatility of SH resin, which adsorbed efficiently many polar as well as apolar compounds [104]. However, in a study of isoquinoline alkaloids the trapping efficiency of silica-based C18 stationary phase was superior compared to polymeric GP-resins [105]. It has been shown that the loadability of medium-polar compounds onto SH resin cartridges is 1-1.5 mg [104]. A study comparing efficiency of HPLC-MS-SPE-NMR with loop-storage HPLC-NMR showed similar S/N ratios when employing C18 cartridges and desorption with CD$_3$CN. A change to GP resin resulted in a decrease in the S/N ratio, also in the case when desorption was performed with CD$_3$OD [29]. The results of these studies generally support the rationale behind the routine use of resin based GP cartridges for HPLC-SPE-NMR analysis, but also stress the fact that for most compounds optimization of trapping conditions, including choice of SPE sorbent, might benefit the quality of NMR spectra.

5.4. Applications of HPLC-SPE-NMR

HPLC-SPE-NMR, often extended with MS, has primarily been applied in studies of natural products. The technique proved its superiority in discovery of novel natural products from plants, fungi and marine organisms, in assisting bioactivity-guided natural product isolation, and as a preparatory step for preparative-scale isolation [20, 29, 31, 34, 36-41, 44, 46-51, 105]. Many major classes of secondary metabolites, including terpenoids, flavonoids, polyketides and alkaloids were shown to be compatible with this technique.

Figure 6. 600 MHz NMR spectra of a drug metabolite acquired in HPLC-MS-SPE-NMR mode. A) $^1$H NMR, B) TOCSY C) NOESY (reproduced from ref. [32] with permission of the copyright owner).
However, HPLC-SPE-NMR is also popular in the pharmaceutical industry, both in drug degradation studies and in early R&D stages \[32, 106, 107\]. In an elegant study, Ceccarelli \textit{et al.} used HPLC-SPE-NMR to determine the structure of a metabolite of a metabotropic glutamate receptor antagonist and used this information to improve metabolic stability of the drug. Trappings allowed for acquisition of 1D as well as TOCSY and NOESY spectra, see Figure 6 \[32\]. In another application the technique was used for analysis of pollutants in groundwater from a former ammunition destruction site \[35\]. HPLC-SPE-NMR has also great potential in assisting metabolomic studies \[33, 108\].

5.5. Extended hyphenation of HPLC-SPE-NMR

Although NOESY spectra acquired in the HPLC-SPE-NMR mode provide valuable information about relative configuration, absolute configuration cannot be assigned directly. This problem can be solved in a number of ways. When dealing with secondary alcohols, the absolute configuration can be determined through comparison of spectra of certain diastereomeric esters – the so-called Mosher method \[109\]. Seger \textit{et al.} have determined the absolute configuration of eight naturally occurring acetylenic alcohols by combination of Mosher’s method with HPLC-SPE-NMR. One mg of each alcohol was derivatized with \((R)\)- and \((S)\)-MTPA-Cl to give the corresponding esters. The reaction mixture was separated on a 150 mm \(\times\) 4.6 mm HPLC column, and the formed products were isolated by a single trapping on GP resin SPE cartridges. \(^1\)H NMR spectra of all compounds, as well as \(^{19}\)F spectra of selected compounds were acquired with an acquisition time for each spectrum of maximum 15 minutes, using a 500 MHz NMR spectrometer equipped with a 60 \(\mu\)L active volume flow probe. The results of this study show that HPLC-SPE-NMR can be employed in the analysis of small-sized reaction mixtures without risking loss of sample during handling and analysis \[110\].

More generally, the absolute configuration can be determined or characterized by acquiring chiroptical data. The advantage of HPLC-SPE for analyte separation and focusing for CD in parallel with NMR was recently demonstrated in characterization of enantiomeric purity of gossypol in \textit{Thespesia danis} \[34\].

One interesting perspective is coupling of HPLC-SPE-NMR to on-line determination of bioactivity. A method for on-line antioxidant activity analysis of plant extracts (HPLC-UV-DPPH•-ABTS•+) has been coupled to HPLC-SPE-NMR for rapid identification of active compounds. Constituents that showed activity were subsequently analyzed using HPLC-UV-SPE-NMR which, combined with HPLC-MS, led to identification of 14 antioxidants from four extracts \[46\]. A rosemary extract was analysed for antioxidant activity by dividing the eluent from the HPLC into two streams. One was trapped on SPE cartridges while the second was used for radical scavenging detection. Active compounds were subsequently desorbed from the SPE cartridges for NMR analysis \[111\].

6. NMR hyphenation with other techniques

In addition to HPLC, various other separation techniques have been hyphenated with NMR detection, and HPLC-NMR systems have been extended with other spectroscopic methods. For example, Louden \textit{et al.} reported a HPLC-DAD-MS-NMR
system supplemented with an IR spectrometer. The eluate from HPLC was directed via a DAD detector to the IR spectrometer and then, through a flow splitter, to NMR and MS [112, 113], or alternatively the IR spectrometer was placed after the splitter, online with UV detector followed by NMR spectrometer [114, 115]. Using these setups, polymer additives, non-steroidal antiinflammatory drugs, and plant extracts containing ecdysteroids [113, 114] have been investigated, obtaining high-quality spectra from all hyphenated instruments [112-115].

A combination of size-exclusion chromatography, UV, MS and IR detection has been reported in the analysis of polymer additives, where IR, however, was performed off-line [116] as opposed to the work of Louden et al [112-115]. The system was used to demonstrate that the coupling of many techniques was feasible, but introduces compatibility problems with respect to the solvents employed [116].

Combination of ion-exchange chromatography and NMR has been the subject of several studies. Reports on the analysis of protein mixtures and drug metabolites have been published in the last decade, employing loop-storage as well as stopped-flow mode, enabling acquisition of 1D as well as 2D NMR data [87, 117].

Another chromatographic technique, which has been combined with on-line NMR detection, is supercritical fluid chromatography (SFC). An advantage of coupling of supercritical fluid chromatography to NMR is that the use of a hydrogen-free solvent circumvents the problems normally associated with solvent signals. Use of special pressure-stable flow probes is necessary, as discussed in section 3. Already in 1997 Albert noted that resolution of 1H NMR spectra obtained using SFC-NMR and SFE-NMR (supercritical fluid extraction-NMR) approached the resolution of conventionally recorded NMR spectra [26]. Braumann et al. have investigated vitamin A acetate isomers in the first real-life application of SFC-NMR. A mixture of five cis/trans isomers was separated using supercritical CO2 as the eluent, using a 400 MHz NMR spectrometer with a specially designed pressure-proof probe for detection. All five isomers were separated and identified on the basis of SFC-NMR analysis. This would not have been possible using normal RP HPLC separation, or detecting the isomers based on UV or MS data [118]. Gel permeation chromatography (GPC) has also been successfully coupled to NMR in the analysis of polymers and oligomers [119-122]. In the most recent report by Hiller et al., [122] high-temperature GPC has been coupled on-line to both on-flow and stopped-flow NMR. The possibility of conducting the experiments at elevated temperatures allows for better dissolution of the analyzed polymers in standard NMR solvents [122].

The combination of capillary separations and NMR detection has been widely used, but the sensitivity issue remains to be a problem. However, since the amounts of stationary phase and solvent consumption are dramatically decreased compared to conventional HPLC (5 µL/min for CapLC compared to 0.5-1 mL/min employed in classical HPLC), it is possible to perform the separation using fully deuterated solvents. This renders solvent suppression schemes in the acquisition of NMR data redundant, which in turn leads to higher quality NMR spectra. The lower volumes of elution bands also lead to higher concentrations of analytes, thereby increasing S/N ratios in the NMR spectra. The use of microcoil NMR probes also leads to increase in the quality of NMR spectra [123]. The advantage of CapLC-NMR with respect to mass-limited samples has been demonstrated [23, 43, 124, 125]. Superior resolution was obtained in the stopped-flow mode compared to spectra acquired in 5 mm NMR tubes in the analysis of an extract of Radix astragali [43].
Lewis et al. compared CapLC-NMR to HPLC-SPE-MS-NMR in the analysis of drug metabolites in urine at 600 MHz. For CapLC-NMR, a 150 × 1 mm column and 5 µL total volume inverse probe were used. For HPLC-SPE-MS-NMR, the separation was achieved on a 150 × 4.6 mm column, analytes were trapped on GP resin cartridges, and eluted into 5 mm tubes for NMR analysis with a cryoprobe. The results indicated that a greater recovery of sample was obtained when using CapLC-NMR compared to HPLC-SPE-MS-NMR, but a higher S/N ratio was obtained in the SPE mode. This, combined with the limited sample-loading capacity of capillary LC systems and the versatility of HPLC-SPE-MS-NMR led to the conclusion that CapLC-NMR is best suited to samples that are truly mass limited [30].

Both capillary electrophoresis (CE) and capillary electrochromatography (CEC) have been hyphenated with NMR and offer outstanding separation capability, although the smaller volumes and shorter residence time of analytes can make the NMR detection problematic. Pusecker et al. have investigated paracetamol metabolites in human urine using a combination of CZE-NMR (capillary-zone-electrophoresis-NMR) and CEC-NMR with the chromatographic systems coupled directly to the NMR spectrometer in the continuous-flow mode. In both experiments three compounds were identified, and it was estimated that in the CZE-NMR experiment, which employed the smallest sample, approximately 10 ng of each compound was detected with S/N of 3 [126].

Besides column chromatography, other chromatographic separation techniques have been applied in a hyphenated mode with NMR, one of them being CPC (centrifugal separation chromatography). Spraul et al. reported the separation of three N-2,4-dinitrophenyl amino acids [127]. The instrumentation was very similar to the instrumentation normally used for HPLC-NMR, and one advantage of CPC is that it has the ability to separate very polar and fragile compounds, and that there is no degradation of material or irreversible adsorption to a solid support, hence avoiding problems related to saturation of a solid-state stationary phase [127].

Gas chromatography has an outstanding separation power and is widely employed in combination with mass spectrometry [25, 128-130]. However, the compounds need to be volatile and because GC-NMR is normally operated in continuous-flow mode, the optimum flow rate has to be a compromise between sensitivity, chromatographic resolution and spectral resolution. In a study of stereoisomeric hydrocarbons a custom-built double-resonant solenoidal microprobe was used for measurements even in stopped-flow mode [25]. In another study using similar NMR equipment, separation and detection of three model compounds was investigated. Although overlap of two chromatographic peaks was observed, clear distinction between different analytes based on the NMR data was still possible [131].

7. Conclusions and future directions

The improvements in NMR hyphenation achieved during the recent three decades, involving a wide range of separation methods, have made NMR analysis of single components of complex mixtures achievable rapidly and without laborious preparative-scale separation and purification procedures. Current HPLC-NMR techniques can operate routinely at the 10-100 µg level. The great versatility of various hyphenated techniques is exemplified by the diversity of samples analyzed, as discussed in this chapter. This versatility is continuously expanding and it is expected that further
technological progress will make an increased number of hyphenated NMR techniques commercially available. Development of practically useful and cost-effective hyphenated HPLC-NMR systems requires that problems with NMR detector sensitivity, related to loading capacities in the chromatographic step, are solved. Especially the development of cryogenically cooled probes has entailed a dramatic leap in routinely achievable S/N ratios for analyte amounts delivered with conventional HPLC columns. A combination of cryogenic technology, miniaturization, and analyte focusing by SPE (and whenever possible also analyte accumulation by multiple trapping) offers further improvements in sensitivity, enabling acquisition of high-quality 1D and 2D NMR data of mixture components present at nanogram levels. Several laboratories are involved in work aimed at extending the applicability of HPLC-SPE-NMR to very polar analytes, which at present can only be analyzed in stopped-flow or loop-storage mode.

In the future, automated liquid handling devices will perhaps shift the emphasis in routine NMR hyphenation from data acquisition using flow probes to NMR microtubes. However, ultrafast data acquisition techniques may render on-flow LC-NMR very attractive in some areas. Extended hyphenations involving more specialized detectors, such as CD detector for characterization of chirality, is of considerable interest. While present-day hyphenated NMR systems usually employ low-resolution MS, high-resolution spectrometers are increasingly affordable and will undoubtedly be routinely included in future systems. Finally, combination of structure elucidation by hyphenated NMR with bioactivity detectors, such as flow-through bioreactors with immobilized proteins or automated bioassay systems that employ well plates, is another exciting possibility. One of the rapidly developing areas of NMR is sensitivity enhancement via hyperpolarization. While current areas of application of NMR with hyperpolarized nuclei remain highly specialized, it is interesting to note that combination of dynamic nuclear polarization with chromatographic separation was proposed as far back as 1994 [132].

As the ability to acquire wide range of high-quality 1D and 2D NMR spectra from multiple chromatographic peaks increases, the bottleneck shifts from sample preparation and data acquisition to data interpretation. To overcome this obstacle, development of NMR spectral databases and intelligent spectra interpretation systems for on-flight structure elucidation or classification is mandatory.

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Short communication

Identification of reaction products between drug substances and excipients by HPLC–SPE–NMR: Ester and amide formation between citric acid and 5-aminosalicylic acid

Jesper Larsena,∗ Dan Staerkb,c, Claus Cornettaa, Steen H. Hансena, Jerzy W. Jaroszewskib

a Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Copenhagen, Denmark
b Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Copenhagen, Denmark
c Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Copenhagen, Denmark

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A B S T R A C T

The reaction between the high-dose drug substance 5-aminosalicylic acid (5-ASA) and the excipient citric acid during storage of an experimental enema preparation has been studied and three isobaric reaction products, i.e., an ester and an amide with non-symmetrically substituted citric acid moieties and a symmetrical amide, were identified by combined use of HPLC–SPE–NMR and HPLC–MS. After storage for 1 week at 70 °C, approximately 5% of the 5-ASA present in the formulation was transformed into these impurities. Storage of the enema for 32 months at room temperature led to loss of approximately 10% of the original amount of 5-ASA, with the ester as the main reaction product.

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1. Introduction

The development of methods based on hyphenation of separation techniques and nuclear magnetic resonance (NMR) have greatly improved possibilities of identification of mixture components without the need of prior isolation and purification of individual compounds [1]. Thus, in contrast to well-established HPLC–MS methods, which are characterised by speed and high sensitivity but also by limited information content with respect to details of molecular structure, HPLC–NMR allows unambiguous on-line identification of even very complex structures. A recent extension of the original HPLC–NMR repertoire of experiments (comprising on-flow, stopped flow and loop-storage techniques [1,2]) is HPLC–SPE–NMR, where the analytes are separated from the HPLC mobile phase by means of post-column solid-phase extraction and then submitted, in an automated fashion, to NMR measurements in a deuterated solvent. Combined advantages of analyte focusing, accumulation by multiple SPE collections and the use of deuterated solvents makes HPLC–SPE–NMR capable of providing NMR data of higher quality than what is usually achievable by direct HPLC–NMR methods, i.e., methods where NMR data are obtained with analytes dissolved in the HPLC mobile phase [2–4]. However, the HPLC–SPE–NMR technique is inherently dependent on the effectiveness of the post-column SPE process [5]. The HPLC–SPE–NMR technique is becoming increasingly popular, especially in natural products research [6–10], food and food supplement analysis [11,12], and pharmaceutical analysis [13], including analysis of drug degradation [14] and drug metabolism [15,16]. In this work, we describe the first example of application of HPLC–SPE–NMR to analysis of reaction products formed by interactions between a drug substance and an excipient upon storage of a pharmaceutical formulation.

For more than half a century, 5-aminosalicylic acid (5-ASA) has been used in the treatment of chronic inflammatory bowel diseases, originally in the form of the prodrug sulphasalazine [17] and subsequently as 5-ASA itself, and in the beginning of the 1980’s the development of 5-ASA drugs accelerated [18–20]. Enemas are among the popular formulation forms for colonic delivery and are still very important in the treatment of distal colitis and proctitis [21,22]. The present text deals with the formation of impurities in an enema formulation of 5-ASA, that has been under development by a pharmaceutical company. It was important to elucidate the structures of the impurities in order to eliminate them from the final formulation. 5-ASA is used in relatively large doses and therefore has a potential of formation of relatively high amounts of potentially harmful impurities.
Because 5-ASA is prone to oxidation, especially in alkaline solution, a number of excipients are usually added to 5-ASA solutions, including ethylenediaminetetraacetic acid, sulfite, and citric acid. The latter is widely used in pharmaceutical formulations as an acidifier, complexing agent or buffer substance. However, citric acid and other di- and polycarboxylic acids can form cyclic anhydrides, which can significantly increase their reactivity in the liquid as well as the solid state [23–25]. Recently, a reaction between 5-ASA and citric acid in a formulation produced by hot-melt technology has been reported, but no final conclusion about the structure of the reaction product was reached [26].

In high dose drugs, an impurity present in an amount of 0.1% or more relative to the active substance has to be conclusively identified. This identification threshold often poses a considerable analytical challenge. Here, we describe identification of reaction products formed in liquid formulations from 5-ASA and citric acid using HPLC–SPE–NMR supported by HPLC–MS. The study thus addresses the problem of distinction between isomeric products formed with this widely used pharmaceutical excipient and is of potential interest for product control of liquid pharmaceutical formulations of drugs containing hydroxy and amino groups.

2. Experimental

2.1. Materials

Citric acid monohydrate and formic acid were purchased from Sigma–Aldrich Chemie (Steinhem, Germany). Methanol and phosphoric acid were purchased from VWR (Leicester, England). 5-Aminosalicylic acid (5-ASA) and N-acetyl-5-aminosalicylic acid were obtained from Ferring Pharmaceuticals (Copenhagen, Denmark).

2.2. High-performance liquid chromatography–mass spectrometry (HPLC–MS)

HPLC–MS experiments were performed on an Agilent 1100 HPLC/MSD system (Agilent Technologies, Palo Alto, CA, USA). The chromatographic system consisted of a Phenomenex Luna 2 × 100 mm 3 μm C18 column kept at 40 °C and the mobile phases A (10% MeOH in water, added 0.1% HCOOH) and B (90% MeOH in water, added 0.1% HCOOH). The gradient elution profile was 0% B at 0 min, 20% B at 10 min, 50% B at 20 to 24 min, 0% B at 24.1 min, with a flow-rate of 0.3 mL/min and a total runtime of 42 min. UV traces were recorded at 240 nm. The MSD was used in positive electrospray mode, drying gas flow 12 L/min, nebuliser pressure 40 psi, drying gas temperature 350 °C, capillary voltage 4 kV and fragmentor 80 V. The MSD was programmed to scan from 100–1200 a.m.u. with a cycle time of 1.08 s. Samples were diluted appropriately with water; the total concentration was typically 0.5 mg/mL and the injection volume was 5 μL.

2.3. High-performance liquid chromatography–solid-phase extraction–nuclear magnetic resonance (HPLC–SPE–NMR)

The HPLC–SPE–NMR system consisted of an Agilent 1100 series chromatograph (quaternary pump, autosampler, column oven, photodiode array detector), a Knauer K100 Wellchrom post-column solvent delivery pump (Berlin, Germany), a Spark Prospekt 2 device (Emmen, Holland), and a Bruker Avance 600 MHz NMR spectrom-

![Fig. 1. Structures of 5-ASA, citric acid, their esters and amides (1–4), and the model compound N-acetyl-5-ASA.](image)
Fig. 2. HPLC–UV–MS chromatograms of enema formulation containing 5-ASA and citric acid after storage for 32 months at ambient temperature. (A) UV trace at 240 nm. (B) Total ion chromatogram. (C) Extracted ion chromatogram m/z 328. (D) Extracted ion chromatogram m/z 310. Chromatographic conditions: Phenomenex Luna C18 column eluted with a gradient of methanol in water (both containing HCOOH) with UV and positive-ion electrospray MS detection (for details, see Section 2.2).

3. Results and discussion

In a pharmaceutical formulation containing both 5-ASA and citric acid, at least four primary reaction products 1–4, i.e., two amides (1 and 3), and two phenol esters (2 and 4), can be expected to be formed (Fig. 1). Formation of an ester of 5-ASA with the hydroxy group of citric acid is not expected, as tertiary alcohols do not form esters by direct esterification.

During a stability study of an enema formulation containing 5-ASA as well as citric acid, formation of three new peaks (Fig. 2) was observed by HPLC–MS (retention times 7.6, 8.3 and 10.9 min), and the same three products were formed upon treatment of the enema at 70 °C for a week. All the peaks had m/z 328, corresponding to reaction products between 5-ASA and citric acid, although a water loss (m/z 310) was so pronounced for the peak with retention time 8.3 min that intensity of the quasi-molecular ion was low (Fig. 2).

In order to elucidate structures of the reaction products, a mixture of 5-ASA and citric acid was heated for 3 h at 70 °C and the supernatant analysed by HPLC–SPE–NMR.

### 3.4. Stability of 5-ASA formulations

An enema formulation containing 5.00 g 5-ASA, 0.10 g sodium edetate, 1.00 g sodium pyrosulfite, 5.00 g citric acid monohydrate, 1.75 g sodium hydroxide and 490 g purified water was investigated. The pH of the suspension was 4.8. The formulation was stored for 8 months at 70 °C and for authentic

<table>
<thead>
<tr>
<th>Compound</th>
<th>1H NMR spectroscopic data (δ values with intensities, multiplicities and coupling constants in Hz in parentheses) for main reaction products between 5-ASA and citric acid, and for authentic N-acetyl-5-amino salisylic acid (methanol-d₄).</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H-3: 6.90 (1H; d; 8.8); 7.62 (1H; dd; 8.8, 1.9)</td>
</tr>
<tr>
<td>2</td>
<td>H-3: 7.05 (1H; d; 8.8); 7.40 (1H; dd; 8.8, 2.4)</td>
</tr>
<tr>
<td>3</td>
<td>H-3: 6.88 (1H; d; 8.9); 7.60 (1H; dd; 8.9, 2.1)</td>
</tr>
<tr>
<td>N-Ac-5-ASA</td>
<td>H-3: 6.88 (1H; dd; 8.9, 0.3)</td>
</tr>
</tbody>
</table>
It is apparent that the $^1$H NMR spectrum recorded with the first of the three impurity peaks shows the presence of a symmetrically substituted citric acid moiety, as the spectrum contains one AB spin–spin coupling system with a geminal coupling constant of the three impurity peaks shows the presence of a symmetrical ester in methanol-d$_4$, six trappings on C$_5$ HD SPE cartridges.

The remaining two impurity peaks must thus correspond to compounds 2 and 3 having non-symmetrically substituted citric acid moiety. Again, comparison of their $^1$H NMR spectra with that of N-acetyl-5-aminosalicylic acid (Table 1) demonstrated that the chemical shifts of the benzene ring protons of the two compounds are almost identical (Fig. 1). Therefore, the reaction product with the shortest retention time is the symmetric citric acid amide (1).

In the aged enema formulation (32 months), the amount of 5-ASA converted to 1, 2 and 3 was approximately 1.1%, 6.6% and 1.7%, respectively (estimated by HPLC assuming the same absorptivity of the products at 240 nm). Thus, almost 10% of the original amount of 5-ASA was lost via the reaction with citric acid. In the accelerated experiment (1 week at 70 °C), the approximate amount of impurities was 5%, distributed as 0.5%, 4.0%, and 0.5% of 1, 2 and 3, respectively.

4. Conclusions

The impurity profile for an enema formulation of 5-ASA with citric acid was investigated and the impurities formed upon storage were identified as compounds 1, 2 and 3. Formation of the symmetrical ester 4 was not observed, presumably because of steric hindrance around the central carboxy group of citric acid and thus negligible equilibrium concentration of this phenol ester. The identity of the products was established using the HPLC–SPE–NMR technique. Use of this hyphenated technique allowed acquisition of $^1$H NMR data with a model formulation subjected to accelerated decomposition, leading to identification of the isoboric reaction products.

Because of a relatively rapid and extensive formation of reaction products between 5-ASA and citric acid, the use of citric acid in liquid 5-ASA formulations is not recommended. Thus, based on these findings, further development of a formulation of 5-ASA containing citric acid was abandoned and replaced with another formulation.

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References

High-Resolution NMR Spectroscopy of Sample Volumes from 1 nL to 10 µL

Michael E. Lacey,†,§ Raju Subramanian,†,§ Dean L. Olson,†,§,‖ Andrew G. Webb,†,§ and Jonathan V. Sweedler*,†,§

Department of Chemistry, Department of Electrical and Computer Engineering, and the Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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I. Introduction

Complete structural elucidation of an unknown compound, whether it is the product of a multistep synthesis, a potent extract from a natural source, or even a component of a waste stream, generally requires a battery of analytical techniques. Among common methods, infrared spectroscopy (IR), mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy typically provide the most extensive chemical information. However, as shown in Table 1, NMR significantly trails these other techniques in terms of the minimum sample amount for an analysis. Nonetheless, NMR spectroscopy fulfills a critical role through its ability to produce unmatched structural information and also to provide data on both intermolecular and intramolecular dynamics. Applications of this technique range from determination of three-dimensional structures of large proteins to the analysis of very small amounts of products from combinatorial syntheses. Furthermore, NMR spectroscopy features an experimental versatility virtually unsurpassed in a nondestructive, analytical method. Although NMR data would prove extremely valuable in many important mass-limited situations, its poor mass sensitivity has, until recently, precluded its routine use in trace analysis.

To accommodate the particular research needs of an analyst, NMR methods vary in complexity from simple one-dimensional proton spectra to multi-nuclear, multidimensional data sets which typically take over 1 week to acquire. Considerable effort in the past two decades has centered on the development of pulse sequences to determine various physical, chemical, and dynamical properties of analytes. While a thorough background and theoretical treatment of NMR and its myriad experiments lies beyond the scope of this review, the interested reader is encouraged to explore this literature.

Despite continuous improvements in the homogeneity and field strength of superconducting magnets during the last 15 years, currently available magnetic fields yield NMR transitions of very low energy. Since the transition energy is small with respect to $kT$ at room temperature (where $k$ is the Boltzmann constant), the population difference between the upper and lower energy states represents a minuscule fraction (typically <0.01%) of the total number of molecules. Since the NMR signal depends on this difference in populations of the two energy states, it is a relatively insensitive detection method.

Many technical advances have been developed to increase the intrinsic NMR sensitivity. One approach utilizes higher magnetic field strengths since the NMR sensitivity increases as the $7/4$th power of the magnetic field strength. High-resolution NMR spectrometers employ superconducting magnets which now reach field strengths as high as 21.1 T (a proton precession frequency of 900 MHz). However, this field strength represents the upper limit of current su-
nuclear Overhauser enhancement (SPINOE). \(^8\)–\(^{10}\) With this method of sensitivity improvement, signal enhancements of ~50-fold for \(^1\)H and greater than 70-fold for \(^13\)C have been realized for soluble compounds in liquid xenon.\(^{10}\) While the SPINOE technique is not limited to a particular NMR sample size regime, substantial sensitivity increases are not realized for all analytes. A third approach limits the receiver coil noise and increases the coil quality factor by using either high-temperature superconducting materials\(^{11,12}\) or cryogenically cooled coils.\(^{13,14}\) While this method improves the NMR sensitivity for all analytes, the large thermal gradient which must be maintained between the coil and liquid samples makes its application to the smallest radiofrequency (RF) coils difficult. By saturating electron spin systems which are coupled to nuclear spins, dynamic nuclear polarization (DNP) represents still another approach to increase the NMR signal intensity.\(^{15,16}\)

Flowing systems avoid several undesirable effects of static DNP analyses.\(^{17}\) and examples of \(^13\)C-DNP detectors for recycled-flow NMR and chromatographic separations have been reported.\(^{18,19}\)

Although the magnet constitutes the most expensive part of a spectrometer, the overall system performance is usually dictated by the performance specifications of the NMR probe.\(^3\) The probe consists of one or more RF coils which both excite transitions between energy levels via an oscillating magnetic field and also detect the weak signals produced by the precessing nuclei. NMR probe manufacturers offer a variety of designs and experimental capabilities which range in complexity from simple proton-detect probes to inverse triple resonance probes which feature RF coils designed for multiple nuclei, with circuits tuned to the proton, carbon, nitrogen, and deuterium precessional frequencies.

Recently, much commercial and academic research has focused on the optimization of RF coils for the analysis of trace materials. Significant increases in NMR sensitivity have been achieved through several different instrumental improvements. This article provides a comprehensive review of the application of NMR spectroscopy to nanoliter volumes for both static analyses and detection in capillary separations. Since no commercially available NMR probes currently have nanoliter-volume capabilities, we have also included descriptions of several high-sensitivity, high-resolution commercial probes which require sample volumes in the low tens of microliters. These microliter-volume probes represent the state of the art for commercially available NMR microprobes. First, we provide a brief overview of the major advances in NMR which have improved performance for mass-limited samples. Next, figures of merit...
which aid in the comparison of NMR probes are presented. Additionally, a variety of NMR approaches to characterize microliter- to nanoliter-volume samples are described along with examples of both static homonuclear and heteronuclear experiments on mass-limited quantities. Finally, the coupling of capillary separation techniques with improved on-line NMR detection is examined.

II. Historical Perspective

The first detailed description of NMR spectroscopy using small, specialized RF coils for mass-limited samples was by Odelblad in 1966 who used continuous wave (CW) techniques to study the physical chemistry of mucus secreted from cells in the human cervix. This study utilized a set of solenoidal microcoils with diameters from 200 to 1000 μm, with a fixed length of just over 1 mm. With these NMR probes in a high-strength permanent magnet (3.7 T), the proton chemical shift and spin–lattice (T1) relaxation times of cervical secretions were measured. In 1979, Shoolery was the first to show high-resolution NMR spectra from a reduced-diameter RF coil. Using RF coils which were designed for direct-observe 13C NMR and closely fitted to the sample container in each case, a decrease in the diameter of the sample vial from 10 mm (1.5 mL volume) to 1.7 mm (15 μL) reduced data acquisition time by a factor of 40 for a given signal-to-noise (S/N) from a fixed sample mass of 5 mg of cholesterol. In this study, 13C spectra were also obtained from as little as 1 mg in 16 h. For 1H spectra, approximately the same data acquisition time was sufficient to detect 1 μg of cortisol acetate. Another application of solenoidal coils (2-mm diameter) in NMR spectroscopy allowed spectra to be acquired in under 3 min from the small superfused hind limb muscles of mice.

Within the realm of magnetic resonance imaging, small diameter RF coils have also been used because of their enhanced sensitivity. As one example, microcoils (diameters as small as 1 mm) have been employed in conjunction with extremely high-strength gradient coils (~800 G/cm) to produce images from 100-μm slices of rat embryos with a 15-μm in-plane resolution. Another approach to NMR microscopy utilizes an inductively coupled coil configuration in which a 1-mm solenoid (mounted directly beneath the sample) serves as the primary coil and a 2-mm solenoid acts as the secondary coil. This arrangement allowed imaging of a single layer (40–70 μm thickness) of onion cells with a 4.5-μm in-plane resolution. Within the past several years, microcoils have been used to acquire magnetic resonance images from single biological cells. It should be noted that small RF coils have also been used extensively in electron paramagnetic resonance experiments. For these applications, intrinsic line widths are several orders of magnitude greater than those for NMR spectroscopy, and so magnetic susceptibility effects can be largely ignored.

III. NMR Figures of Merit

In examining the extension of NMR techniques to mass-limited samples for both static analyses and on-line detection of capillary separations, several figures of merit are important to determine the feasibility of a particular experiment using a particular coil. For example, the structural characterization of a minute amount of a precious sample demands long acquisition times for a variety of experiments. On the other hand, the use of NMR as an on-line detector for separations places strict limits upon analyte observation time and thus the information content of the spectra. In the following two sections, figures of merit for NMR spectral resolution, mass and concentration sensitivity, and limits of detection are presented and evaluated for both static and flowing situations.

A. Spectral Resolution

Spectral resolution represents a very important figure of merit since it reflects the information content that can be extracted from an NMR experiment. Ideally, resonances should have Lorentzian shapes and narrow line widths (defined as the full width at half-maximum). To further characterize line shape, the width of the resonance signals at 0.55% and 0.11% of the maximum peak height are also often reported. The former is the height of the 13C satellite peaks, and the latter represents 20% of this height. Since the height of a Lorentzian function is inversely proportional to its line width (LW), narrower resonance signals also result in higher sensitivity. For simple one-pulse experiments with liquid samples, factors such as thermal gradients and the homogeneity of magnetic field in the sample region often limit the line width of the resonances in a spectrum. On the other hand, the digital resolution of the data set generally imposes the resolution limit for multidimensional experiments. For cases in which the digital resolution of the data has a significant impact on the apparent line width in the spectrum, resolution enhancement techniques such as zero-filling and linear prediction can be employed to improve the appearance of the spectrum. In fabricating probes with more sensitive, reduced-diameter RF coils to examine smaller analyte amounts, the proximity of the probe materials to the sample becomes more detrimental to spectral resolution. As a result, good line width specifications for small-volume probes pose a significant challenge. However, flowing systems can impose a limit upon spectral resolution which originates from short observation times rather than poor magnetic field homogeneity. Clearly, an acceptable LW for an NMR spectrum depends on the experimental conditions.

B. Signal-to-Noise, Sensitivity, and Limits of Detection

While the S/N of an individual peak in an NMR spectrum is usually defined as the height of the peak divided by the root-mean-square (RMS) noise, in the NMR literature the RMS noise is usually multiplied by 2 for reasons that can be attributed only to tradition. Several factors contribute to the signal strength, including the nuclear precession frequency, the RF receiver coil size and geometry, the sample
concentration and volume, the number of magnetically equivalent nuclei which give rise to a particular resonance, and the line width of the resonance. Furthermore, parameters such as the number of acquisitions (NA) and total acquisition time (\( t_{\text{acq}} \)) can significantly affect spectral results. As the noise in an NMR spectrum increases as \( \text{NA}^{1/2} \) while the signal is directly proportional to NA (for a fully relaxed system), \( S/N \) varies with \( \text{NA}^{1/2} \). Because NA is directly proportional to \( t_{\text{acq}} \), \( S/N \) also increases as \( t_{\text{acq}}^{1/2} \).

Of course, \( S/N \) in the frequency domain depends heavily upon the degree and type of data processing employed in the time domain. Throughout the history of FT-NMR spectroscopy, various mathematical operations (e.g., exponential, Lorentz-Gaussian, sinebell, trapezoidal multiplication) have been developed which can enhance \( S/N \) at the expense of spectral resolution and vice-versa.\(^{33}\) While NMR performance depends on acquisition and processing parameters for a particular sample and set of conditions, probe comparisons mandate specification of standard conditions for data collection and processing. However, many NMR spectroscopists and instrument manufacturers have developed measures of performance which are used only in NMR. In contrast, we prefer figures of merit which facilitate comparisons among NMR probes and evaluation of NMR with respect to other analytical methods.

At this point, a distinction should be made between the total sample volume required for an NMR analysis, \( V_{\text{tot}} \), and the volume of sample observed by the RF coil, \( V_{\text{obs}} \). The fraction \( V_{\text{obs}}/V_{\text{tot}} \) reflects the sample observation efficiency, and ideally this observe factor, \( f_{\text{obs}} \), should equal 1 (\( V_{\text{obs}} = V_{\text{tot}} \)). Because of magnetic susceptibility discontinuities at air and container interfaces, the sample usually extends beyond the coil region to provide quality data; in such cases, \( V_{\text{obs}} < V_{\text{tot}} \). For instance, 5-mm tubes used for conventional NMR probes typically require 500–700 \( \mu L \), only about 1/3 of which resides in the \( V_{\text{obs}} \) region of the NMR coil. Clearly, a limit of detection (LOD), defined in terms of the concentration or mass of sample which yields a \( S/N \) of 3, for the entire sampling system can be significant for analyte-limited applications. However, the signal from the sample within the RF coil will be used as the basis for comparisons of probe sensitivity in this paper. By focusing on \( V_{\text{obs}} \) factors associated with sample loading are isolated and the fundamental limits of probe performance can be assessed more directly.

In general, NMR users are accustomed to an expression of \( S/N \) for a given analyte concentration. For example, the standard test for \(^1H\) NMR sensitivity of high-field instruments consists of a single scan of 0.1% ethylbenzene in CDCl\(_3\).\(^{37}\) Although the results of this test are generally reported as the \( S/N \), such a method is not appropriate for very small \( V_{\text{obs}} \) where analyte amounts may be 5 orders of magnitude less than that for a 5-mm probe. In these cases, a number of acquisitions must be accumulated or the concentration must be increased to give a meaningful result. Consequently, this performance parameter can be more explicitly defined as time-normalized concentration sensitivity

\[
S_c = \frac{S/N}{C \cdot t_{\text{acq}}^{1/2}} \quad (1)
\]

where \( C \) is the sample concentration and \( t_{\text{acq}}^{1/2} \) accounts for the total experiment time. Such a definition, however, may not accurately reflect the performance of a probe which has been designed for mass-limited samples. In such a case, a more appropriate indicator of probe performance is the mass sensitivity

\[
S_m = \frac{S/N}{\text{mol} \cdot t_{\text{acq}}^{1/2}} \quad (2)
\]

where the mole amount refers to the portion of the sample which resides within \( V_{\text{obs}} \). The \( V_{\text{obs}} \) and sample concentration are used to compute the moles of observed sample. In contrast to \( S_c \), \( S_m \) does not require designation of the tube diameter for a sensitivity test but instead incorporates the observed volume into the computation of sample amount. In both cases, the analyst can trade gains in \( S/N \) for reduced data acquisition time or sample quantity.

To emphasize the distinction between \( S/N \) and the intrinsically instrumental nature of sensitivity, suppose that NMR spectra are acquired and processed under identical conditions for 5- and 10-mm diameter cylindrical tubes in a fixed RF coil with the same analyte at equal concentrations. Because the number of spins in the 10-mm tube is 4-fold greater than in the 5-mm tube, the resultant signal magnitude, the corresponding \( S/N \), and \( S_c \) exhibit a 4-fold increase. However, since \( S_m \) does not depend on the volume of sample examined,\(^3,38\) utilization of \( S_m \) instead of \( S_c \) can yield more appropriate probe comparisons for mass-limited samples. In essence, the sensitivity of an NMR probe results from factors which include diameter, geometry, and quality factor of the RF coil, as well as other hardware specifications.

For a fixed coil size and line width, \( S_c \) is directly proportional to the fraction of volume within the coil which is occupied by sample. This parameter, called the coil filling factor, \( f_c \), is readily computed from the inner diameter of the sample container and the NMR coil diameter (\( d_i \)). If the NMR coil is wound directly on the sample container, \( f_c = (i.d./o.d.)^2 \). These considerations establish both \( S_m \) and \( S_c \) as useful figures of merit in NMR probe evaluation.

An additional figure of merit to consider especially when comparing NMR probes for use as detectors in separations is the normalized limit of detection (nLOD). These values are affected by the experiment time as well so that

\[
n\text{LOD}_c = \frac{3C t_{\text{acq}}^{1/2}}{S/N} \quad (3)
\]

and

\[
n\text{LOD}_m = \frac{3\text{mol} \cdot t_{\text{acq}}^{1/2}}{S/N} \quad (4)
\]
where the mole quantity again refers to that amount of sample in $V_{\text{obs}}$ and the S/N corresponds to the peak of interest. The nLODs not only reflect probe sensitivity but also allow the user to compute the approximate mass or concentration of sample needed to acquire a desired S/N for a specific spectral peak using a particular probe and acquisition time.

Performance comparisons of NMR probes employed under different analytical situations are rendered more easily using the figures of merit described here. The relative importance of these figures of merit depends on the amount of sample available and the acceptable concentration range for a given experiment. These performance criteria are readily obtained and very useful in choosing a probe for a particular analysis. For instance, in the cases of many probes, only a fraction of the total sample lies within $V_{\text{obs}}$. A mass-limited condition generally calls for the greatest $S_m$, which is usually the probe with the smallest coil. A situation which is not mass-limited or where the sample already exists in a relatively large volume and cannot be concentrated benefits by using the probe with the largest $S_c$, which usually has the biggest $V_{\text{obs}}$.

**IV. Approaches To Augment NMR Probe Performance**

**A. Microsample Tubes and Magnetic Susceptibility Plugs**

The simplest approach to increasing the signal-to-noise of the NMR experiment, without having to redesign the RF coil, utilizes smaller sample tubes (which may even feature glass which is matched to the magnetic susceptibility of the solvent) or tube inserts to decrease the volume required for an experiment. If the sample volume is reduced by a factor of 4, for example, the concentration can be correspondingly increased by a factor of 4, maintaining the same total number of spins. For analytes dissolved in conductive media, the decrease in $V_{\text{tot}}$ can reduce the noise contribution from the sample and the S/N of the spectrum will increase. Alternatively, the sample can be contained in a spherical microcell. This places all of the sample within the active region of the RF coil and increases the observe factor to unity. The spherical shape of the cell is necessary to minimize the magnetic susceptibility induced line broadening that occurs since the sample/air and glass/air interfaces are now all contained within the coil. Such a strategy is straightforward and easily implemented, but the lower volume limit for commercial products is currently 18 $\mu$L.

Another approach to small-volume NMR spectroscopy is to insert plastic plugs (matched to the susceptibility of the solvent) within a tube designed and optimized for larger volumes. These plugs are intended to restrict the sample to the active region of the RF coil, again increasing the observe factor so that it approaches unity. For a 3-mm o.d. tube, for example, plugs allow a minimum sample volume of ~60 $\mu$L. No such susceptibility-matched inserts are commercially available for the nanoliter regime, although liquid perfluorocarbon susceptibility-matched plugs have recently been demonstrated for solenoidal microcoils with observe volumes less than 1 $\mu$L. While these approaches increase the sample observation efficiency, the overall performance of the NMR probe can be further improved by making changes to the RF coil.

**B. Reduction in Saddle-Type Coil Size**

For the most part, the RF coils of standard high-resolution NMR probes are based upon a “saddle”-shaped geometry (see diagram in Figure 1A). From consideration of the spatial configuration of magnetic vector potentials, Hout and Richards theoretically determined the sensitivity (as assessed by the RF field, $B_1$, per unit current, i) of this coil type as:

$$
\frac{B_1}{i} = \frac{n\mu_0 \sqrt{3}}{\pi} \left[ \frac{2dh}{(d^2 + h^2)^{3/2}} + \frac{2h}{d(d^2 + h^2)} \right]
$$

Figure 1. Examples of two RF coil geometries: (A) saddle type, (B) solenoid.

where $n$ is the number of turns, $\mu_0$ is the permeability of free space, $d$ is the diameter of the coil, and $h$ is its length. Clearly, reduction of the diameter and length of the coil increases sensitivity. In addition to the theoretical derivation of eq 5, Hout and Richards also demonstrated that the length of the 90° pulse provides a direct measure of the sensitivity for a single RF coil system. That is, shorter 90° pulse widths correspond to higher sensitivity RF coils. Virtually all commercial NMR probe manufacturers now offer probes which utilize the increased sensitivity of small saddle coils. The first significant development involved Varian’s use of 1.7-mm tubes for both 1H and 13C NMR of mass-limited samples. The introduction of an inverse-detection microprobe designed for 3-mm diameter tubes with total sample volumes of ~140 $\mu$L and observe volumes of ~60 $\mu$L added further capabilities to small-volume NMR.

By reducing the sample size to a 1.7-mm diameter tube, rapid data acquisition on reduced sample amounts was enabled for inverse-detection microprobes.

**C. Solenoidal Coils and Magic Angle Spinning**

For solenoidal RF coils (see Figure 1B), the sensitivity is given by
Therefore, assuming that the length-to-diameter ratio (h/d) is kept constant, the coil sensitivity will increase as the inverse of the diameter of the coil. For coils of diameters less than ~3 mm, the major noise source, even for lossy biological samples, arises from the resistance of the coil itself. Resistance depends on both the winding geometry (including wire diameter, number of turns, and turn spacing) and the resistivity of the conductor.

Varian introduced a series of probe designs termed "Nano-NMR probes" which use a 4-mm solenoidal RF coil which is oriented at the magic angle (54.7°) with respect to the static magnetic field (B_0). For a given length and diameter, the solenoidal design is approximately 2.5–3 times more sensitive than the saddle coil geometry described previously. This design remedies the analyte wasted as a result of poor observe factors by placing the entire sample within the active region of the coil (i.e., f_0 = 1). By spinning at the magic angle, the otherwise severe magnetic susceptibility effects are reduced and high-resolution spectra can be produced. In contrast to conventional static NMR probes, rapid spinning of the sample at the magic angle also enables one to collect data from heterogeneous samples. To further improve the spectral quality, the coil is constructed using “zero-susceptibility” materials (as are most RF coils in high-resolution, high-sensitivity commercial probes). The maximum volume that can be accommodated is 40 μL, although good line shapes can be achieved for considerably lower volumes. For a spinning liquid sample, this probe obtains line widths that are only a few tenths of a Hertz greater than conventional high-resolution probes.

D. Microcoils

For solenoidal coils of diameter greater than about 100 μm, eq 6 can be expressed as the S/N per unit volume of sample

\[
\frac{B_1}{i} = \frac{\mu_0 N}{d \sqrt{1 + (h/d)^2}}
\]

\[
(S/N)_{puv} = \frac{\alpha_0^2 \frac{[n/d_c \sqrt{1 + (h/d_c)^2}]}{\sqrt{n^2 d_c \alpha_0 1/2 / h}}}{\alpha_0} \propto \frac{\alpha_0^{7/4}}{d_c}
\]

where \(\alpha_0\) is the nuclear precession frequency. Since the mass sensitivity is dependent on the S/N_{puv}, the above expression demonstrates that \(S_m\) improves for a microcoil with a fixed length-to-diameter ratio as the coil diameter decreases (i.e., \(S_m \propto 1/d_c\)). Though the coil quality factor and inductance go down as \(d_c\) is reduced, a key performance parameter is the inductance per unit volume which increases as \(d_c\) decreases and produces the enhanced mass sensitivity of NMR microcoils. Since thermal noise in the coil windings rather than sample noise is the primary contributor in microscopic studies, minimizing coil resistance also improves performance. For coils of diameter below 100 μm, the sensitivity dependence on the coil diameter is reduced to a square root relationship with the diameter. Since the 4-mm diameter solenoid used in the Varian probe is not limited by fabrication considerations, the sensitivity of the NMR experiment can be substantially improved by reducing the coil diameter. Figure 2 demonstrates the agreement between the theoretical and experimental increase in S/N per unit volume as the coil diameter decreases in size. To date, NMR microcoils generally have been wound directly onto a capillary which functions as both sample container and coil form. According to electromagnetic field theory, a sample enclosed by a perfectly uniform and infinitely long hollow cylinder (e.g., fused silica capillary) experiences a uniform static magnetic field. However, the susceptibility variation in the materials near the sample (largely copper and its coating, adhesive, and air) can lead to localized distortions of the static magnetic field in the sample region. Furthermore, as the sample more closely approaches the coil windings, capillary coating, and surrounding environment, magnetic field inhomogeneities become more pronounced. The resultant line broadening decreases S/N and resolution. As a result, substantial attention has been devoted to studying the effects of coil proximity to the sample, especially considering the diminutive dimensions of microcoil NMR compared to traditional configurations.

While conventional microliter-scale probes use fabrication materials with near-zero magnetic susceptibility or sample plugs matched to the susceptibility of the solvent, the approach undertaken for microcoils surrounds the coil and capillary region with a fluid of magnetic susceptibility close to that of copper, the coil material. As shown in Figure 3, this has proved very effective in decreasing LW, improving line shape, and increasing S/N. Through the use of a susceptibility-matching fluid and a magic angle orientation of the solenoid relative to B_0, LW, line shape, and sensitivity can be improved significantly. This inexpensive and easily implemented strategy avoids problems associated with attempts to create materials of zero magnetic susceptibility for small dimensions.

To carry out complete structural elucidation (especially for complex molecules), the RF probe should have the capability of performing a variety of two-dimensional heteronuclear NMR techniques. In par-
but line shape is irregular. The effect of improved micro-matching fluid, the line width is reduced to about 11 Hz coherence (HMQC) and heteronuclear single quantum (HSQC) require the probe to operate at considerably more sophisticated NMR pulse sequences. For larger molecules such as proteins, deuterium lock channel, are required for such experiments. A single-tuned proton coil. In practice, some reduction must be accepted. Major design criteria for such inverse-detection probes include (a) minimization of losses of the multiple-tuned circuitry such that the heteronuclear channels are also as efficient as possible. High efficiency results in less power being deposited during decoupling, the decoupling bandwidth is maximized, and pulse widths are minimized. (b) isolation among the individual channels must be maximized: strategies include designs which yield geometric and/or electrical orthogonality and shielding using ground planes. Inverse-detection experiments involve proton detection while simultaneously using high-powered decoupling on the heteronuclear channel. Any cross-talk between the channels will lead to severe degradation of S/N.

The last criterion poses a particular challenge for microcoils since the lumped elements which comprise the multiple frequency impedance matching circuits are typically positioned in much closer proximity than for traditional NMR probes. Electrical characterization of the probes includes the measurement of the S12, scattering parameter, defined as the reverse insertion voltage gain when source and load impedances are 50 Ω. That is, for 1 V applied to the inputs of the proton channel, S12 is a measure of the voltage detected at the output of the carbon channel if both channels have been impedance-matched to 50 Ω. For perfectly isolated channels, the scattering parameter will be 0. In reality, S12 values range from 0.1% (–30 dB) to 0.01% (–40 dB).

E. Microfabrication

Given the increased sensitivity which arises from miniaturized RF coils, microfabrication techniques can prove effective in establishing accurate geometries and good mechanical stability for smaller dimensions. As shown in Figure 4A, solenoidal microcoils have been fabricated by microcontact printing. While high-resolution 1H NMR spectra of ethylenediyne and acetone were demonstrated, the sensitivity of the printed microcoil was lower than others made by hand from copper wire. This decreased sensitivity was attributed to higher resistance which resulted from a lower cross-sectional area and higher resistivity of the electrodeposited copper compared to annealed copper. Planar coils with inner diameters less than 50 μm have been fabricated for aluminum and gold deposited on glass substrates. However, because of the potential for coupling dedicated preamplifiers and microcoils on a single substrate, gallium arsenide (GaAs) offers a more attractive RF coil material. Planar receiver coils with 4.5 turns, a trace width of 3.5–10 μm, a trace separation of 3.5–10 μm, an i.d. of 97 μm, and an o.d. of 200 μm were fabricated. Through a combination of lift-off and electropolishing technologies, the pattern was transferred from a mask written by conventional electron-beam lithography to a GaAs substrate. A 1.5-cm RF transmitter coil, constructed from a single turn of 18-gauge copper wire, was mounted orthogonally to the microfabricated receiver coil on the circuit board. Figure 4B shows a scanning electron micrograph of a planar microcoil.

For a simple model of n concentric turns and constant interturn spacing and trace width, the S/N per unit volume is...
where $z$ refers to the on-axis distance from the coil, $r$ corresponds to the resistivity, $D$ is the outer diameter of the spiral, and $d$ is the diameter of the conductor. According to this equation, the S/N varies slowly with coil diameter. While fewer turns require less space and fabrication effort, a greater number of turns yields increased inductance and a reduction in the capacitance required for impedance matching. Currently, the use of microfabrication techniques is in an inchoate stage. Although NMR spectra with line widths under 2 Hz have been achieved and microfabricated fluidic systems have been coupled to chip-based NMR detectors, magnetic susceptibility remains a significant issue. Additionally, planar microcoils must overcome the additional challenge of the extremely low signal strengths from subnanoliter observe volumes. Nevertheless, these techniques should prove essential as NMR spectroscopy examines continually smaller dimensions.

\[
(S/N)_{puv} = \sum_{n} \frac{\mu_0}{D[1 + (2z/D)^{3/2}]} \sqrt{\sum_{n} \frac{4\rho D}{d^2}}
\]  

(9)

Figure 4. (A) Photograph of a 325-μm diameter solenoidal microcoil fabricated by microcontact printing. (Reproduced with permission from ref 65. Copyright 1997 American Institute of Physics.) (B) Scanning electron micrograph of a planar microcoil with an o.d. of 200 μm and an i.d. of 97 μm.

F. Direct Comparisons of Probe Sensitivity

An important question for an analyst is which NMR probe to use. We consider cases in which the sample is either mass-limited or concentration-limited but do not include planar microcoil probes in this comparison because their sensitivity is currently lower than the other small-volume probes. Since a valid comparison requires careful attention to sample preparation and NMR processing conditions for a particular analyte, a comprehensive survey of available commercial probes could not be garnered from the literature. The selected examples are provided to illustrate the general effects of RF coil design on mass and concentration sensitivity. In all cases, results were processed with a line broadening value equivalent to the line width of the resonance signals.

The first example derives from product literature for a probe designed for trace analysis. With a reduced-diameter saddle coil probe from Nalorac, a 1H NMR spectrum was acquired from a total sample mass of 172 μg of sucrose in 23 μL of D2O (21.8 mM sucrose) in a 1.7-mm o.d. sample tube. The single-scan S/N is 193 for the anomeric proton on a 600 MHz spectrometer with an acquisition time of 4.1 s. Since $V_{obs}$ is approximately 60% of the total sample volume, the sensitivities (with appropriate units) are easily computed and appear in Table 2.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sensitivity (S/N$^{1/2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nalorac</td>
<td>222 μL (2.26 mM sucrose)</td>
</tr>
<tr>
<td>Varian</td>
<td>134 μL (300 MHz)</td>
</tr>
<tr>
<td>Microcoil probe A</td>
<td>5580 μL (500 MHz)</td>
</tr>
<tr>
<td>Microcoil probe B</td>
<td>110 μL (600 MHz)</td>
</tr>
</tbody>
</table>

Since $V_{obs}$ is approximately 60% of the total sample volume, the sensitivities (with appropriate units) are easily computed and appear in Table 2. Suppose an analyst wants to know what the result would be for the same sample mass in a 5-mm probe. A good comparison is to acquire a spectrum under identical NMR conditions with the same mass of sucrose (172 μg) dissolved in the observe volume of the 5-mm probe. On the basis of a coil length of 16 mm and a tube i.d. of 4.2 mm, $V_{obs} = 222$ μL (2.26 mM sucrose). The anomic proton S/N is 136 for a single scan on a 600 MHz Varian INOVA spectrometer using a Varian 5-mm proton detection probe. For the same data acquisition and processing parameters as well as comparable line widths and shapes, the resultant sensitivities from the 5-mm Varian probe appear in Table 2. Clearly, the mass sensitivity is better for the Nalorac probe, but the concentration sensitivity is superior for the 5-mm Varian probe.

To illustrate more clearly the effects of design parameters on performance, two microcoil probes are also included in Table 2. Microcoil probe A is 1-mm long and is wound on a capillary with a 75-μm i.d./360-μm o.d. (Vobs of 5 nL); microcoil probe B is 1.6-mm long and has a coil diameter of 850 μm and a Vobs of 620 nL. The performance indicators in Table 2 are computed and appear in Table 2. Suppose an analyst wants to know what the result would be for the same sample mass in a 5-mm probe. A good comparison is to acquire a spectrum under identical NMR conditions with the same mass of sucrose (172 μg) dissolved in the observe volume of the 5-mm probe. On the basis of a coil length of 16 mm and a tube i.d. of 4.2 mm, $V_{obs} = 222$ μL (2.26 mM sucrose). The anomic proton S/N is 136 for a single scan on a 600 MHz Varian INOVA spectrometer using a Varian 5-mm proton detection probe. For the same data acquisition and processing parameters as well as comparable line widths and shapes, the resultant sensitivities from the 5-mm Varian probe appear in Table 2. Clearly, the mass sensitivity is better for the Nalorac probe, but the concentration sensitivity is superior for the 5-mm Varian probe.

Table 2. Performance Comparisons of Several NMR Probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sensitivity (S/N$^{1/2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Varian</td>
<td>30 μL (300 MHz)</td>
</tr>
<tr>
<td>Nalorac</td>
<td>134 μL (300 MHz)</td>
</tr>
<tr>
<td>Microcoil probe A</td>
<td>5580 μL (500 MHz)</td>
</tr>
<tr>
<td>Microcoil probe B</td>
<td>110 μL (600 MHz)</td>
</tr>
</tbody>
</table>

Data for microcoil probes A and B were collected at 300 and 500 MHz, respectively. These results were normalized to 600 MHz according to the relationship $[B_j]^{500}$.
2 show that the microcoil probe A achieves the highest \( S_m \) and requires the smallest sample size but is limited in \( S_c \). The results for microcoil probe B confirm that a significant improvement in \( S_c \) and a proportionate decrease in LOD, for the microcoil, can be attained by increasing \( f_c \).\(^6\) Clearly, design parameters can be manipulated to suit particular experimental requirements.

In another example, a comparison was made for the same mass of material (400 ng of menthol) in the observed region of three NMR coils with \( V_{obs} = 230 \mu L \) (5 mm tube), 40 \( \mu L \) (Varian nanoprobe),\(^55\) and 31 nL (microcoil; capillary i.d., 340 \( \mu m \) o.d.).\(^70\) For the same mass of menthol dissolved in \( V_{obs} \), the increase in mass sensitivity for the microcoil compared to the Varian microprobe allows acquisition of a comparable spectrum in 10-fold less time (9 min compared to 15 h).\(^70\) Consequently, the \( S_m \) for the microcoil exhibits a 10-fold enhancement (100\(^{1/2} \)) compared to the Varian nanoprobe.

Consider a promising natural product of which only 100 pmol of material has been isolated. If the sample is dissolved in a \( V_{obs} \) of 5 nL, the sample concentration is 20 mM. In contrast, dissolution of the material in 230 \( \mu L \) (\( V_{obs} \) for a typical 5-mm spinning tube) yields a concentration of only 440 nM. Each NMR probe contains the same mass-limited amount of sample in its respective \( V_{obs} \) regions. In this case, the microcoil obtains a significantly higher \( S/N \) for a given \( t_{eq} \).\(^3\) A mass-limited condition generally calls for the greatest \( S_m \) which is usually the probe with the smallest coil.

Alternatively, consider NMR binding studies of neuropeptide receptors which typically require low concentrations (\( \mu M \)) to determine the point at which strong interaction ceases. In this situation, one has to sacrifice \( S_m \) to gain overall \( S/N \) by using a larger-volume sample. A situation which is not mass-limited or where the sample already exists in a relatively large volume and cannot be concentrated benefits by using the probe with the largest \( S_c \), which usually has the biggest \( V_{obs} \). Clearly, no single probe fulfills all needs, and one must employ the right tool for a particular application. Considerations of probe sensitivity are weighed against the amount of sample available for analysis and the acceptable concentration range for the particular experiment. Although the use of small sample volumes can be limited by such factors as sample loading and solubility, the microcoil approach clearly offers impressive performance gains.\(^57\) Given the superior mass sensitivity of microcoil probes, their advantages in mass-limited applications are obvious.

\section*{V. Microliter-Volume Static NMR Spectroscopy}

\subsection*{A. Natural Product Extracts}

In many cases, compounds obtained from natural sources have contributed to advances in medicine, materials, and many other areas. Since these compounds are typically isolated in small amounts, NMR probes with high mass sensitivity are required. As one example, the 3-mm Nalorac probe was used along with a variety of other analytical techniques to solve the structure of cryptolepicarboline, a new indoloquinoline-\( \beta \)-carboline dimeric alkaloid isolated from Cryptolepis sanguinolenta, a shrub known for its medicinal properties.\(^51\) For a total amount of sample of 250 nmol (100 \( \mu g \)), a one-dimensional \( ^{13}C \) spectrum required NMR data collection over the period of a weekend. In another application of this inverse-detection microprobe, HMOC experiments were performed on less than 100 nmol of a potent Caribbean ciguatoxin at 500 MHz.\(^52\) With the standard 120 \( \mu L \) cell, total acquisition time for the HMOC spectrum was 231 h. The use of a Shigemi microcell reduced the volume to 67 \( \mu L \) and the total data acquisition time to 101 h. With a 1.7-mm Nalorac probe, 550 nmol of cryptolepine dissolved in 25 \( \mu L \) gave gradient-HSQC (GHSQC) and gradient-HMBC (GHMBC) in 12 min and 1.1 h, respectively.\(^50\) Figure 5 shows the aromatic region of the GHSQC spectrum. In further analysis of the sample, an 8\% impurity, corresponding to less than 50 nmol, was characterized from GHSQC and GHMBC spectra acquired in 25.5 and 56.5 h, respectively.\(^53\) Long-range proton—nitrogen GHMBC spectra have also been obtained from a 1 mg sample (3 \( \mu mol \)) of the alkaloid strychnine dissolved in 30 \( \mu L \) of CDCl\(_3\) in 18 h.\(^54\) For weekend runs, sample requirements decrease to 1 \( \mu mol \).

With regard to structural elucidation of unknown compounds, the Varian Nano-NMR probe has also proven useful in a variety of cases. As one example, the complete carbon skeleton of a novel steroid, 2-hydroxyandrosta-1,4-diene-3,16-dione, was determined using the \( ^{13}C-^{13}C \) INADEQUATE experiment with approximately 40 \( \mu mol \) of material: the total data acquisition time was 62 h.\(^71\) In a second study, a novel glycosaminoglycan molecule was investigated.\(^72\) Using a spin rate of 1850 Hz, TOCSY spectra were acquired in 30 h, DQF—COSY in 20 h, and ROESY in 60 h using 10 \( \mu g \) of material. Proteins and peptides have also been characterized with this technology. For instance, a study was conducted on
renatured lysozyme obtained from fully reduced lysozyme under folding/oxidation conditions.\textsuperscript{73} One-dimensional spectra from only 6 \( \muL \), corresponding to 50 nmol, were obtained in 32 scans. Two-dimensional TOCSY spectra were acquired in 3–7 h. Finally, the Nano-NMR probe was used to obtain the full three-dimensional solution structure of a novel peptide, Pi1, purified from the venom of the scorpion Pandinus imperator.\textsuperscript{74} In this case, a limited sample of 150 \( \mug \) (50 nmol) precluded the use of standard NMR probe technology. Using 40 \( \muL \) of sample at a concentration of 0.8–1 mM, a range of two-dimensional experiments provided the requisite information for complete structural characterization. Figure 6 shows a contour plot of a TOCSY spectrum of Pi1.

B. Analysis of Combinatorial Chemistry Products

Through the combination of magic angle spinning (MAS) and high-resolution probe fabrication, the Nano-NMR probe enables on-bead studies of products from solid-phase syntheses. The first application to combinatorial chemistry examined 1.5 mg (3 \( \mumol \)) of a test compound bound to a Tentagel solid-phase-synthesis resin in DMSO-\( d_6 \).\textsuperscript{75} By spinning the sample at several kilohertz, significant improvements in the spectral resolution are achieved.\textsuperscript{75,76} In another report, MAS and spin-echo spectroscopy were used to follow the lithium aluminum hydride reduction of a resin-bound methyl benzoate to the corresponding benzyl alcohol.\textsuperscript{77} Short T\textsubscript{2} signals from the polystyrene were efficiently suppressed by using the spin-echo sequence, and high-resolution proton spectra at 500 MHz using the Nano-NMR probe clearly showed the difference between the reactants and products. In a similar vein, Sarkar et al. used a combination of proton and heteronuclear techniques in identifying moieties while still bound to a solid-phase resin bead.\textsuperscript{78} Proton spectra were obtained at 500 MHz from [3,5-dimethoxy-\( ^{13}C \)] benzoic acid coupled to a single 100-\( \mum \) diameter resin bead. The sample amount was estimated to be 800 pmol per bead. However, considerable interferences from the solvent, impurities, and a background from the polystyrene bead itself were evident. By using a one-dimensional HMQC sequence with a \( ^{13}C \)-labeled compound, the \( ^{13}C \)-filtered proton spectrum of the labeled compound showed signals only from the protons of the 3,5-dimethoxy-\( ^{13}C \) moiety, with excellent suppression of the other protons. The total data acquisition time was approximately 3 h. A two-dimensional \( ^1H-^{13}C \) HMQC spectrum was also run, with an experiment time of 17.5 h. For these latter experiments the solenoidal coil of the probe was tuned to \( ^{13}C \) and an outer decoupler coil tuned to the proton frequency. Since this Nano-NMR probe is designed for \( ^{13}C \) direct detection, some loss of efficiency compared to inverse detection is expected.

VI. Nanoliter-Volume Static NMR Spectroscopy

While the examples above show the utility of NMR for static analyses in the microliter regime, downscaling experiments to nanoliter volumes presents additional challenges. While the early reports\textsuperscript{79–81} of nanoliter-volume NMR spectroscopy illustrated the potential of submillimeter coils, the relatively broad line widths (7–11 Hz) were too great to resolve most scalar couplings. In the initial report of high-resolution \( ^1H \) NMR of nanoliter-volume samples, a 130-fold improvement in mass sensitivity (relative to a conventional probe) was demonstrated for a microcoil with a 5-nL observe volume.\textsuperscript{38} Figure 7 shows a spectrum acquired from 3.3 nmol of \( \alpha \)-bag cell peptide from the sea slug Aplysia californica; the LOD is 124 pmol for an acquisition time of 11.8 min.\textsuperscript{38} While this initial work was performed at 300 MHz, further refinements of such microcoil probes have included experiments at higher fields and the incorporation of a lock channel.\textsuperscript{82}

While the majority of microcoil work has focused on \( ^1H \) NMR, nanoliter-volume probes have been fabricated for both direct and inverse detection of \( ^{13}C \).\textsuperscript{83,84} Since the S/N of inverse-detection experiments is less than that of homonuclear proton experiments, typical sample volumes used are larger than those described earlier. With optimized coil geometries and volumes of between 700 nL and 1.2 \( \muL \), with concentrations of 30–100 mM, high-quality HMQC and HSQC spectra have been obtained in a few hours.\textsuperscript{84}

Figure 6. Contour plot of a TOCSY spectrum of Pi1 obtained at 35 °C. Spin systems are labeled by sequential residue position; a line connects the full spin system. (Reprinted with permission from ref 74. Copyright 1997 American Chemical Society.)

Figure 7. 300 MHz \( ^1H \) NMR spectrum of Aplysia californica \( \alpha \)-bag cell peptide (residues 1–7). The 5-nL detection cell contains 3.0 \( \mug \) (3.3 nmol) of peptide. The LOD is 112 ng (124 pmol) for an acquisition time of 11.8 min. (Reproduced with permission from ref 38. Copyright 1995 American Association for the Advancement of Science.)
Figure 8 depicts a circuit diagram of the inverse-detection probe used for either $^1$H-$^{15}$N or $^1$H-$^{13}$C experiments. The inner solenoidal coil is tuned to protons and the outer surface coil to the heteronucleus. The surface coil consists of a single circular loop, 7 mm in diameter, and is mounted 1 mm from the microcoil edge. A chip capacitor is placed across the coil leads as close to the loop as possible. Although surface coils have a high sensitivity and are simple to construct, they are traditionally not used in high-resolution NMR due to a spatially inhomogeneous magnetic field profile. However, since the volume enclosed by microcoils is so small, the surface coils prove to be highly efficient both for pulsing and decoupling. Furthermore, the $B_1$ homogeneity is sufficiently high to produce a 180° pulse which inverts the magnetization cleanly during pulse-width calibration. The inductor and capacitor values used for proton operation at 500 MHz are shown in Table 3. The surface coil and solenoidal microcoil are housed in a plastic bottle which also contains the magnetic susceptibility matching fluid, as described previously. All the circuit elements, including the coils, are positioned on a 3 cm x 5 cm double-sided printed circuit board, with the impedance matching circuitry for protons and heteronuclear frequencies on opposite sides.

When impedance matched, the $S_{11}$ parameters for $^1$H and $^13$C channels were each less than $-30$ dB, meaning that less than 0.1% of the power separately transmitted to each channel is reflected from the probe back through each individual channel. The $S_{12}$ parameter, described earlier, was measured to be less than $-25$ dB at both frequencies. The typical $^1$H and $^{13}$C 90° pulse widths were 1.7 and 11.5 $\mu$s with 8 and 0 dB transmitter attenuations (amplifier rated outputs were 50 and 300 W, respectively). Full $^13$C decoupling over a spectral width of 150 ppm was achieved using WALTZ-16 scheme with 14 dB decoupler transmitter attenuation. This probe has been used to measure $^1$H-$^{13}$C correlations on several low molecular weight organic compounds. As one example, Figure 9 shows a two-dimensional HMQC spectrum of 54 mM chloroquine diphosphate in D$_2$O acquired with an inverse-detection microcoil probe. The 745-nL $V_{obs}$ contains 40 nmol (13 $\mu$g) of chloroquine. The data, 32 transients per slice, 1024 x 128 ($\times$2, hypercomplex) points, are acquired in 3.6 h. The data were zero-filled to 256 points in the $^{13}$C dimension. A 40° shifted sinebell function was applied followed by Gaussian multiplication prior to Fourier transformation.

![Figure 9.](image_url) $^13$C-decoupled HMQC spectrum of 54 mM chloroquine diphosphate in D$_2$O acquired with an inverse-detection microcoil probe. The 745-nL $V_{obs}$ contains 40 nmol (13 $\mu$g) of chloroquine. The data, 32 transients per slice, 1024 x 128 ($\times$2, hypercomplex) points, are acquired in 3.6 h. The data were zero-filled to 256 points in the $^{13}$C dimension. A 40° shifted sinebell function was applied followed by Gaussian multiplication prior to Fourier transformation.

Figure 10. Example of liquid susceptibility matching plugs used with microcoils for limited-volume samples.

Table 3. Values of Circuit Elements Used in Inverse-Detection Probes

<table>
<thead>
<tr>
<th></th>
<th>$^1$H (500 MHz)</th>
<th>$^{13}$C (125.7 MHz)</th>
<th>$^{15}$N (50.7 MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L$_1$</td>
<td>42 nH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L$_2$</td>
<td>14 nH</td>
<td>14 nH</td>
<td></td>
</tr>
<tr>
<td>C$_1$</td>
<td>5.6 pF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_2$</td>
<td>5.6 pF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_3$</td>
<td>3.2 pF</td>
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<td>C$_4$</td>
<td>6.5 pF</td>
<td></td>
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<td>C$_5$</td>
<td>3.8 pF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_6$</td>
<td>100 pF</td>
<td>100 pF</td>
<td></td>
</tr>
<tr>
<td>C$_7$</td>
<td>10.2 pF</td>
<td>330 pF</td>
<td></td>
</tr>
<tr>
<td>C$_8$</td>
<td>11 pF</td>
<td>20 pF</td>
<td></td>
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</tbody>
</table>
permeation chromatography (GPC) as well as liquid chromatography (HPLC), capillary electrophoresis (CE), NMR has been coupled to separation and geochemical analysis of planetary samples. In chemical communication within living organisms, strategies are requisite in such diverse areas as characterization of pharmaceutical candidates, understanding chemical communication within living organisms, and geochemical analysis of planetary samples. In particular, NMR has been coupled to separation methods such as gas chromatography (GC), supercritical fluid chromatography (SFC), gel-permeation chromatography (GPC) as well as liquid chromatography (HPLC), capillary electrophoresis (CE), and capillary electrochromatography (CEC). Some of the primary advantages of on-line (versus off-line) NMR detection in flowing systems include improved chromatographic resolution, consistent detector response, on-line data analysis, and faster data acquisition. Capillary separations offer the additional benefit of reduced solvent consumption. The experimental versatility of NMR spectroscopy as a detector for separations has been applied successfully to solve a wide variety of problems. However, this review focuses on the combination of NMR and capillary-scale (i.d. of less than 1 mm) separations. While the recent advances in NMR microprobe development outlined above have enabled NMR detection of microseparations, many of the capabilities of conventional NMR have not yet been applied to the capillary scale. The interested reader is encouraged to explore the literature for reviews of NMR coupled to conventional-scale separations.

The joining of capillary electroseparation methods and capillary HPLC with NMR detection provides information complementary to existing methods. While Table 1 illustrates that LODs for CE with microcoil NMR detection are currently poorer than other on-line detection techniques, the ability to identify compounds on the basis of their NMR spectra and to spectrally discriminate among unknown peaks using multidimensional data merits exploration. Moreover, improvements in LODs will continue to accrue through further hardware developments. In contrast to conventional NMR probes, the enhanced mass sensitivity of reduced-diameter RF transceivers enables the coupling of one of the most structurally-rich detection schemes and highly efficient capillary separations.

VII. NMR Detection of Capillary Separations

As practitioners of the chemical sciences continue to blur disciplinary boundaries through investigations of truly complex systems, analytical methods must correspondingly increase in their degree of sophistication. During the past two decades, hyphenated analytical techniques have become rather commonplace. Multimode separation and detection strategies are requisite in such diverse areas as characterization of pharmaceutical candidates, understanding chemical communication within living organisms, and geochemical analysis of planetary samples. In particular, NMR has been coupled to separation methods such as gas chromatography (GC), supercritical fluid chromatography (SFC), and gel-permeation chromatography (GPC) as well as liquid chromatography (HPLC), capillary electrophoresis (CE), and capillary electrochromatography (CEC). Some of the primary advantages of on-line (versus off-line) NMR detection in flowing systems include improved chromatographic resolution, consistent detector response, on-line data analysis, and faster data acquisition. Capillary separations offer the additional benefit of reduced solvent consumption. The experimental versatility of NMR spectroscopy as a detector for separations has been applied successfully to solve a wide variety of problems. However, this review focuses on the combination of NMR and capillary-scale (i.d. of less than 1 mm) separations. While the recent advances in NMR microprobe development outlined above have enabled NMR detection of microseparations, many of the capabilities of conventional NMR have not yet been applied to the capillary scale. The interested reader is encouraged to explore the literature for reviews of NMR coupled to conventional-scale separations.

A. Flow Effects

In designing NMR detection strategies for flowing separation systems, the effects of solution flow on the NMR line shape, S/N, and LW must be considered. In contrast to stopped-flow conditions which allow longer NMR acquisition times, the continuous flow associated with typical column separations imposes certain restrictions on NMR data. Flow of analyte through the detection coil reduces the effective longitudinal relaxation time of the sample by replacing pulsed spins with unpulsed spins, thereby allowing more rapid pulse repetition and improved S/N per unit time. In 1984, Laude and Wilkins reported a systematic study of the effects of flow rates on both NMR sensitivity and resolution for analytical-scale HPLC–NMR. Wu et al. developed a model for microcoils and compared the S/N obtained with optimized pulse repetition rates as a function of coil diameter.

In a similar fashion, the effective transverse relaxation time ($T_{2,\text{flow}}$) may also be shortened. The decrease in $T_{2,\text{flow}}$ due to the limited spin residence time ($t$) may increase signal LW for any given spin not observed for a period long enough to allow full signal decay of the analyte within the observe volume of the NMR probe. In these cases, the effective transverse relaxation time of the analyte decreases in a flowing system and the signal decays more rapidly than for a static analysis. The resulting increase in line width due to flow is inversely proportional to $t$. Consequently, while higher flow rates allow faster pulse repetition and a possible increase in S/N, the degradation in LW may conceal important spectral information. As a result, the optimum flow rate for on-line NMR detection is a compromise among S/N, LW, and chromatographic resolution.

In addition to the effects of flow on spectral resolution and data acquisition strategies, NMR possesses the capability of in situ flow characterization during a separation. Although not yet demonstrated on the capillary scale, pulsed field gradient (PFG) NMR techniques have been used to study...
dispersion in packed HPLC columns. From the average values of the axial and transverse apparent diffusion coefficients in a packed column over a certain volume of the bed, Tallarek et al. related the local value of the height equivalent to a theoretical plate of a chromatographic column to the velocity of the liquid. Furthermore, excellent agreement between theoretical studies of the plate height equation and the PFG-NMR results were demonstrated. Given this initial demonstration, the use of gradient NMR techniques for the in situ analysis of capillary separations holds great promise.

B. HPLC–NMR

Though demonstration of both stopped-flow and continuous-flow HPLC–NMR first appeared in the late 1970s, this hyphenated technique has only recently begun to experience widespread use. The development of suitable flow cells and techniques to optimize NMR acquisition conditions have laid the foundation for today's commercial HPLC–NMR instruments. Sensitivity enhancements through higher-field magnets, smaller-diameter transceiver coils, and increased dynamic range of receivers, combined with the availability of versatile solvent suppression techniques, have also alleviated many of the initial problems of HPLC–NMR. Nonetheless, the relatively poor mass sensitivity of conventional NMR detection schemes, compounded by limited observation time for each analyte, has remained the primary challenge to on-line NMR detection of nanoliter volumes.

HPLC column eluent clearly represents a mass-limited condition. Typical HPLC–NMR detection cells have RF coil observe volumes in the range of 60–250 μL and are used in conjunction with conventional 2–4.6-mm i.d. columns. NMR probes designed for small amounts of material are well-suited to microbore and capillary HPLC because the higher efficiency separations result in higher sample concentrations eluting from the column. Typically, the analyte concentration in the peak maximum is inversely proportional to the square of the column i.d. Thus, the smaller the column i.d., the less a given sample is diluted prior to detection. As an example, Table 4 shows typical peak volumes and expected analyte peak concentrations for a 1 nmol injection. Note how capillary HPLC increases the analyte concentration more than 2 orders of magnitude, so that the combination of smaller and more efficient NMR probes coupled with smaller HPLC columns may represent a superior HPLC–NMR configuration. The improved $S/N$ of reduced-diameter transceivers yields lower mass LODs and allows coupling of NMR to the more efficient microbore and capillary HPLC columns. However, downsampling HPLC–NMR 3 orders of magnitude to nanoliter regimes introduces several unique considerations.

Conventional HPLC–NMR employs separation columns which are connected to NMR detection cells via open tubular capillaries with internal diameters substantially smaller than the column to avoid extracolumn band broadening. Since the band broadening introduced by the connections and transfer lines causes significant peak dispersion in capillary separations, on-column detection for small-volume HPLC is desirable. One technique employs a solenoidal transceiver coil wrapped directly around the separation column. Attachment of the coil to the flow cell itself creates a detector with a high $f_c$, thereby improving concentration sensitivity. Such a design positions the detection cell perpendicular to $B_0$ and takes advantage of the augmented sensitivity of solenoidal geometry. An alternative approach utilizes saddle-type coils produced by Bruker with parallel alignment of the separation column with respect to the magnetic field. With this arrangement, HPLC columns are inserted into the magnet so column types and diameters can be changed without altering the size of the transceiver coil. This experimental configuration allows effective shimming and permits column changes to occur without noticeable perturbation of magnetic field homogeneity. In both cases, to avoid negative effects of the HPLC apparatus on the magnetic field and vice versa, the pump and the solvent reservoir remain more than 1 m from the magnet. To maximize signal intensity, prepolarization of the nuclei through residence in the magnetic field for greater than 5 $T_1$ intervals establishes a Boltzmann distribution of nuclear spins.

The relatively large volumes and flow rates used with conventional (4.6-mm i.d.) analytical HPLC columns make the deuterated solvents conventionally used for NMR spectroscopy a significant part of the total experiment cost (except $D_2O$). Although halo-carbons can eliminate $^1H$ NMR background signals, these solvents are not amenable to the majority of separations. To minimize the interference of solvent peaks, one can employ either solvent suppression techniques or deuterated solvents and smaller-diameter chromatographic columns to minimize solvent consumption. For continuous-flow experiments, solvent presaturation is not very effective because a fraction of the spins is constantly replaced in the $V_{obs}$ region by unsaturated spins. The change in resonance frequencies for signals which accompany gradient elution poses an additional challenge. Despite considerable improvements in these methods, solvent suppression usually renders spectral regions near the solvent signals unobservable. In contrast, the small volume requirements of microbore and capillary HPLC columns not only permit the use of gradient elution with completely deuterated solvents but also fulfill the desire for higher separation efficiencies and reduction of solvent consumption.

Table 4. Typical Column Separation Parameters and Resultant Peak Concentrations for 1 nmol of Analyte

<table>
<thead>
<tr>
<th>column i.d. (mm) and mode</th>
<th>flow rate (μL/min)</th>
<th>peak volume (μL)</th>
<th>Peak concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6 (standard)</td>
<td>1000</td>
<td>200</td>
<td>0.005</td>
</tr>
<tr>
<td>1 (microbore)</td>
<td>30</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>0.32 (capillary)</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.18 (capillary)</td>
<td>1</td>
<td>0.3</td>
<td>3</td>
</tr>
</tbody>
</table>
around a fused silica capillary (250 μm i.d.; 360 μm o.d.) to create a \( V_{\text{obs}} \) of 50 nL and an \( f_c \) of 48%. For a separation of three amino acids and two peptides, submicrogram LODs were obtained on a 300 MHz spectrometer. However, this system represented a 100-fold mismatch between the estimated analyte peak volume of \( \sim 5 \mu L \) and the 50-nL observe volume of the detector, a condition far from ideal. The use of fast pulse repetition rates partially compensated for this problem (by taking advantage of new spins brought in by flow).

In another report of capillary HPLC–NMR coupling,\(^{111} \) a 180-μm i.d. packed capillary was inserted within a 2.0-mm NMR probe to create a \( V_{\text{obs}} \) of 200 nL (\( f_c \) = 0.9%). For stopped-flow NMR data acquisition, the spectral resolution obtainable with this arrangement was \( \sim 1.5 \) Hz. This system was applied to the characterization of vitamin A derivatives, which tend to decompose or isomerize with exposure to air or light. A contour plot of the capillary HPLC–NMR chromatogram illustrated the \(^1\)H NMR spectral differences between vitamin A acetate and its reaction products. However, no LODs or information about separation efficiency were included in this report.

Continuous- and stopped-flow NMR coupled to gradient capillary HPLC was first reported in 1996.\(^{110} \) In this report, a 315-μm i.d. capillary was packed with reversed-phase particles before the NMR detection window to separate a mixture of dansylated amino acids. Mounted vertically within a \(^1\)H/\(^{13}\)C 2.5-mm inverse-detection Bruker microprobe, \( V_{\text{obs}} \) for the capillary was 900 nL. Gradient elution yields a more efficient separation and offers a convenient means of sample preconcentration. The use of highly efficient capillary HPLC and a higher magnetic field strength (600 MHz) also provided improved performance. Though the capillary and NMR coil arrangement was not optimal (\( f_c \) = 1.6%), LODs of about 70 pmol were obtained under stopped-flow conditions for \( t_{\text{acq}} = 20 \) min.

Elucidation of the structure of a previously unknown kitol isomer (natural retinol dimer) represents a practical application of capillary HPLC with nanoliter-volume NMR detection.\(^{112} \) Despite a decrease in the NMR coil diameter from 2.5 to 2.0 mm compared to their previous study,\(^{110} \) \( f_c \) decreased to 0.8%. With a \( V_{\text{obs}} \) of 200 nL, the LOD was 150 pmol for a model compound in 7.7 min. Figure 12 illustrates the use of fully deuterated solvents for acquisition of spectra unhindered by solvent suppression. As shown in Figure 12A, the one-dimensional spectral data combined with the two-dimensional TOCSY experiment in Figure 12B allow unambiguous structural determination. Spectra were recorded when the chromatographic peak maximum had entered \( V_{\text{obs}} \), as signaled by continuous monitoring of the \(^1\)H NMR signal.

Another approach to joining capillary HPLC and NMR utilizes a fixed capillary NMR detection cell and allows fassile coupling to a variety of microseparation methods.\(^{88} \) By treating a 60-μm i.d. fused silica capillary with HF and localized heat, Pusecker et al. increased the i.d. to 190 μm for a specific region of the capillary. In this report, the 240-nL detection cell was placed within a 2-mm Bruker microprobe and connected to a packed HPLC capillary via Teflon tubing. Such a configuration yields an \( f_c \) of 0.9% and line widths on the order of 2.5 Hz, after shimming on a chloroform signal for the hump test (3% chloroform in 97% acetone-\( d_6 \)). In addition to increasing analyte observation times (for a flowing system), the enlarged detection cell facilitates shimming through a more stable lock and better S/N for the hump test. Capillary HPLC–NMR was demonstrated with this arrangement for both a mixture of alkyl benzoates and a 20% solution of hop bitter acids. Stopped-flow \(^1\)H–\(^1\)H COSY data were presented for two of the hop bitter acids (\( t_{\text{acq}} = 13 \) h). Unfortunately, information about LODs was not included. These separations were performed in completely deuterated solvents and, as such, prevented solvent signals from overlapping with analyte signals in the NMR spectrum. Given the wide applicability of HPLC, many more examples of capillary HPLC–NMR will certainly appear in the coming years.

![Figure 12](image-url)
C. SFC–NMR

Though the union of supercritical fluid chromatography and supercritical fluid extraction with \(^1\)H NMR has been reported,\(^{113–117}\) this combination has never been demonstrated on the nanoliter scale. SFC–NMR employing CO\(_2\) or various other supercritical solvents without a \(^1\)H NMR background signal as the mobile phase eliminates the need for solvent suppression. Capillaries can withstand the elevated temperatures and pressures associated with supercritical fluids. Additionally, their diminutive volumes allow the economical use of deuterated organic polar modifiers. For these reasons, nanoliter SFC–NMR may appear soon.

D. CE–NMR

As a collection of efficient and flexible separation methods, capillary electrophoretic techniques have found widespread application in the analysis of charged and neutral species.\(^{118,119}\) In contrast to HPLC where column dimensions can be manipulated over a substantial range, the i.d. of electrophoresis capillaries is generally restricted to less than about 100 \(\mu\)m to avoid problems from dissipation of Joule heat. This upper limit on capillary i.d., combined with the high separation efficiency and short observation times of CE, necessarily requires a detection method which can identify the presence of small amounts of material. As listed in Table 1, myriad techniques have been implemented as CE detectors.\(^{120,121}\) Though these detection schemes generally exhibit good sensitivity, mass spectrometry is presently the only CE detector which yields rich chemical information about the analyte. Since NMR spectroscopy provides both confirmatory and complementary chemical information, it enables unprecedented levels of structural elucidation for CE detection. In addition, because of the ability of CE to concentrate analytes, samples with lower initial concentrations can utilize the enhanced \(S_m\) of improved NMR microprobes.

Although NMR can provide a wealth of unique structural information and CE has impressive separation capabilities, the coupling of CE with NMR detection has been demonstrated only recently.\(^{58,79,80,88–92}\) Among the various separation techniques, CE is particularly amenable to solenoidal NMR microcells because the transceiver coil can be wrapped directly around the same fused silica capillary used in the separation. Sampling may occur via electrokinetic, gravimetric, or pressure injection. As such, NMR detection requires minimal modification of most existing CE equipment. In general, two different CE–NMR configurations have been demonstrated. In one arrangement,\(^{58,79,80,91}\) both the inlet and outlet vials are housed within the NMR probe head (see Figure 13A). Presently, this setup requires that sample injection occur externally, followed by insertion into the magnet bore. In the other reported CE–NMR configuration,\(^{88–92}\) the inlet vial remains outside of the magnet so that the probe remains stationary within the magnet during sample injection and separation (see Figure 13B). Future improvements will include the development of CE–NMR systems which allow automated injection, separation, and detection completely within the magnet.

Despite relatively simple instrumental requirements, CE–NMR data reflects a complex interdependence on flow rate, electric field, and current. For instance, the current which passes through the capillary produces a magnetic field gradient that may perturb the uniformity of the \(B_0\) field if it cannot be counteracted through shimming. For a cylindrical conductor which carries current \(i\) uniformly distributed over the cross-sectional area of the wire, Ampere’s Law yields

\[
B_i = \frac{\mu_0 i r}{2\pi R^2}
\]

where \(B_i\) represents the current-induced magnetic field within the cylinder, \(\mu_0\) is the permeability constant, \(r\) is the radial distance from the center of the capillary, and \(R\) corresponds to the capillary inner diameter.\(^{122}\) Because slight temperature variations within the capillary lead to minor changes in buffer conductivity, the current may not be exactly uniformly distributed across the cross-sectional area of the capillary. Nevertheless, this radial gradient varies linearly in magnitude to the extent that the current has a component in the \(B_0\) direction. As shown in Figure 14, electrophoretic currents can
cause significant NMR signal degradation for a capillary configuration which is not parallel to $B_0$. In addition to NMR spectral broadening due to current-induced magnetic field gradients, the high electric fields applied in capillary electroseparation techniques can partially align large molecules, thereby broadening signals. Although field strengths of 90 V/cm have been shown to have a negligible effect on the LW of $^1H$ NMR spectra of amino acids, the effects of higher electric field strengths on larger molecules has not been explored within the context of CE-NMR.

Investigations which compare pressure-driven and electroosmotic flow can isolate the effects of flow versus electrophoretic migration. In contrast to flowing HPLC-NMR experiments, CE has the characteristic that each separated analyte migrates at a different velocity and, therefore, has a different detector residence time. For example, an electropherogram with peaks at 3 and 30 min exhibits detector residence times which differ by an order of magnitude. Therefore, a strategy of variable data acquisition time may be most suitable for optimizing S/N in on-line electrophoretic-NMR. For instance, limited observation time may be improved by decreasing or discontinuing the voltage to reduce or stop flow as the analyte enters the $V_{obs}$ region. In this way, longer observation times can be applied to faster migrating analytes so that either a greater number of scans or a longer relaxation time between each scan can be used to enhance S/N. To eliminate both flow-induced and current-induced distortions of line width and shape, a unique stopped-flow strategy has been demonstrated where the CE voltage is periodically halted so that NMR information is obtained only under quiescent conditions. This method of data acquisition cancels the effect of magnetic field gradients, increases the NMR S/N (due to longer observation times), and allows separation efficiencies on the order of 50 000. Additionally, preconcentration (stacking) methods extend the usable sample concentration range of the method but do not generally affect NMR mass LODs. Figure 15 demonstrates the enhanced S/N which results when stacking conditions are utilized and illustrates the capability of NMR to monitor the concentration event directly and obtain spectra from lower concentrations of analytes.

While initial reports of CE-NMR demonstrated the potential of coupling the high separation efficiency of CE with the capacity of structural analysis provided by NMR, original spectral LWs were larger than 7 Hz. Broad LWs make characterization of unknowns difficult, and the ~100 mM LODs precluded most assays. Subsequent improvements in microcoil NMR probe design have resulted in high-resolution spectra and improved LODs. In addition to the solenoidal microcoil transceivers, saddle-type coils have recently been employed for CE-NMR. More specifically, the 240-nL NMR detection cell described earlier has been utilized in conjunction with a 60-μm i.d. separation capillary for CE-NMR. With this arrangement, a separation of lysine and histidine was achieved with an esti-
mated LOD of \( \approx 2 \) mM for lysine. On the basis of the aliphatic and aromatic NMR signals, the amino acids could be distinguished. In two other reports of CE-NMR with saddle-type coils, separation and detection of the major metabolic products of the analgesic drug paracetamol was demonstrated from an extract of human urine. Because the metabolism of paracetamol is well established and has been studied extensively with NMR spectroscopy, this analyte and its metabolic products provide an attractive biological model system to evaluate the performance of capillary electroseparation systems coupled to NMR microprobes. Though an 8-nL injection volume was used for these CE-NMR experiments, a larger 400-nL NMR detection cell was created from an 80-μm i.d. capillary. As illustrated in Figure 16, the glucuronide and sulfate conjugates of the drug as well as a major endogenous species were identified from the 1H NMR spectra of the electropherogram. LODs for each of the analytes were estimated to be \( \approx 10 \) ng, but no indication of separation efficiency was given.

Since the electrophoretic current in the capillary is parallel to \( B_0 \) for saddle-type coils, the current-induced magnetic field and its deleterious effects on the NMR signals is minimized. On the other hand, the relatively large detector zones used in conjunction with these coils introduce significant band broadening and thus limit separation efficiency. With advances in the sensitivity of NMR probes and a better understanding of the challenges involved in coupling CE and NMR, this hyphenated technique is becoming practical in situations which require separation and structural determination of mass-limited unknowns.

In addition to structural identification of separated analytes, CE-NMR provides many unique opportunities to study fundamental electrophoretic phenomena in a nondestructive manner. Though not yet performed for capillaries, demonstrated capabilities of electrophoretic-NMR include direct measurement of pH, selective detection of charged species, diffusion coefficients, mobilities, and flow imaging. As one example of the diagnostic information which is available in CE-NMR data, electroosmotic flow and solvent composition can be monitored directly. For an injection plug of 10% H2O in D2O, Figure 17 shows a leading parabolic-type concentration profile (originating from a gravimetric injection) and a trailing flat profile (caused by the application of an electrical potential after the injection). The length of the plug is consistent with contributions from injection and diffusion and indicates that other sources of zone spreading such as mixing are not significant.

Other preliminary experiments have established an essentially linear relationship between LW and electroosmotic flow rate. In this fashion, LW can be used to measure the electroosmotic flow rate of a solvent and the electrophoretic migration rate of a charged species. By examining the LW, one can compute the coil residence time of the analyte and hence flow rates for all components in a CE separation as they migrate through the detector. Such information is difficult to obtain with other detection methods without perturbing the separation. This aspect of NMR detection in CE certainly has not yet been fully explored. A greater understanding of the fundamental separation mechanisms among the many CE modes of operation would aid in the development of improved or novel approaches.

### E. CEC-NMR

As a hybrid of CE and HPLC, capillary electrochromatography has found increasing utility within...
the past decade. With high electrical potentials applied across packed capillary columns, CEC features the selectivity of a stationary phase and eliminates the parabolic flow profile of pressure-driven separations. As a result, CEC has enjoyed significant success in the separation of a variety of charged and neutral species with high efficiencies. Presently, only four reports of CEC–NMR have appeared in the literature. In one of these examples, the 240-nL NMR detection capillary described previously was attached in series with a 20-cm length of 250-μm i.d. capillary packed with 5-μm particles. The total capillary length was 220 cm with 110 cm from inlet to detector. As shown in Figure 18, five alkyl benzoates were separated and identified on-line with 1H NMR detection. Compared to a separation of the same five alkyl benzoates by capillary HPLC–NMR, the combination of pressure assistance and electroosmotic flow reduced the elution time in CEC–NMR by a factor of 2. Additionally, the improved chromatographic resolution of CEC–NMR (with respect to capillary HPLC–NMR) was attributed to the plug flow profile of electroosmosis. In stopped-flow mode, a two-dimensional 1H–1H COSY spectrum was acquired for pentyl benzoate to verify the coupling pattern of the monosubstituted aromatic hydrocarbon. Though not reported for CEC–NMR, LODs can be estimated in the hundreds of picomoles based upon the performance of the probe for a detection cell filled with analyte.

In two other accounts, CEC–NMR was applied to the separation of metabolites of paracetamol. Optimal separation conditions were first established by conventional CEC with UV absorbance detection. For these CEC–NMR experiments, a 400-nL NMR detection cell was used and connected to a 250-μm i.d. capillary packed with 20 cm of stationary phase. While the observe volume was increased from the 240 nL used in the other CEC–NMR example, the 1H remains below 2% for this configuration. Because of the relatively low electric field used (~100 V/cm) and the long distance from inlet to detector, the separation required almost 1 h to complete. Nevertheless, on-line CEC–NMR data acquisition allowed the 1H NMR detection and identification of the two major metabolites (i.e., the glucuronide and sulfate conjugates of the drug) as well as the endogenous material hippurate from an injection volume of 500 nL. In addition, a two-dimensional TOCSY experiment was performed overnight in stopped-flow CEC–NMR mode to provide confirmatory evidence of the glucuronide paracetamol conjugate. Given the metabolite concentration in urine and the observed NMR S/N, it was estimated that amounts in the low nanogram range were detected for each metabolite. Although the benefits of greater loading capacity and consequently improved NMR S/N for CEC compared to CE are evident, the authors make note of the technical difficulty of coupling capillary electroseparations and NMR detection. Despite these challenges, CEC–NMR offers the combination of highly efficient separations, increased sample loading capacity compared to CZE, and information-rich detection. As a result, this hyphenated technique will find extensive use in the future.

VIII. Conclusions

As techniques for chemical analysis are used in continually smaller domains, experimental challenges for inherently insensitive methods such as NMR become increasingly severe. Among the various schemes to increase the intrinsic sensitivity of an NMR experiment, the development of small-volume RF probes has experienced a renaissance during the past decade. Commercial NMR probes now allow analyses of nanomole quantities in microliter volumes from natural product extracts and combinatorial chemical syntheses. Although the development of nanoliter-volume probes is still in the research phase, the enhancements in sensitivity which have resulted from submillimeter-diameter coils are exciting. Through the fabrication of nanoliter-volume NMR probes and their coupling to microseparation strategies, mass-limited analytes in complex matrixes are becoming viable samples for NMR analysis. Recent demonstrations of on-line HPLC–NMR–MS have combined one of the most widely used separation methods with two of the most information-rich techniques of chemical characterization. The extension of this doubly hyphenated method to the capillary scale will enable rapid, chemically rich screening of mass-limited samples with enhanced mass sensitivity.

While the increased sensitivity of reduced-diameter RF probes provides a widely applicable benefit for NMR spectroscopy, microcoils offer several additional advantages which have not been fully explored. As one example, the diminutive spatial dimensions of microcoil probes enable their use in low-homogeneity high-field magnets. One study reported increased spectral resolution from a series of microcoils with decreasing diameters within a magnet of poor (100 ppm/cm) homogeneity. This property of microcoils enables their potential use as part of benchtop high-resolution NMR spectrometers. Additionally, since the self-resonance frequency of microcoils lies in the GHz range, their use within very high-field (i.e.,
> 30 T) magnets is possible. Microcoils can also be applied to solid-state NMR. The short pulse widths of these coils are beneficial for wide-line spectroscopy and multipulse line-narrowing techniques. Since a rudimentary capillary spinner has been developed for high-resolution microcoil studies, microcoil NMR can be expanded to include magic angle spinning of solid or semisolid samples. The reduced dimensions of the rotor would allow higher spin rates and thus yield more effective proton dipolar decoupling. Finally, the use of reduced-diameter RF probes for NMR spectroscopic characterization of cellular samples has only begun to receive attention. Numerous areas in the biological sciences will undoubtedly benefit from improved NMR spectroscopy of small volumes in the coming decade.

IX. Acknowledgments

It is a pleasure to acknowledge Wayne Kelley and Andrew Wolters for their suggestions and helpful discussions in the preparation of this manuscript. We acknowledge support from the National Institutes of Health (GM 53030) and the Camille and Henry Dreyfus Foundation (J.V.S.). M.E.L. appreciates the financial support of an NSF Graduate Fellowship. A.G.W. acknowledges the National Science Foundation for the support of a CAREER award (DBI 97-22320).

X. References

Introduction

Today, systems biology is rapidly developing in the various areas of biomedical research, including human oncology. So far, functional genomic strategies have largely centred on gene-expression studies (genomics and transcriptomics) or protein level (proteomics). In addition, the biochemistry of a tumour, especially glucose uptake and metabolism, is very different from that of a normal cell: mitochondrial metabolism is impaired and cytosolic glycolysis is elevated with a subsequent increase in glucose uptake (Warburg’s effect). In the past years, various specific metabolites have been reported to be associated with cancer development and progression, including citrate, myo-inositol, poly-unsaturated fatty acids (PUFA), nucleotides, phosphocholine and other cell membrane constituents [1]. This makes nuclear magnetic resonance (NMR) (1H, 31P and 13C NMR spectroscopy) one of the most valuable techniques to evaluate cancer metabolism and efficacy of the treatment since multiple metabolic pathways can be assessed simultaneously [2–5]. Understanding tumour-related processes through metabolic profiling has been widely used in the last decades to differentiate between different cancer cell lines and to monitor metabolic processes that occur in cancer cells during events such as apoptosis, down-stream-pathway regulation and enzyme abnormalities [6–8]. Despite the successful use of high-resolution NMR-based metabolic analysis in cell culture models as well as in human blood or urine, clinical application of NMR is often limited by the fact that this technology has rather low sensitivity, compared with other approaches. In the clinical setting, the volume of collected samples is rather low. For examples, fine needle aspirates, solid tumour biopsies or specific body fluids often do not exceed 10–20 µl volumes. For conventional high-resolution (liquid) methods, which are based on the use of 3–5 mm probes, this amount of study sample is below the limit of detection, not to mention their limits of quantification. Various novel technological advances are developed to overcome the ‘small volume sample’ problem [9], such as

Use of the 1-mm micro-probe for metabolic analysis on small volume biological samples

Natalie J. Serkova a, Amy S. Freund b, Jaimi L. Brown a, Douglas J. Kominsky a, *

aBiomedical MRI/MRS Cancer Center Core, University of Colorado Health Sciences Center, Denver, CO, USA
bBruker BioSpin Corporation, Billerica, MA , USA

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Abstract

Endogenous metabolites are promising diagnostic end-points in cancer research. Clinical application of high-resolution NMR spectroscopy is often limited by extremely low volumes of human specimens. In the present study, the use of the Bruker 1-mm high-resolution TXI micro-probe was evaluated in the elucidation of metabolic profiles for three different clinical applications with limited sample sizes (body fluids, isolated cells and tissue biopsies). Sample preparation and 1H-NMR metabolite quantification protocols were optimized for following oncology-oriented applications: (i) to validate the absolute concentrations of citrate and spermine in human expressed prostatic specimens (EPS volumes 5 to 10 µl: prostate cancer application); (ii) to establish the metabolic profile of isolated human lymphocytes (total cell count 4 x 10^6: chronic myelogenous leukaemia application); (iii) to assess the metabolic composition of human head-and-neck cancers from mouse xenografts (biopsy weights 20 to 70 mg: anti-cancer treatment application). In this study, the use of the Bruker 1-mm micro-probe provides a convenient way to measure and quantify endogenous metabolic profiles of samples with a very low volume/weight/cell count.

Keywords: quantitative 1H-NMR • micro-probe • body fluids • biopsy extract • cell extract • oncology
high-resolution magic angle spinning (HR-MAS) probes for solid state NMR, cryo-probes or micro-probes.

In the present article, we report of the use of Bruker 1-mm TXI (triple-resonance $^{1}H^{13}C^{31}P$ inverse) micro-probe (Bruker Biospin, Billerica, MA, USA) for small volume biological samples. We present our sample preparation protocols and quantitative metabolic results for three different study designs, including small volume human body fluids, small volume human cell samples and small volume biopsy samples. All NMR spectra were obtained using high-resolution Bruker 500 MHz Avance and DRX spectrometers equipped with Bruker high-resolution inverse 1-mm TXI probes with or without automated tuning and matching (ATM) option.

**Experimental**

All human and animal studies were approved by the Institutional Review Board (IRB) of Human Research and the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado, Health Sciences Center, respectively.

**Sample preparation protocol on human prostatic fluids**

Five to 10 ml of prostatic fluids were collected after prostatic massage and immediately put onto dry ice (−78.5°C). Thirty ml of deuterium oxide (D$_2$O) with ca. 0.03 wt.% 3-(trimethylsilyl)propionic-2,2,3,3,-d4 acid sodium salt (TMSP, Aldrich, Milwaukee, WI, USA) was added to each sample, resulting in the final volume of 35–40 µl. Samples were centrifuged at 4000 × g for 10 min. (4°C) to remove proteins. Thirty µl of supernatant were transferred into a Bruker 1-mm glass capillary using 1-ml syringes with thin epidural needles. The glass capillaries were sealed and inserted into the magnet using a 1-mm NMR spinner.

**Cell isolation and acid extraction protocol for human lymphocytes**

Twenty millilitre whole blood was collected from healthy subjects into plastic heparin preserved serological tubes. Lymphocytes were isolated from the fresh whole blood by Ficoll gradient centrifugation using a Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ, USA). Briefly, the blood was mixed with an equal amount (1: 1 vol/vol) of balanced salt solution. In another centrifuge tube, an appropriate amount (3: 4 vol/vol of blood/salt mixture) of Ficoll-Paque Plus solution was added to the bottom of a centrifuge tube. The blood/salt mixture was carefully layered on top of the Ficoll-Paque Plus and centrifuged at 400 × g for 40 min. at 20°C. The top layer of plasma was removed. The layer of lymphocytes was transferred to a new centrifuge tube and re-suspended in three-times volume of balanced salt solution. The cells were centrifuged at 60 × g for 10 min. at 20°C to wash the leucocytes and remove platelets. Isolated lymphocytes in the supernatant (4 × 10$^6$ cells) were subsequently re-suspended in 10 ml of RPMI 1640 medium containing 5 mmol/l [1–13C] labelled glucose and 10% foetal bovine serum (FBS) and incubated at 37°C for 4 hrs. The lymphocytes were washed twice with 1 ml ice-cold isotonic NaCl, centrifuged (5 min. at 400 × g and 4°C) and frozen in liquid nitrogen. The cells were then extracted with 2 ml of ice-cold PCA (12%) [10]. The samples were centrifuged (15 min. at 1300 × g and 4°C) and the aqueous phase was removed and neutralized (pH 5 7) using KOH. The samples were centrifuged again and lyophilized overnight. The lyophilized leucocytes extracts were dissolved in 30 µl D$_2$O with TMSP and centrifuged at 8000 × g for 5 min. at 4°C. The supernatants were transferred into Bruker 1-mm glass capillaries using 1-ml syringes with thin epidural needles. The glass capillaries were sealed and inserted into the magnet using a 1-mm NMR spinner.

**Methanol/chloroform and acid extraction protocols for tissue biopsies**

Two human head-and-neck squamous cell carcinoma (HNSSC) cell lines – UMSCC2 and HN31 – were used in a nude mouse xenograft model. After 28 days, tumour xenografts were harvested, immediately frozen in liquid nitrogen and subsequently underwent dual methanol/chloroform or acid extraction for protein precipitation and separation of water-soluble from lipid metabolites. The tumour mass was between 20 and 80 mg per sample. Two extraction protocols were tested: a dual methanol/chloroform extraction for HN31 tumours and a 12% perchloric acid extraction for UMSCC2 tumours. Methanol/chloroform extraction: Frozen tissues were weighed, powdered in the presence of liquid nitrogen and homogenized in 0.5 ml of ice-cold methanol. Ice-cold chloroform (0.5 ml) was added and samples were vortexed. Additional 0.5 ml of ice-cold water was added to the samples and vortexed. The samples were kept at 4°C overnight. The samples were centrifuged for 30 min. at 1400 × g at 4°C. Upper methanol phase (water soluble extract) was transferred into a lyophilizing glass and freeze-dried overnight. Lipids in the lower organic phase were evaporated to dryness under a stream of nitrogen or, alternatively, using a vacuum speed centrifuge.

Perchloric acid extract: Again, frozen tissues were weighed, powdered in the presence of liquid nitrogen and homogenized in 2 ml ice-cold 12% PCA. The extracts were then vortexed and centrifuged at 1300 × g for 10 min. at 4 degree. The supernatant was transferred to a new centrifuge tube and 1 ml ice-cold 12% PCA was added to the pellet, vortexed and centrifuged again. The supernatants were then combined and neutralized with 1.0M KOH to a pH of 7.2. The extracts were centrifuged again at 1300 × g for 10 min. at 4 degree. The supernatant (water-soluble phase) was transferred into a lyophilizing glass and freeze-dried overnight. The pellets (lipid fraction) were re-dissolved in 2 ml water, the pH was adjusted to 7.2 and freeze-dried overnight.

All dried water-soluble extracts were dissolved in 40 µl D$_2$O with TMSP and centrifuged at 8000 × g for 5 min. at 4°C. The lipid extracts were dissolved in 40 µl of deuterated chloroform+0.03 wt.% TMSP/deuterated methanol mixture (2: 1 vol/vol). The re-dissolved water-soluble and lipid extracts were transferred into Bruker 1-mm glass capillaries using 1-ml syringes with thin epidural needles. The glass capillaries were sealed and inserted into the magnet using a 1-mm NMR spinner.

**NMR experiments**

All NMR experiments were performed on Bruker 500 MHz spectrometers (operating frequency 500.15 MHz) using Bruker 1-mm high-resolution inverse TXI ($^{1}H^{13}C^{31}P$) Z-gradient micro-probes (Bruker BioSpin, Billerica, MA, USA). The samples were maintained at 287 K, as measured.
with a thermocouple internal system. Field homogeneity was achieved by coil-shimming using 1D water pre-saturation experiment in interactive mode as control. To assist 1H-NMR peak assignment and metabolite identification in expressed prostatic specimen (EPS), leucocyte extracts and biopsy extracts, two-dimensional gradient (2D)-H/H-COSY (correlation spectroscopy) and (2D)-H/C-HSQC (heteronuclear single quantum correlation) NMR techniques were used for metabolite identification. The COSY acquisition parameters were: standard Bruker pulse sequence 'cosygpqf'; ns/H/11005 64 scans across 256 increments with ds/H/11005 16 dummy scans, spectral width SW (F1) = SW (F2) = 6666 Hz and TD = 2048 data points; using 90 degree pulse and recovery delay of d1 = 1.5 sec. HSQC spectra were also acquired, with a standard Bruker pulse sequence 'hsqcetgp', with 512 increments (echo/anti-echo) and ns/H/11005 320 and ds/H/11005 16 scans per increment; SW (F1, 13C) = 17,608 Hz and SW (F2, 1H) = 5000 Hz; TD = 2048 data points; using 90 degree pulse and a recovery delay of 1 sec. All spectra were Fourier transformed and lactate (Lac3, CH3) was used as an internal chemical shift reference for both carbon (21 ppm) and proton (1.32 ppm) axes. Methanol was used as an internal chemical shift reference for both carbon (21 ppm) and proton (1.32 ppm) axes.

**Table 1** TMSP concentration calculations based on the 1H-NMR spectrum of 20 mM metabolite solution

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Start(ppm)</th>
<th>Start(Hz)</th>
<th>End(ppm)</th>
<th>End(Hz)</th>
<th>Integral</th>
<th>TMSP</th>
<th>Mean</th>
<th>±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol</td>
<td>4.1091</td>
<td>2055.53</td>
<td>4.035</td>
<td>2018.49</td>
<td>959.3</td>
<td>2.08</td>
<td>2.11</td>
<td>0.07</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.818</td>
<td>1909.92</td>
<td>3.7425</td>
<td>1872.14</td>
<td>897.35</td>
<td>2.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inositol</td>
<td>3.6736</td>
<td>1837.66</td>
<td>3.5922</td>
<td>1796.94</td>
<td>1919.22</td>
<td>2.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inositol</td>
<td>3.5782</td>
<td>1789.97</td>
<td>3.513</td>
<td>1757.33</td>
<td>1885.62</td>
<td>2.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inositol</td>
<td>3.3282</td>
<td>1664.9</td>
<td>3.2512</td>
<td>1626.38</td>
<td>925.94</td>
<td>2.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine</td>
<td>3.0899</td>
<td>1545.69</td>
<td>3.0034</td>
<td>1502.4</td>
<td>2984.37</td>
<td>2.01</td>
<td></td>
<td></td>
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<tr>
<td>Citrate</td>
<td>2.7093</td>
<td>1355.32</td>
<td>2.6199</td>
<td>1310.57</td>
<td>1864.53</td>
<td>2.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>2.5949</td>
<td>1298.1</td>
<td>2.5128</td>
<td>1257.02</td>
<td>1858.63</td>
<td>2.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>1.5251</td>
<td>762.94</td>
<td>1.443</td>
<td>721.86</td>
<td>2813.2</td>
<td>2.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMSP</td>
<td>0.0301</td>
<td>15.04</td>
<td>-0.0213</td>
<td>-10.64</td>
<td>900</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Alanine, citrate, creatine and myo-inositol (each 20 mM) were used to calculate the final concentrations of TMSP in D2O in this experiment.

1H-NMR spectrum is presented in Fig. 1.
reference for lipid spectra (3.35 ppm). For metabolite quantification, one-dimensional $^1$H-NMR spectra were obtained from each sample, with a standard water pre-saturation pulse program 'zgpr'. The total number of acquisitions varied from 40 to 128, depending on the sample volume, with 2 scans were collected into TD = 32K data points, resulting in total acquisition time of 10–32 min. Conventional $^1$H acquisition parameters were: power level $p_{11} = 20$ dB; power angle $p_{1} = 6.3$ msec (90 degree pulse); power level for water pre-saturation $p_{9} = 77$ dB; water suppression at $0_{1P} = 4.76$ ppm; spectral width $SW = 5000$ MHz; and the pulse delay of 12.75 sec. (calculated as $5^\ast T_1$) was applied between acquisitions for fully relaxed $^1$H-NMR spectra. The external standard TMSP was used as a chemical shift reference (0 ppm).

In addition, for precise calculation of TMSP concentrations in D$_2$O, we recorded $^1$H-NMR spectra of the 20 mM metabolite mixture in 40 µl D$_2$O with TMSP. The $^1$H-NMR acquisition parameters were identical to the experimental set-up with the study samples, the number of transients for metabolite/ D$_2$O/ TMSP standard solutions was $ns = 40$.

**Spectral analysis and metabolite quantification**

All 2D data were processed using XWINNMR 3.5 or TopSpin software. Tissue metabolites were identified based on the results from our chemical
shift database and/or referred to the Human Metabolome Database from the University of Alberta (http://www.hmdb.ca/). After performing Fourier transformation (with line broadening LB / H11005 0.2 Hz) on one-dimensional 1H-NMR spectra and making phase and baseline corrections, each identified 1H peak was integrated using Bruker 1D WINNMR program. The final TMSP concentration in the capillary was calculated prior to metabolite calculations in the study extracts using a 20 mM metabolite standard solution. TMSP concentrations were calculated based on NMR intensities (from the fully relaxed 1H-NMR spectra) of each metabolite according to equation (1) (Fig. 1) and the final concentration of TMSP (given as mmol/ml) represented a mean value (Table 1).

\[
C = \frac{20\text{mM} \times \text{Itmsp} \times \text{Nmet}}{\text{Imet} \times \text{Ntmsp}} \quad (1)
\]

where
- \( C \) = TMSP concentration
- \( \text{Itmsp} \) = integral of 1H TMSP peak at 0 ppm
- \( \text{Ntmsp} \) = number of protons in 1H TMSP peak (N = 9)
- \( \text{Imet} \) = integral of 1H peak of a metabolite
- \( \text{Nmet} \) = number of protons in 1H peak of a metabolite
- \( 20 \text{mM} \) = metabolite concentration in the standard solution

The absolute concentrations of single endogenous metabolites in the study sample were then referred to the TMSP integral and calculated according to the equation (2):

\[
C_x = \frac{1xN_x \times C_{1:9}}{V : \text{Msample}} \quad (2)
\]

where
- \( C_x \) = metabolite concentration
- \( 1x \) = integral of endogenous metabolite 1H peak
- \( N_x \) = number of protons in metabolite 1H peak (from CH, CH2, CH3, etc.)
- \( C \) = TMSP concentration (see above for TMSP concentration calculation)

The intra-sample variation (multiple sample preparations from the aliquots of the same sample) yielded error of \( \leq 5\% \) [17, 18].

**Table 2** Absolute concentrations [μmol/ml] of endogenous metabolites in human EPS fluids from healthy male volunteers calculated from 1H-NMR spectra

<table>
<thead>
<tr>
<th></th>
<th>Alanine</th>
<th>Citrate</th>
<th>Inositol</th>
<th>Lactate</th>
<th>PCholine</th>
<th>Spermine</th>
<th>Valine</th>
</tr>
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<tbody>
<tr>
<td>EPS #1</td>
<td>1.65</td>
<td>270.51</td>
<td>19.18</td>
<td>2.01</td>
<td>&lt;LLQ</td>
<td>47.83</td>
<td>11.59</td>
</tr>
<tr>
<td>EPS #2</td>
<td>1.86</td>
<td>376.63</td>
<td>16.92</td>
<td>2.27</td>
<td>0.55</td>
<td>79.92</td>
<td>19.54</td>
</tr>
<tr>
<td>EPS #3</td>
<td>1.50</td>
<td>339.20</td>
<td>17.16</td>
<td>1.10</td>
<td>&lt;LLQ</td>
<td>50.09</td>
<td>15.34</td>
</tr>
<tr>
<td>EPS #4</td>
<td>0.10</td>
<td>764.47</td>
<td>41.85</td>
<td>0.57</td>
<td>0.39</td>
<td>168.24</td>
<td>37.70</td>
</tr>
<tr>
<td>EPS #5</td>
<td>0.82</td>
<td>326.86</td>
<td>21.24</td>
<td>1.26</td>
<td>0.19</td>
<td>36.62</td>
<td>23.36</td>
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<tr>
<td>EPS #6</td>
<td>3.29</td>
<td>309.93</td>
<td>18.72</td>
<td>3.61</td>
<td>0.14</td>
<td>56.88</td>
<td>18.66</td>
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<tr>
<td>EPS #7</td>
<td>2.27</td>
<td>125.88</td>
<td>11.87</td>
<td>2.07</td>
<td>0.16</td>
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<td>EPS #8</td>
<td>0.47</td>
<td>292.34</td>
<td>17.36</td>
<td>0.28</td>
<td>0.24</td>
<td>53.26</td>
<td>7.72</td>
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<tr>
<td>EPS #9</td>
<td>&lt;LLQ</td>
<td>161.59</td>
<td>7.70</td>
<td>&lt;LLQ</td>
<td>&lt;LLQ</td>
<td>18.87</td>
<td>10.97</td>
</tr>
</tbody>
</table>

Volume of EPS samples was between 5 and 10 μl, and diluted with D2O to the final volumes of 30–40 μl (based on the ‘sensitive volume’ and the lowest limit of quantification of the TXI micro-probe). Abbreviations: LLQ, low limit of quantification (0.10 μmol/ml)

**Results and discussion**

Metabolic analysis on human prostatic fluids: potential application for prostate cancer research

Total of nine randomized EPS samples from healthy male volunteers were analysed by 1H-NMR using a Bruker 1-mm TXI micro-probe. The sample volume was between 5 and 10 μl. We compared the signal to noise ratios of the CH2-signal of citrate at 2.65 ppm in an EPS sample using a 1-mm TXI micro-probe versus a conventional 5-mm TXI probe. The signal to noise ratio for 10 μl EPS in 30 μl D2O (1 scan, line-broadening 0.2 Hz applied) was significantly decreased because of a dilution factor. In addition, the use of the 1-mm TXI micro-probe also facilitated a better solvent suppression.

All men were presumed cancer-free at the time of EPS collection. Using two-dimensional HSQC and COSY spectra, the
peaks for alanine, citrate, myo-inositol, lactate, phosphocholine, spermine, valine were identified and quantified on one-dimensional 1H-NMR spectra (Fig. 2A and B). Specifically, the ‘normal’ EPS profile had pronounced NMR peaks for the amino acid citrate (concentrations range 161.6 to 764.5 µmol/ml), as well as the polyamine spermine (18.9 to 168 µmol/ml) and the osmolyte myo-inositol (7.7–42 µmol/ml), and diminished peaks for alanine, lactate, phosphocholine and valine. There was some age-dependency for higher concentration ranges of citrate and spermine corresponding to younger subjects – a tendency which will be confirmed and validated in a large ongoing clinical study.

In prostate cancer research, previous ex vivo as well as in vivo NMR / MRSI studies have demonstrated a linear correlation between the pathological Gleason and the magnitude of the decrease of citrate and the elevation of choline in prostatic gland [11]. The advantage of using EPS versus prostate biopsies for cancer detection and characterization would be that the sampling procedure for EPS is relatively non-invasive, and can be performed multiple times without the risk of bleeding or infection. Moreover, EPS analysis may lead to a more ‘global’ sample of the prostate gland relative to the ‘hit-or-miss’ approach of random biopsy sampling. One of the shortcomings for clinical use of
Metabolic analysis of isolated human lymphocytes: chronic myelogenous leukaemia application

Chronic myelogenous leukaemia (CML) has served as a prototype neoplasm for basic research as well as for clinical studies designed to develop curative cancer treatment. Beside their genetic instability (Philadelphia chromosome) and molecular abnormality (activity of BCR-Abl oncprotein), human CML cells show an abnormal...
The previous data on human cell lines were obtained using 108 cells and higher [10]. While cell numbers are not limiting for clonal cells, this amount of cell material is difficult to obtain from the peripheral blood of human subjects. Currently, we are validating these metabolic pathways in lymphocytes, isolated from the peripheral blood of CML patients prior and upon imatinib treatment. The future studies on low cell count extracts from human lymphocytes will allow us to establish the metabolic phenotype of imatinib responsiveness and early detection of resistance development – the major clinical obstacle in current CML treatment strategies.

### Metabolic analysis on human head-and-neck cancer biopsies: potential application for anti-cancer therapies

Metabolic cancer markers can be assessed not only in tumour cells in vitro, but can be quantified in human biopsies ex vivo. Unfortunately, the tumour mass that can be obtained during clinical biopsy sampling or from the orthotopic or xenograft animal models is usually very limited. In this study, we analysed 20–70 mg human HNSCC biopsies from mouse xenograft models. Metabolite quantification was not possible using a 5-mm conventional probe due to low signal-to-noise ratios (below 3:1) and low spectral resolution. Using a 1-mm TXI micro-probe for methanol/chloroform or acid extracts, high-quality one-dimensional (1H-NMR, Fig. 4A and B) and two-dimensional (COSY, Fig. 4C) spectra were obtained. Absolute concentrations of endogenous metabolites from two different HNSCC tumour types, calculated from 1H-NMR spectra, are presented in Table 3. Important markers, such as phosphocholine (HN31: 0.94 ± 0.21 µmol/g; UMSCC2: 2.15 ± 1.30 µmol/g), glycerophosphocholine (0.24 ± 0.05 and

### Table 3  Absolute metabolite concentrations (µmol/g) calculated from 1H-NMR spectra (Fig. 4A and B) from HNSCC biopsy extracts (both water-soluble and lipid fractions) using Bruker 1-mm TXI micro-probe

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>NH31 (n = 4)</th>
<th>UMSCC2 (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH3-Acetyl groups (peak 12)</td>
<td>9.25 ± 0.34</td>
<td>4.99 ± 0.61</td>
</tr>
<tr>
<td>Alanine (11)</td>
<td>3.12 ± 1.78</td>
<td>1.62 ± 0.42</td>
</tr>
<tr>
<td>Aspartate (17)</td>
<td>0.33 ± 0.15</td>
<td>0.32 ± 0.15</td>
</tr>
<tr>
<td>Cholesterol (1)</td>
<td>4.43 ± 0.56</td>
<td>3.12 ± 1.08</td>
</tr>
<tr>
<td>Creatine+PCreatine (18)</td>
<td>1.65 ± 0.91</td>
<td>1.14 ± 0.09</td>
</tr>
<tr>
<td>Total fatty acids (2)</td>
<td>91.00 ± 3.25</td>
<td>77.24 ± 4.99</td>
</tr>
<tr>
<td>Glucose (24)</td>
<td>1.46 ± 0.49</td>
<td>0.47 ± 0.18</td>
</tr>
<tr>
<td>Glutamate (13)</td>
<td>6.24 ± 2.43</td>
<td>3.56 ± 0.61</td>
</tr>
<tr>
<td>Glutamine (15)</td>
<td>0.53 ± 0.22</td>
<td>1.37 ± 0.05</td>
</tr>
<tr>
<td>Glutathione (16)</td>
<td>0.68 ± 0.24</td>
<td>1.30 ± 0.25</td>
</tr>
<tr>
<td>Glycine (22)</td>
<td>0.43 ± 0.19</td>
<td>1.30 ± 0.08</td>
</tr>
<tr>
<td>Glycerophosphocholine (20)</td>
<td>0.44 ± 0.05</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>myo-Inositol (23)</td>
<td>0.31 ± 0.12</td>
<td>0.76 ± 0.65</td>
</tr>
<tr>
<td>Lactate (10)</td>
<td>5.66 ± 1.02</td>
<td>7.51 ± 1.77</td>
</tr>
<tr>
<td>Mono-unsaturated fatty acids (8)</td>
<td>3.23 ± 0.78</td>
<td>1.22 ± 0.33</td>
</tr>
<tr>
<td>Phosphocholine (21)</td>
<td>0.94 ± 0.21</td>
<td>2.15 ± 1.30</td>
</tr>
<tr>
<td>Phospholipids (6)</td>
<td>13.21 ± 1.02</td>
<td>7.11 ± 1.77</td>
</tr>
<tr>
<td>Poly-unsaturated fatty acids (5)</td>
<td>5.25 ± 0.66</td>
<td>3.23 ± 0.89</td>
</tr>
<tr>
<td>Succinate (14)</td>
<td>1.94 ± 0.46</td>
<td>1.26 ± 0.10</td>
</tr>
<tr>
<td>Taurine (19)</td>
<td>2.61 ± 0.17</td>
<td>2.58 ± 0.17</td>
</tr>
<tr>
<td>Triacylglycerol (7)</td>
<td>9.23 ± 1.07</td>
<td>6.23 ± 1.01</td>
</tr>
<tr>
<td>Valine, Leucine, Ile (9)</td>
<td>3.90 ± 0.23</td>
<td>4.06 ± 0.06</td>
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</table>

The data are given mean ± S.D. with n = 4 for HN31 and n = 3 for UMSCC2 cells. All metabolite assignments were made based on two-dimensional NMR experiments.

The previous data on human CML cells and clonal cell lines were obtained using high cell counts of 5 × 10^6 cells and higher [10]. While cell numbers are not limiting for clonal cells, this amount of cell material is difficult to obtain from the peripheral blood of human subjects. The total lymphocyte count in ml blood is 1 × 10^6 cells, with the recovery rate after isolation being below 50%. Using a 1-mm TXI micro-probe, we analysed cell extracts from 4 × 10^6 isolated lymphocytes, which we obtained from 20 ml of whole peripheral blood. Even though the cell count was 100-fold lower than our previous studies on clonal cell lines [10], we were able to obtain good quality 1H-NMR spectra with total acquisitions of 128 (Fig. 3A). Major cellular metabolites, including glucose, lactate (with 13C-satellite peaks), cholesters, amino acids were detectable in lymphocytes extracts. Compared to our previous metabolic data on human CML cells K-562 and CML-T1 (Fig. 3B), isolated human lymphocytes from healthy subjects had higher intracellular levels of glucose (0.78 ± 0.32 µmol/g versus 0.032 ± 0.01 and 0.25 ± 0.01, P < 0.0001) and significantly decreased lactate concentrations, including de novo formation of 13C-lactate (0.25 ± 0.07 µmol/g versus 0.61 ± 0.11 and 1.08 ± 0.22, P < 0.01). Interestingly, the total glutamate concentrations were higher in lymphocytes compared to clonal cells (2.43 ± 0.35 µmol/g versus 1.78 ± 0.22 and 1.25 ± 0.12, P < 0.02). Finally, phosphocholine concentrations were significantly lower in lymphocytes versus transformed cells (0.49 ± 0.12 µmol/g versus 1.92 ± 0.22 and 2.12 ± 0.17, P < 0.001) with no differences in glycerophosphocholine concentrations among the three cell types.

These metabolic differences between human un-transformed lymphocytes and clonal CML cell lines reveal increased glucose utilization through glycolysis with increased lactate production as well as increased phospholipids turnover in cancer cells. Currently, we are validating these metabolic pathways in lymphocytes, isolated from the peripheral blood of CML patients prior and upon imatinib treatment. The future studies on low cell count extracts from human lymphocytes will allow us to establish the metabolic phenotype of imatinib responsiveness and early detection of resistance development – the major clinical obstacle in current CML treatment strategies.
0.20 ± 0.03 μmol/g, glucose (1.46 ± 0.49 and 0.47 ± 0.18 μmol/g), lactate (5.66 ± 1.02 and 7.51 ± 1.77 μmol/g) and glutathione (0.68 ± 0.25 and 1.30 ± 0.25 μmol/g) were easily detected and quantified using the 1-mm TXI micro-probe. Both tumour types are EGFR over-expressed head-and-neck tumours. Since a lot of attention to targeting of down-stream pathways in cancer cells has been shown in the last 5 years [14], currently we are investigating metabolic consequences of targeting EGFR in HN31 and UMSCC2 tumours using the same xenograft model and NMR approaches.

Conclusions

The use of a Bruker 1-mm TXI micro-probe provides a more convenient way to measure and quantify samples with a very small volume/weight/cell count in biological samples. The micro-probe allows for a remarkable increase in the signal to noise ratio and therefore a significant decrease in the experiment time while improving spectral resolution and solvent suppression. The signal overlap – one of the major limiting factors in NMR-based metabolomics – was in the same range as for conventional 5-mm NMR probes and can be further addressed by applying modern NMR sequences [15]. Alternatively, spectral segmentation for multivariate analysis (PCA, PLS-DA) can be performed in a similar way as for conventional NMR probes, to overcome individual metabolite separation and identification [16]. In the present study, this first capillary NMR probe for discrete samples was applied for three different kinds of biological samples and may provide useful future applications:

1. 1H-NMR on small volume biofluids (e.g. prostatic fluids), after an appropriate validation of prostate-specific metabolites, e.g. citrate, inositol and spermine, may serve as an alternative non-invasive assay for early detection of prostate cancer;
2. 1H-NMR spectroscopy on low cell number extracts from small blood volumes can be used as a clinical assay for early metabolic detection of CML and treatment failure in CML patients;
3. 1H-NMR-based quantitative assessment on small volume tumour biopsies may be used to metabolically monitor the efficacy of anti-tumour and anti-angiogenic treatment and therefore improve the treatment regiments with expensive targeted agents.

Acknowledgements

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References