DOI: 10.1002/anie.200601789

NMR Spectroscopy

Extending the Scope of NMR Spectroscopy with Microcoil Probes**

Frank C. Schroeder and Matthew Gronquist*

Keywords:
analytical methods · capillary NMR spectroscopy · mass-sensitivity · NMR spectroscopy · structure elucidation

Dedicated to Dr. Volker Sinnwell on the occasion of his 63rd birthday

Capillary NMR (CapNMR) spectroscopy has emerged as a major breakthrough for increasing the mass-sensitivity of NMR spectroscopic analysis and enabling the combination of NMR spectroscopy with other analytical techniques. Not only is the acquisition of high-sensitivity spectra getting easier but the quality of CapNMR spectra obtained in many small-molecule applications exceeds what can be accomplished with conventional designs. This Minireview discusses current CapNMR technology and its applications for the characterization of mass-limited, small-molecule and protein samples, the rapid screening of small-molecule or protein libraries, as well as hyphenated techniques that combine CapNMR with other analytical methods.

1. Introduction

In the spectroscopic characterization of small (or large) molecules, NMR spectra most closely reflect how we casually think about organic structures as ball-and-stick models. First-order interpretation of NMR spectra allows us to think of each NMR signal as representing individual hydrogen or carbon atoms (more precisely, groups of nonequivalent atoms), which comes as close as one can get to "seeing" each individual hydrogen or carbon atom in a molecule. Homo- and heteronuclear correlation spectra such as COSY, HSQC (heteronuclear single-quantum correlation), or HMBC (heteronuclear multiple-bond correlation) then provide additional signals, which, with little abstraction, translate into bonds that connect the atoms.[1] It is this close analogy to molecular models that makes the interpretation of NMR spectra so satisfying and intuitive. A detailed understanding of the physical background of solution NMR spectroscopy is much more difficult to attain but is not required for most small-molecule chemists. Generally, organic chemists and chemical biologists are able to use NMR spectroscopy very effectively without worrying much about quantum mechanics or statistics, as a basic understanding of pulse sequences and data processing is entirely sufficient to maximize the utility of available NMR spectrometers. However, just about anybody who has used NMR spectroscopy for the characterization of molecular structures or dynamics has at one point or another encountered the Achilles' heel of NMR spectroscopy: its limited sensitivity. For example, NMR spectroscopic analyses typically require several orders of magnitude more material than mass spectrometry.[2–4] That NMR spectroscopy, as opposed to mass spectrometry, is nondestructive is of little comfort to those who simply cannot isolate enough material to obtain sufficiently good NMR spectra. Examples of mass-limited applications include the identification of new natural products, the analysis of metabolites or breakdown products of pharmaceuticals and agrochemicals, as well as the characterization of large libraries of synthetic compounds from combinatorial chemistry.

The sensitivity of NMR spectroscopy depends primarily on three instrumental parameters: the strength of the magnetic field (the use of stronger magnets results in higher sensitivity), the size and fill factor of the receiver coil (mass-sensitivity increases with increasing fill factor and decreasing coil diameter), and the amount of noise introduced during detection.[5,6] After NMR spectrometers became widely

[*] Dr. F. C. Schroeder
Biological Chemistry and Molecular Pharmacology
Harvard Medical School
250 Longwood Avenue, Boston, MA 02115 (USA)
Fax: (+1) 617-432-6424
E-mail: frank_schroeder@hms.harvard.edu
Dr. M. Gronquist
Department of Chemistry
SUNY College at Fredonia
Fredonia, NY 14063 (USA)

[**] We thank Professor Jerrold Meinwald and Dr. Ivan Keresztes, Cornell University, as well as Tim Peck and Dean Olson, Protasis-MRM, for helpful suggestions and discussions.

available in the 1960s, the development during the subsequent two decades of superconducting magnets allowed a dramatic increase in field strengths and thus sensitivity. However, in the past 15 years the field strengths of commercially available NMR spectrometers have increased more and more slowly, as current magnet designs approach the limits of existing superconducting-wire technology and as the costs associated with installing higher-field spectrometers rise more than exponentially with field strength. For example, the costs for the purchase and installation of a 900-MHz spectrometer can easily reach $10000000 (ca. €8000000), whereas less than 10% of this amount would be required for a standard configured 600-MHz instrument.[7] Meanwhile, the sensitivity at 900 MHz increases only by a relatively modest factor of roughly two as compared to at 600 MHz.[7] More recently, efforts to improve the sensitivity of NMR spectroscopy have therefore focused on probe design, aimed at reducing thermal noise in the receiver and developing probe geometries that allow for smaller receiver coils with better fill factors. The introduction of cryogenic probes, in which thermal noise is greatly reduced by cooling the receiver coil and preamplifiers to 25 K or below, resulted in sensitivity gains of roughly a factor of three to four as compared to conventional probe designs.[8,9] However, the design of cryogenic probes places extremely high demands on thermal insulation and cooling capacity, as the 25-K receiver coil is separated from the room-temperature NMR sample by only a few millimeters. The required refrigerated helium-based cooling system makes the acquisition and maintenance of cryogenic probes rather expensive, and installation of a cryogenic probe usually necessitates that the spectrometer be dedicated exclusively to this probe.

Cryogenic probes offer significant sensitivity gains and therefore, despite the aforementioned drawbacks, have become indispensable for the characterization of biological macromolecules by NMR spectroscopy. For the analysis of mass-limited samples, however, microcoil probe designs[10] offer a viable alternative by providing significant gains in mass-sensitivity at a fraction of the cost of cryogenic probes. In this Minireview, we describe some of the physical concepts relevant to understanding the advantages and limitations of microcoil probe designs and provide a general estimate of how the mass-sensitivity of microcoil probes compares with that of conventional probes. We also discuss the applications of microcoil NMR spectroscopy as a stand-alone technique and in conjunction with other analytical techniques such as HPLC or electrophoresis, and consider recent progress towards incorporating microcoil probes in high-throughput applications.

2. Microcoil NMR Probes

2.1. Background and Design

The rationale for the development of microcoil NMR probes was presented by the fact that for a given mass of analyte, a reduction in the diameter of the receiver coil increases the signal-to-noise ratio (S/N).[5,10–12] Because a reduction in coil diameter inevitably results in a decrease in the sample volume, microcoil NMR probes will only provide a sensitivity advantage in cases where the mass-limited sample is fully soluble in the smaller volume of solvent. On the other hand, for concentration-limited samples microcoil designs offer no advantage, as a smaller probe accommodates much less of a concentration-limited sample than conventional designs. Accordingly, the concentration sensitivity of microcoil NMR probes is generally lower than that of conventional 5-mm probes (Table 1).[10]

Designing NMR probes with smaller receiver coils has been a challenging task. A smaller coil requires the sample to be in closer proximity to the coil, which can result in

Table 1: Comparison of the concentration-sensitivity (S_c) and mass-sensitivity (S_m) for a test sample of sucrose using a CapNMR microcoil probe and a 5-mm H{C,N} probe at 600 MHz with a Shigemi tube.[a]

Probe	Sample volume [μL]	S_c (S/N per mM) (normalized)	S_m (S/N per μmol) (normalized)
5 mm	260[b]	10.4	1
CapNMR	5	1	5.0

[a] A single scan was acquired for samples of a solution of sucrose (17.2 μg, 50 nmol) in D_2O.[18] Signal-to-noise ratios were determined using the anomeric proton and VNMR (VARIAN) software. For a similar comparison see Ref. [12]. [b] A slightly smaller volume of 200–220 μL could have been used for the Shigemi tube, which could have increased the mass-sensitivity for the 5-mm probe by up to 25% and thus reduce the advantage of CapNMR in mass-sensitivity to a factor of roughly four.

Frank C. Schroeder studied chemistry and physics at the University of Hamburg and completed his PhD in 1997 with Wittko Francke. He carried out postdoctoral studies with Jerrold Meinwald at Cornell University, where he further developed his interests in the spectroscopic characterization of small molecules and their role in chemical biology. In 2004, he took his current position as Director of the Natural Products Initiative at Harvard Medical School. His research encompasses NMR spectroscopic approaches for the characterization of small-molecule mixtures and identification and function of signaling molecules in plants and higher animals.

Matthew Gronquist completed his PhD in 2002 at Cornell University, where he carried out studies under the guidance of Jerrold Meinwald into the characterization and synthesis of natural products. Following graduation he moved to the State University of New York (SUNY) College at Cortland, where he was appointed an assistant professor. In 2006, he moved to the SUNY College at Fredonia and is currently an assistant professor of chemistry. His research interests include small-molecule signaling in bacteria and the chemical ecology of arthropods.

significant line-broadening as a result of differences in magnetic susceptibility between the sample, coil material, and surrounding environment.[13–16] Furthermore, smaller coil diameters also require reconsideration of how the sample is introduced into the probe because conventional sample tube designs cannot be miniaturized indefinitely and maintaining a high fill factor for the coil is necessary to take full advantage of the sensitivity gain that results from reducing its size. A major breakthrough came with the introduction of probes that feature a receiver coil directly wrapped around a capillary-scale flow cell, which is embedded in a fluid whose magnetic susceptibility closely matches that of the coil material.[17] Careful susceptibility matching made it possible to drastically reduce magnetic field inhomogeneities in the vicinity of the coil and resulted in greatly improved lineshapes suitable for high-resolution NMR spectroscopy. Linewidths of the most recent generation of commercial capillary microcoil NMR (CapNMR) probes now easily match those of 5-mm cryogenic probes. As CapNMR flow cells are extremely small, they can be oriented horizontally, perpendicular to the magnetic field, which allows the use of solenoidal receiver coils (Figure 1). Compared to the "saddle"-type coil designs necessary for vertically oriented samples, solenoidal coils

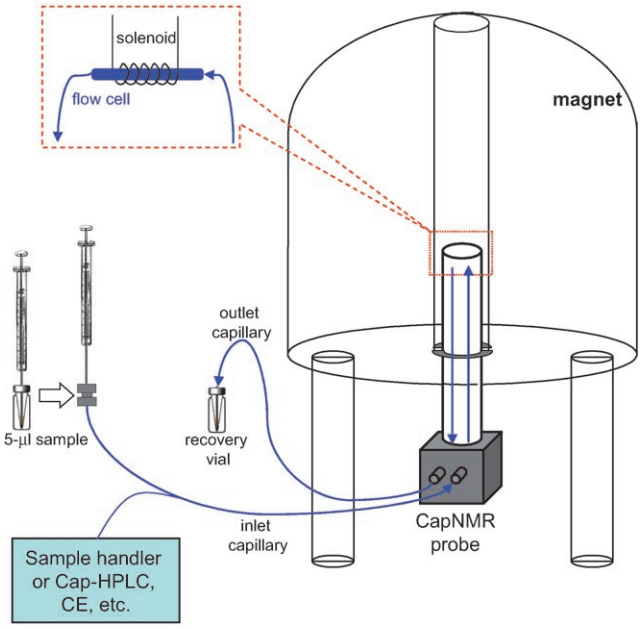

Figure 1. Schematic of the setup and probe design for CapNMR spectroscopy. CE = capillary electrophoresis.

provide roughly twofold higher intrinsic sensitivity as a result of their stronger coupling to the sample.[10] The solenoidal design thus further increases overall sensitivity of the probe, in addition to the improvement gained from miniaturizing the coil. Another consequence of working with an extremely small sample volume is that shimming is very fast and usually involves little more than adjusting a few first-order shims.[12] Frequently, when one is dealing with a series of samples in the same solvent, shimming is needed for the first sample only.

Microcoil NMR probes typically employ a single coil for the deuterium lock, proton, and X-nuclei ($X = {}^{13}C$, ${}^{15}N$) channels. In its most common—and most mass-sensitive—design, the CapNMR probe features a very small 5-µL flow cell with an active volume of only about 2.5 µL.[12] In addition, a slightly less mass-sensitive 10-µL version is available, with an active volume of roughly 5 µL. In either case, the flow cell is connected to gas-chromatography-type fused-silica capillaries or FEP (fluorinated ethylene propylene) tubing (inner diameter (i.d.): 75–100 µm), which serve as the inlet and outlet. For loading of the flow cell, the sample is injected into the inlet line either manually by syringe or automatically by a liquid-handling system. There are many advantages of using a capillary-scale flow path. First, employing flow cells allows somewhat higher fill factors to be achieved than with sample-tube-based microcoil designs. The use of very narrow capillaries virtually eliminates mixing within the outlet and inlet and thus reduces problems associated with sample management, such as peak dispersion and sample carryover, that are commonly encountered with larger-scale flow probes.[12] Furthermore, the inlet and outlet lines can be easily connected to other analytical instrumentation, for example, capillary LC, for "online" hyphenated applications. Perhaps more importantly, the compatibility of CapNMR with standard laboratory titer plates and microvials makes it well-suited for the high-throughput analysis of compound libraries and allows for facile combination with analytical platforms that include analytical-scale HPLC or capillary electrophoresis (CE) and mass spectrometry (MS).

2.2. A Comparison of Mass-Sensitivity

As should be evident from the preceding section, using the CapNMR system requires a new approach to sample preparation and loading. Instead of preparing a glass NMR tube containing the sample as a solution in 200–600 µL of solvent (depending on the type of probe and glass tube), in CapNMR the sample has to be dissolved in only 5–10 µL of solvent, and the solution must subsequently be transferred, without significant losses, manually by syringe or under automation using a liquid-handling system for injection into the probe. To compare the utility of CapNMR as a flow-cell design to that of traditional sample-tube-based probes, these differences must be carefully considered, especially if one is primarily interested in using CapNMR for manual injection of samples that otherwise could be analyzed by using traditional probes. The primary motivation for using CapNMR in this case would be its significantly higher mass-sensitivity,[12] but exactly how much of a difference does it make compared to using a "normal" 5-mm probe for real samples? The use of solvent-specific susceptibility-matched NMR tubes (e.g. Shigemi tubes) in 5-mm probes already results in a two- to threefold net increase in mass-sensitivity. To assess the degree to which using CapNMR could improve the sensitivity of NMR spectroscopy beyond that achieved by using Shigemi-type tubes, Gronquist et al. directly compared spectra obtained with both probe designs by using a 10 mM sucrose solution and a series of natural product extracts as test samples.[18] In

one series of experiments, one-dimensional ^{1}H NMR spectra as well as two-dimensional (^{1}H,^{13}C) HMQC and (^{1}H,^{13}C) HMBC[19] spectra were acquired for a 5-μL sample of a solution of sucrose (10 mM), which was injected into the CapNMR probe by syringe. In a second series of experiments, the same amount of sucrose was dissolved in the volume of solvent (D_2O) required for using a 5-mm Shigemi tube, followed by acquisition of an equivalent set of spectra using a conventional 5-mm H{C,N} probe and the same spectrometer. A comparison of the ^{1}H spectra reveals that the signal-to-noise ratio is about five times better for the CapNMR spectrum (Figure 2). In addition, the HDO solvent peak in the CapNMR spectrum is smaller by several orders of magnitude than in the spectrum obtained using a 5-mm probe as a consequence of the fact that the active volume of the 5-mm probe is more than 100-times greater than that of the CapNMR probe. The much smaller solvent signal allowed the use of a higher receiver gain for acquisition of the CapNMR spectrum, which contributed to the observed sensitivity gain.

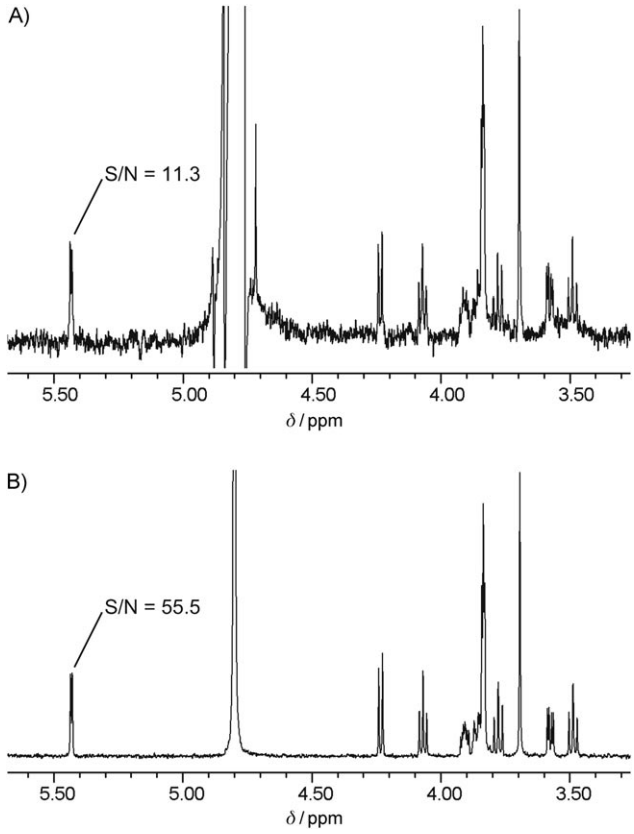

Figure 2. ^{1}H NMR spectra (1 scan each) of sucrose (17.2 μg) dissolved in A) D_2O (260 μL) using a Shigemi tube and a 5-mm H{C,N} probe, and B) 5 μL of D_2O using a 5-μL CapNMR probe. S/N ratios were determined by using VNMR (Varian Inc.) software.

Of particular relevance for the NMR spectroscopic characterization of small molecules are the indirect-detection carbon–proton correlations HMQC, HSQC, and HMBC. Owing to the low natural abundance of ^{13}C the sensitivity of these experiments is significantly lower than that of proton–proton correlations, and insufficient signal-to-noise ratios of HMQC/HSQC and especially HMBC spectra is what ultimately limits structure determination of mass-limited, small-molecule samples. CapNMR probes are commonly equipped with gradient coils and thus allow the acquisition of both the gradient and the slightly more sensitive nongradient versions of HMQC and HMBC. For these spectra, CapNMR probes can also provide significant sensitivity advantages, as shown for the nongradient HMBC spectra in Figure 3.[18] The nongradient versions of indirect-detection carbon–proton correlations, in which signals from ^{12}C-bound protons are suppressed through phase-cycling and which, in some cases, can provide higher sensitivity than the corresponding gradient-based versions[20] (see also Ref. [21]), especially benefit from the much smaller solvent signals acquired with CapNMR probes.

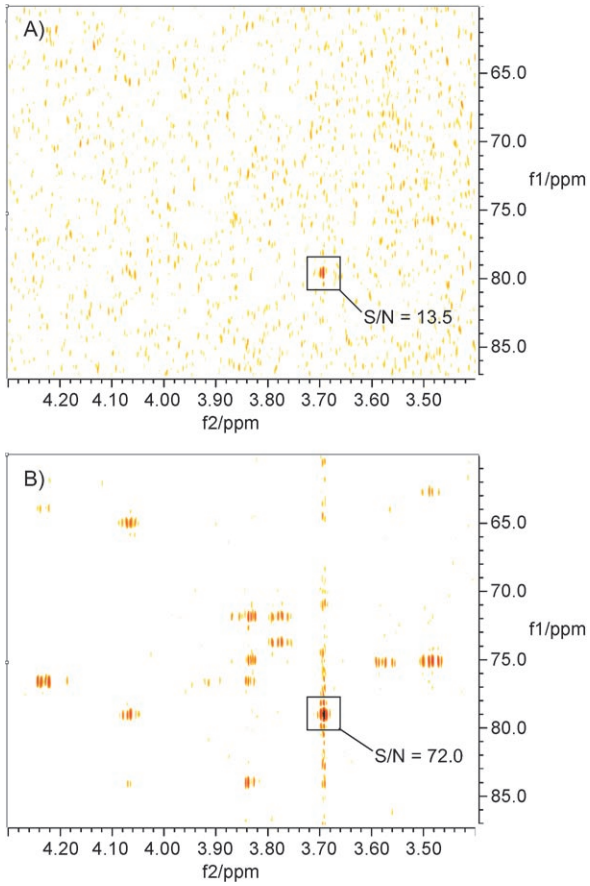

Figure 3. A) Nongradient (^{1}H,^{13}C) HMBC spectrum of sucrose (17.2 μg) dissolved in D_2O (260 μL) using a Shigemi tube and a 5-mm H{C,N} probe. B) Nongradient (^{1}H,^{13}C) HMBC spectrum of sucrose (17.2 μg) dissolved in D_2O (5 μL) using a 5-μL CapNMR probe. The total acquisition time for each spectrum was 14 h.[18] S/N ratios were determined on slices in f2 by using VNMR (Varian Inc.) software.

3. Stand-Alone Applications

3.1. Offline Characterization of Small Molecules

There are relatively few reports on the use of microcoil NMR probes for the offline characterization of mass-limited small-molecule samples, such as precious natural products or other biologically active materials that cannot be obtained in sufficient quantity to enable structure determination by using conventional NMR probes. Nonetheless, natural product chemists can expect to benefit greatly from the introduction of microcoil probes. Simply because of their scarcity, many organisms with potentially interesting secondary metabolites are effectively placed off limits for natural product exploration based on conventional NMR spectroscopy, as the amounts of sample isolated would likely not be sufficient to obtain spectra of suitable quality for identification. The relatively slow adoption of microcoil NMR technology for natural products applications might in part be related to its flow-cell design, which requires a dedicated approach to sample preparation and might deter chemists who are used to using glass NMR tubes. However, manual injection[18,22] or automated loading[23] of individual samples into the CapNMR probes is entirely feasible. For loading of a 5-μL CapNMR flow cell, the sample dissolved in 5 μL of deuterated solvent is injected into the inlet line, either by using a dedicated liquid-handling system or simply by syringe. The injection of the sample is then followed by injection of a small amount of additional deuterated solvent, which serves to push the sample through the inlet line into the flow cell. The volume of solvent needed to push the sample into the flow cell depends on the length of the inlet line and is easily calibrated upon initial installation of the probe by using a test sample. For recovery of the sample after completion of NMR spectroscopic analysis, the sample is simply flushed from the probe into a recovery vial by injecting a larger volume of solvent (typically around 50 μL). Offline sample injection as described here is highly reproducible and can be used for the routine characterization of many kinds of mass-limited samples. However, it requires that all samples be carefully filtered prior to injection to avoid blockages in the capillaries that might result from insoluble contaminants. Accordingly, inline filtration is a standard procedure (standard filter size: 2 μm).

The identification of two antibacterial glycosides **1** and **2** by Hu and co-workers represented the first example of structure elucidation of new natural products by using CapNMR spectroscopy (Figure 4).[24] Glycosides **1** and **2** were isolated by automated high-throughput fractionation of a library of plant extracts. Following fractionation, those fractions that displayed antibacterial activity were characterized offline by using the CapNMR probe with manual injection. Similarly, the antibacterial plant metabolites suaveolindole[25] (**3**) and a group of partially acetylated oligorhamnosides[26] such as **4** were identified by using CapNMR spectroscopy. The amount of glycoside **2** isolated (70 μg) would clearly have been insufficient for identification by using a conventional 5-mm probe, and although **1** and **3** were isolated in amounts large enough to warrant identification by

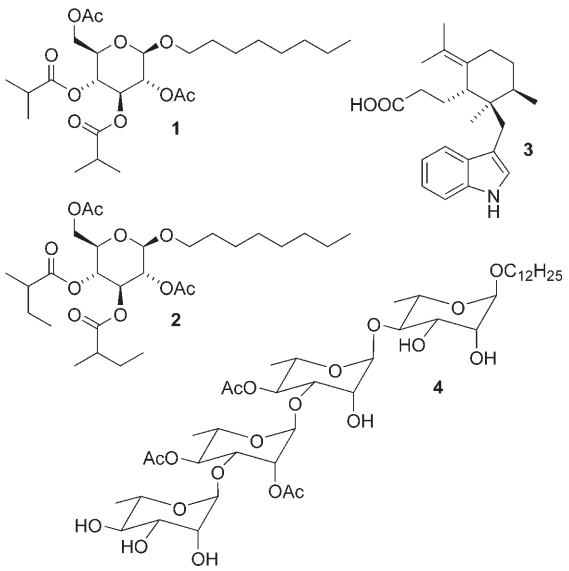

Figure 4. Plant-derived natural products identified by Hu and co-workers by using CapNMR spectroscopy.

using conventional 5-mm probes, use of the CapNMR probe significantly reduced the times required for the acquisition of ^{13}C NMR, gCOSY (gradient-selected COSY), HSQC, and HMBC spectra.

In a study aimed at exploring natural product diversity in rare organisms, Gronquist et al. used a 5-μL CapNMR probe to characterize and identify a series of novel steroidal pyrones isolated from a small collection of specimens of a rare firefly species.[18] Thirteen different cardenolides and related steroids, for example, **5** and **6**, were identified, all of which represented new natural products (Figure 5). The compounds

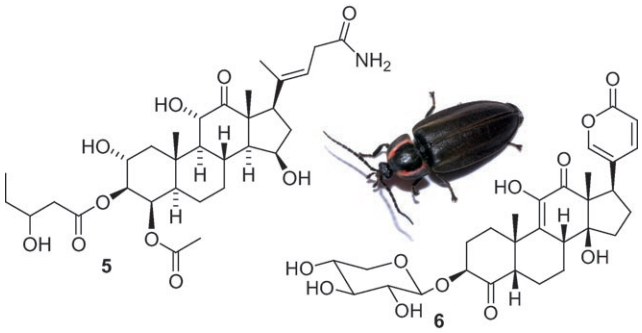

Figure 5. Steroids identified from *Lucidota atra* fireflies by using CapNMR spectroscopy.

were identified on the basis of data from routine dqf-COSY (double quantum filtered COSY), NOESY, HMQC, and HMBC spectra. Nongradient HMBC spectra of sufficient quality for structure determination were obtained for samples containing as little as 40 nmol of material. By directly comparing the sensitivity achieved by CapNMR spectroscopy with that of a 5-mm H{C,N} probe with a Shigemi tube, it was found that CapNMR provided a roughly threefold gain in sensitivity while maintaining very high spectral quality.

Spectral characteristics such as good line shapes and a low level of artifacts were essential for the spectroscopic characterization of the steroid samples, as several of these constituted mixtures of two or three compounds. The observed sensitivity gain of roughly a factor of three is significantly smaller than the increase in sensitivity determined in the sucrose experiments described in Section 2.2. The reason for the lower sensitivity gain in the case of the steroid example lies in the specific details of the sample preparation procedure. When using the Shigemi tube, the sample can be transferred into the tube almost without loss, as the relatively large volume of the NMR tube permits multiple rinses of the original sample vial. With CapNMR, however, a small amount of sample is inevitably left behind in the original sample vial, because the sample has to be transferred by a single 5-μL injection. Nevertheless, the use of the CapNMR probe for characterization of the firefly-derived cardenolides allowed the identification of at least twice as many compounds as would have been possible by using the 5-mm probe/Shigemi tube combination.

In addition to its value for the identification of new natural products, Hu et al. demonstrated the utility of capillary NMR spectroscopy for the dereplication of natural product libraries.[27] As generally only about 10 nmol (about 5 μg for a compound with a molecular weight of 500 $g\,mol^{-1}$) of material is required for the acquisition of 1D ^1H and (^1H,^1H) COSY spectra, even trace amounts of known natural products can easily be identified. CapNMR probes also hold great promise for the identification of known metabolites in pharmacology or of synthetic products in combinatorial chemistry (see Section 5).

3.2. Protein Structure Determination

While CapNMR probes have been commercially available for a few years, their utility for the characterization of biological macromolecules has been realized only recently. The relatively low concentration-sensitivity of microcoil probes makes their use for the characterization of proteins and nucleic acids non-intuitive, because most biological macromolecules are generally much more concentration-limited than organic small molecules. However, in 2004, Peti et al. showed that the use of microcoil probes for protein structure determination offers distinct advantages.[22] NMR spectroscopic characterization of a protein usually starts with assignment of the protein backbone by using well-established triple-resonance experiments such as HNCOCA and HNCA.[28] This step is followed by the acquisition of additional spectra aimed at assignment of the aliphatic and aromatic side chains. Compared to the characterization of the protein backbone, the assignment of the side chain, especially aromatic amino acid side chains, is much less straightforward and often very time-consuming because it relies on interpretation of 2D (^1H,^1H) NOESY, COSY, and TOCSY spectra that suffer from poor signal dispersion and are thus prone to cross-peak overlap, which results in ambiguous assignments. As the hydrophobic aromatic side chains play a key role in protein stability, there is considerable interest in improving methods for their characterization. Peti et al. showed that the distinctive physical properties of microcoil probes allow side-chain assignments to be dramatically simplified. In addition to providing high mass-sensitivity, microcoil probes exhibit excellent radiofrequency (RF) characteristics as a direct result of the solenoidal coil design, and only very low transmitter power is needed to achieve short proton and carbon 90-degree pulses. These specific qualities of microcoils allowed for the first time the acquisition of aliphatic–aromatic HCCH-TOCSY spectra, which can be used to assign the aromatic and aliphatic side chains of a fully ^{13}C-labeled protein in a single experiment (Figure 6). By using conventional room-temperature or cryogenic 5-mm probes the acquisition of such spectra would not be feasible because the RF power required to induce TOCSY transfer over the entire chemical shift range of aromatic and aliphatic carbon atoms would be prohibitively high. Using their full-range HCCH-TOCSY version, Peti et al. completely assigned the side chains for a sample of only 500 μg of a 9.7-kDa protein. The 3D HNCO and HNCOCA spectra required for assignment of the backbone were also obtained by using the CapNMR probe.

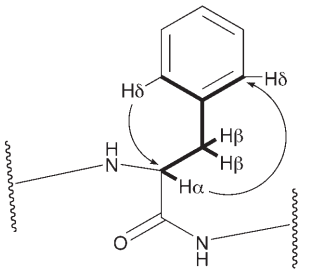

Figure 6. An important step in the NMR spectroscopic characterization of proteins consists of connecting Cα/Hα and Cβ/Hβ atoms of aromatic amino acids with the Hδ protons in the corresponding aromatic ring system. HCCH-TOCSY correlations between Hα and Cδ atoms and between Hδ and Cα atoms in a phenylalanine residue are shown.[22]

In another recent example, Peti et al. outlined the utility of capillary NMR systems in structural genomics.[29] To identify promising targets for structure determination by X-ray crystallography, structural genomics researchers increasingly resort to NMR spectroscopic screening of protein libraries for proteins that exhibit substantial folding in solution. In combination with high-throughput methods for protein expression and purification, implementing CapNMR techniques allows for a substantial miniaturization of structural genomics pipelines. By using ^1H NMR and 2D (^1H,^{15}N) COSY spectra, Peti et al. demonstrated the effectiveness of their miniaturized pipeline by comparing the results obtained for nine mouse homologue protein targets with those obtained by using traditional "large-scale" methodology. This comparison indicated that the quality of structural information obtained through CapNMR-based structural genomics pipelines was similar to that achieved by using conventional methods. Thus, capillary NMR spectroscopy allows implementation of a miniaturized structural

genomics pipeline without compromising data quality, thus significantly reducing the costs associated with protein production as, for example, the requirements for labeled ^{15}N media decrease by about 20-fold.

4. Hyphenated Techniques

The design and performance parameters of capillary probes offer distinct advantages over earlier flow-through NMR probe designs and make them well suited for direct coupling to various chromatographic methods, resulting in so-called "hyphenated" techniques.[30] CapNMR probes are especially well matched to capillary-based separations, which operate with compatible flow rates and give peak volumes similar to the volume of the flow cell, a necessary condition for fully exploiting the increased mass-sensitivity of the microcoil probe. The higher peak concentrations generally realized in capillary separations provide further gains in sensitivity when compared to larger bore separations.[31] The very low flow rates used in capillary-based systems translate into lower consumption of deuterated solvents, thus reducing costs and decreasing the need for solvent suppression. Capillary NMR spectroscopy has been coupled online to capillary HPLC (CapLC-NMR), as well as to capillary electrophoretic techniques such as capillary isotachophoresis (cITP-NMR)[32] and capillary electrochromatography (CEC-NMR).[33]

4.1. Capillary LC-NMR (CapLC-NMR)

Capillary HPLC is the most common separation technique to be coupled online with the CapNMR probe, largely as a result of the popularity and applicability of HPLC as the method of choice for a wide range of separation problems. As with earlier versions of LC-NMR,[34,35] various modes of operation are possible depending on the nature of the sample and specific time requirements. In continuous-flow mode (also referred to as on-flow), transients are collected continuously over the course of the separation. The continuous introduction of fresh polarized nuclei into the flow cell benefits the sensitivity by reducing the need for relaxation delays in the pulse sequences, but also results in additional line-broadening as flow rates increase.[36] As each eluting peak occupies the active volume of the flow cell for only a short period of time, relatively few transients are collected for each component and this mode is thus necessarily relatively insensitive.[33] Sensitivity can be improved by decreasing flow rates[37] and increasing sample loading, but a point of diminishing returns is quickly reached where the chromatographic separation begins to suffer. Nonetheless, the increased mass-sensitivity of the CapNMR probe makes continuous-flow LC-NMR a viable technique for applications in which rapid analysis yielding detailed structural information is required. The feasibility of CapLC-NMR for continuous-flow analysis of mixtures of natural products was demonstrated by Krucker et al.[38] In their analysis, a 200-nL aliquot containing approximately 1.3 μg each of four tocopherol homologues was separated and each elution peak was positively identified based solely on its ^1H NMR spectrum. Note that two of the components, β- and γ-tocopherol, were indistinguishable by their mass spectra. Comparison to previous work by the same authors using classical HPLC-NMR showed the CapLC-NMR system to achieve comparable performance while decreasing the amount of sample required by a factor of 200.[39]

For most mass-limited applications, longer acquisition times are required than can be achieved by using a continuous-flow mode. In stopped-flow mode, selected elution peaks are stored in the flow cell for extended periods of time, allowing the acquisition of longer 1D or 2D spectra. Large improvements in the signal-to-noise ratio are realized, but total run times necessarily become longer. Online UV/Vis detection is generally incorporated immediately upstream from the NMR probe to identify peaks prior to their entry into the capillary probe flow cell, and the proper time delay required to correctly position the eluting analyte bands in the active volume of the flow cell must be calibrated. The continuous-flow-path construction of the narrow diameter (50–100 μm i.d.) inlet/outlet feed lines of the flow cell minimizes sample dilution caused by diffusion, while the small sample volume and relatively larger diameter of the flow cell facilitate rapid equilibration of the samples stored in the flow cell.[12] As a result, acquisitions lasting several hours are possible with negligible sample diffusion. The potential of stopped-flow CapLC-NMR has been demonstrated recently for a variety of mass-limited applications in natural product analysis,[40,41] metabolite identification,[42,43] and as a potential technique for monitoring protein phosphorylation in cellular signaling pathways.[44]

These analyses show CapLC-NMR to be a promising technique for the rapid extraction of detailed structural information from mass-limited samples. However, as previously noted for stand-alone applications, the benefits of using CapLC-NMR as opposed to other online and offline NMR techniques are only fully realized for samples that are truly mass-limited. In a recent comparison of CapLC-NMR with alternate methods, CapLC-NMR was shown to be an effective technique for distinguishing among diastereomeric adamantane metabolites present in a urine sample.[41] However, the authors noted that limitations inherent in the CapLC-NMR system, such as the loading potential of the column and difficulties in targeting the desired elution peaks for stopped-flow acquisitions, will often warrant the use of alternate techniques in cases where the sample size is not severely limited. In these cases, a larger-scale fractionation followed by offline collection of the fractions of interest and subsequent manual or automated injection into the CapNMR probe can provide better results. Although most applications of CapLC-NMR reported to date have been demonstrations of feasibility, the technique seems well on its way to becoming routine, as underscored by the recent introduction of a commercially available CapLC-NMR system that comes complete with autosampler and integrated diode-array UV/Vis detection.[45]

4.2. Capillary Electrophoresis Hyphenated to Capillary NMR (CE-NMR)

Capillary electrophoresis and its many variants have developed into important separation techniques that frequently prove effective in situations where HPLC is not suitable.[46] Although a wide variety of detection strategies are possible (UV/Vis spectroscopy, MS, etc.), the lack of sensitivity inherent to NMR spectroscopy has previously precluded its coupling to capillary electrophoretic separations. Microcoil NMR spectroscopy for the first time provides a viable way to combine the powerful separation capabilities of capillary electrophoresis with the detailed structural and molecular dynamic information afforded by NMR spectroscopy.[14] The most promising variant of microcoil CE-NMR described to date is capillary isotachophoresis-NMR (cITP-NMR).[32] Capillary isotachophoresis (cITP) separates and concentrates charged species based on their electrophoretic mobilities through the application of a high voltage across a capillary containing a two-buffer system comprised of a leading electrolyte (LE) and a trailing electrolyte (TE; Figure 7). Under optimal conditions, cITP has the ability to concentrate charged analytes by two to three orders of magnitude over the course of the separation. When hyphenated to capillary NMR spectroscopy, cITP allows focusing of sample components precisely in the active volume of the capillary probe to maximize the potential of the microcoil design. The focusing of the sample realized with cITP-NMR allows the use of probes with active volumes on the order of tens of nanoliters, thus providing even greater gains in mass-sensitivity and making cITP-NMR the most mass-sensitive NMR technique yet realized. The potential of cITP-NMR for the online separation and analysis of nanomolar quantitities of biologically relevant small molecules was demonstrated by Korir et al.[47] A mixture containing 2.5 nmol each of three sulfated heparin disaccharides was separated, and ^{1}H NMR spectra suitable for identification were acquired for each component as the focused bands passed through the 25-nL active volume of a custom-built microcoil probe.

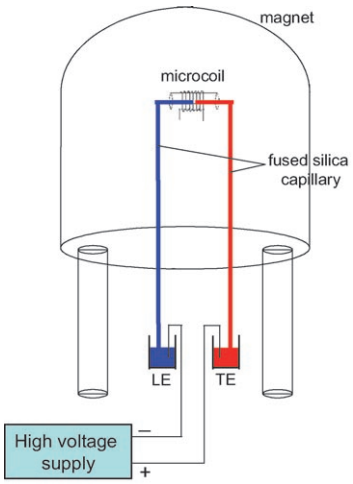

Figure 7. Schematic of cITP-NMR. Charged analytes are focused through the application of high voltage across a capillary that contains a two-buffer system. Focused bands are detected as they move through the microcoil probe.[47,49]

In contrast to CapLC-NMR, which seems to be on the verge of entering the mainstream as a routine analytical method, capillary electrophoresis-NMR (CE-NMR) is still in the early stages of development. Most results reported to date are based on custom-built, "in-house" probes, which often use internally modified capillaries and very small active volumes.[48,49] Nonetheless, the preliminary results seem promising and suggest that CE-NMR will continue to develop and may become the technique of choice for specific applications in chemical, biochemical, and environmental analysis.

5. High-Throughput Capillary NMR Spectroscopy

Capillary NMR spectroscopy holds particular promise for use in high-throughput applications, such as in the analysis of natural product or synthetic compound libraries. A continuing problem in characterizing large libraries has been the lack of a universal detector. Whereas various combinations of MS, UV, and evaporative light-scattering detectors (ELSD) have been used extensively in high-throughput applications, these methods often suffer from response bias or a general lack of structural information. The detailed structural and stereochemical information afforded by NMR spectroscopy is thus often necessary for the comprehensive characterization of natural product and synthetic library compounds.[50] In addition, NMR spectroscopic techniques have been used to screen small-molecule libraries for weak ligand–protein interactions that are difficult to observe with traditional fluorescence-based techniques but which may hold important clues for drug development.[51–53] While automated high-throughput NMR systems are not novel,[54] the increased mass-sensitivity realized with microcoil capillary probes greatly extends the scope and applicability of high-throughput NMR spectroscopy as it facilitates faster acquisitions on smaller amounts of sample while consuming far less deuterated solvent. The flow-through design of capillary probes makes them well suited for automation either hyphenated online to analytical separations or offline as stand-alone analyzers. The general trend appears to be toward offline analyses facilitated through the use of common sample formats across complementary analytical platforms. The ease of shimming and the shim stabilities that are intrinsic to the microcoil design translate into further efficiency for overall sample throughput. Finally, as NMR spectroscopy is non-destructive, incorporating it into an online analysis enables further coupling to a fraction collector or other detection techniques, such as MS, UV, and ELSD, thus allowing the acquisition of complementary structural information.

Preliminary evaluations carried out on test libraries by using microcoil probes with various system configurations were recently reported. Bailey and Marshall recently reported a method utilizing continuous flow for the rapid analysis of a test library comprised of *p*-hydroxybenzoic acid esters.[55] Individual samples were automatically loaded sequentially from a 96-well plate and pumped through the capillary flow

cell as a steady sample stream. Transients were collected continuously and stored as a pseudo-2D file, from which increments corresponding to individual samples were extracted and pooled to give individual 1D spectra. By this means, ^{1}H NMR spectra suitable for structure confirmation were obtained for injection amounts corresponding to approximately 3.4 μg of sample per injection. Analysis of a 96-well plate could be completed in less than 1 hour, and consumption of the deuterated solvent was less than 4.0 mL per plate. Alternatively, Kautz et al. described a stopped-flow method based on segmented flow analysis (SFA).[56] In this method, library compounds are injected as discrete plugs separated by an immiscible, susceptibility-matched fluorocarbon carrier fluid. Sample plugs are detected by their NMR signal upon entering the flow cell and then stopped for spectral acquisition. The carrier fluid serves to wet the capillary walls, decreasing adhesion and limiting the resultant carryover and loss of sample. The immiscible nature of the carrier fluid also limits sample dispersion, allowing 2D acquisitions with durations of up to several days when necessary. The potential of the technique was demonstrated through the unambiguous identification of all members of a test library containing such structurally diverse elements as reserpine, erythromycin, and uracil (green, red, and blue structures, respectively, in Figure 8). Sample amounts were on the order of 10 μg per

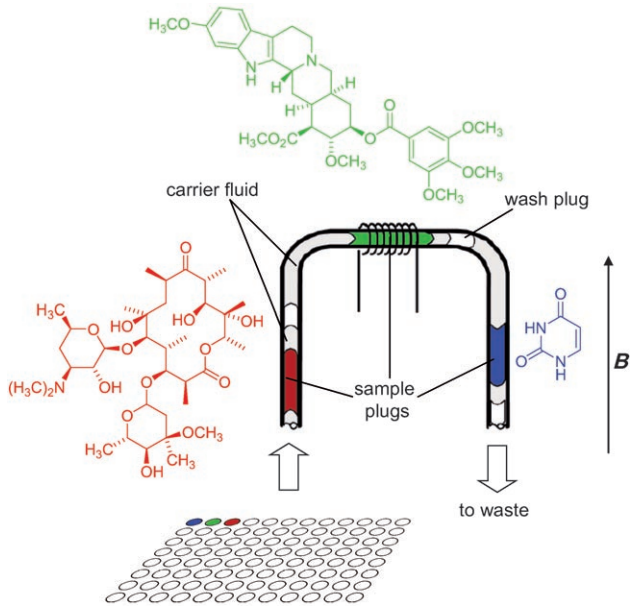

Figure 8. Schematic of SFA-NMR.[56] Library samples are advanced as discrete 1 μL sample plugs, separated by an immiscible carrier fluid. [D_6]DMSO wash plugs containing 1% trimethylsilylpropionate are included to further limit sample carryover and facilitate sample detection and positioning.

injection, and analysis of a 96-well plate could be completed in less than 3 h while consuming less than 0.5 mL of deuterated solvent per plate. Most recently, a flexible automated system capable of accepting both open-access samples in vials as well as library samples prepared in 384-well plates was described and evaluated.[23] The system, which is controlled and monitored by using web-based software, allows walk-up analysis of priority samples prepared in microliter vials anytime during library analyses.

6. Summary and Outlook

NMR spectroscopy is clearly one of the most valuable analytical techniques for organic chemists and biochemists. The introduction of microcoil probes is likely to further extend its scope and utility as they offer improved mass-sensitivity and are easily combined with almost any chromatographic technique. For a technology so radically different from that of conventional NMR probes, current microcoil designs have been astonishingly successful and, within only a few years of their original inception, have evolved into a serious alternative to cryogenic probes, as for many situations they offer comparable sensitivity at a fraction of the cost. Moreover, CapNMR probes can be installed and exchanged with conventional 5- or 3-mm NMR probes very quickly and thus, as opposed to cryogenic probes, do not require a spectrometer to be solely dedicated to their use. As the technology is still young, further improvements in sensitivity and overall design are likely. Chemists interested in microcoil NMR spectroscopy will have to adjust to specific requirements for sample preparation and handling. Current sample injection procedures require careful filtration, and complete transfer of mass-limited samples into the flow cell cannot always be achieved. However, improved routines for both manual and automated sample injection continue to be developed. High-throughput combinatorial chemistry as well as pharmacological applications and high-throughput natural products chemistry will benefit from the availability of probes with two or more flow cells.[57]

At this time, microcoil probes appear particularly well suited for small-molecule NMR spectroscopy, whereas their utility for the characterization of biological macromolecules is somewhat limited because for many proteins or nucleic acids sufficiently high sample concentrations cannot be obtained. For such concentration-limited samples, cryogenic probes will remain the obvious choice. However, as recent examples by Peti et al. have illustrated, the use of microcoil probes for protein structure determination is entirely feasible and in certain situations can offer distinct advantages (see Section 3.2).

There are additional benefits of using miniaturized NMR probes, some of which have not yet become fully apparent. Because of their small sample volumes, microcoil NMR probes require far smaller homogeneous magnetic field regions than traditional 5-mm probes. One consequence is the relative ease of shimming of microcoil probes, which usually requires adjusting only a few first-order shims. More importantly, the less stringent requirements for the homogenous region of the magnetic field may allow the development of smaller magnets, which could lead to significant cost savings, but might also hold promise for the introduction of magnets with higher field strengths or the use of alternative materials for magnet design, including the use of room-temperature superconducting materials. Introduction of

smaller highfield magnets suitable for high-resolution NMR spectroscopy would dramatically reduce space requirements for NMR spectrometers and might ultimately lead to the development of benchtop instruments.

Received: May 8, 2006
Published online: September 22, 2006

[1] R. R. Ernst, *Angew. Chem.* **1992**, *104*, 817; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 805.
[2] A. E. Derome, *Modern NMR Techniques for Chemistry Research*, Pergamon, New York, **1987**.
[3] C. L. Putzig, *Anal. Chem.* **1994**, *66*, 26R.
[4] M. E. Lacey, R. Subramanian, D. L. Olson, A. G. Webb, J. V. Sweedler, *Chem. Rev.* **1999**, *99*, 3133.
[5] D. I. Hoult, R. E. Richards, *J. Magn. Reson.* **1976**, *24*, 71.
[6] A. Abraham, *The Principles of Nuclear Magnetism*, Clarendon Press, Oxford, **1961**.
[7] P. L. Rinaldi, *Analyst* **2004**, *129*, 687.
[8] D. J. Russell, C. E. Hadden, G. E. Martin, A. A. Gibson, A. P. Zens, J. L. Carolan, *J. Nat. Prod.* **2000**, *63*, 1047.
[9] M. Spraul, A. S. Freund, R. E. Nast, R. S. Withers, W. E. Maas, O. Corcoran, *Anal. Chem.* **2003**, *75*, 1546.
[10] A. G. Webb, *Prog. Nucl. Magn. Reson. Spectrosc.* **1997**, *31*, 1.
[11] J. N. Shoolery, *Top. Carbon-13 NMR Spectrosc.* **1979**, *2*, 28.
[12] D. L. Olson, J. A. Norcross, M. O'Neil-Johnson, P. F. Molitor, D. J. Detlefsen, A. G. Wilson, T. L. Peck, *Anal. Chem.* **2004**, *76*, 2966.
[13] N. Wu, T. L. Peck, A. G. Webb, R. L. Magin, J. V. Sweedler, *Anal. Chem.* **1994**, *66*, 3849.
[14] N. Wu, T. L. Peck, A. G. Webb, R. L. Magin, J. V. Sweedler, *J. Am. Chem. Soc.* **1994**, *116*, 7929.
[15] T. L. Peck, R. L. Magin, P. C. Lauterbur, *J. Magn. Reson. Ser. B* **1995**, *108*, 114.
[16] A. G. Webb, S. C. Grant, *J. Magn. Reson. Ser. B* **1996**, *113*, 83.
[17] D. L. Olson, T. L. Peck, A. G. Webb, R. L. Magin, J. V. Sweedler, *Science* **1995**, *270*, 1967.
[18] M. Gronquist, J. Meinwald, T. Eisner, F. C. Schroeder, *J. Am. Chem. Soc.* **2005**, *127*, 10810.
[19] For a detailed explanation of two-dimensional NMR spectroscopy and commonly used NMR spectroscopic acronyms, see: H. Kessler, M. Gehrke, C. Griesinger, *Angew. Chem.* **1988**, *100*, 507; *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 490.
[20] R. Hurd, B. K. John, *J. Magn. Reson.* **1991**, *91*, 648.
[21] P. L. Rinaldi, P. A. Keifer, *J. Magn. Reson. Ser. A* **1994**, *108*, 259.
[22] W. Peti, J. Norcross, G. Eldridge, M. O'Neil-Johnson, *J. Am. Chem. Soc.* **2004**, *126*, 5873.
[23] A. Jansma, T. Chuan, R. W. Albrecht, D. L. Olson, T. L. Peck, B. H. Geierstanger, *Anal. Chem.* **2005**, *77*, 6509.
[24] J.-F. Hu, H.-D. Yoo, C. T. Williams, E. Garo, P. A. Cremin, L. Zeng, H. C. Vervoort, C. M. Lee, S. M. Hart, M. G. Goering, M. O'Neil-Johnson, G. R. Eldridge, *Planta Med.* **2005**, *71*, 176.
[25] H.-D. Yoo, P. A. Cremin, L. Zeng, E. Garo, C. T. Williams, C. M. Lee, M. G. Goering, M. O'Neil-Johnson, G. R. Eldridge, J.-F. Hu, *J. Nat. Prod.* **2005**, *68*, 122.
[26] J.-F. Hu, E. Garo, G. W. Hough, M. G. Goering, M. O'Neil-Johnson, G. R. Eldridge, *J. Nat. Prod.* **2006**, *69*, 585.
[27] J.-F. Hu, E. Garo, H.-D. Yoo, P. A. Cremin, L. Zeng, M. G. Goering, M. O'Neil-Johnson, G. R. Eldridge, *Phytochem. Anal.* **2005**, *16*, 127.
[28] J. Cavanagh, W. J. Fairbrother, A. G. Palmer III, N. J. Skelton, *Protein NMR Spectroscopy*, Academic Press, San Diego, CA, **1996**.
[29] W. Peti, R. Page, K. Moy, M. O'Neil-Johnson, I. A. Wilson, R. C. Stevens, K. Wüthrich, *J. Struct. Funct. Genomics* **2005**, *6*(4), 259.
[30] D. A. Jayawickrama, J. V. Sweedler, *J. Chromatogr. A* **2003**, *1000*, 819.
[31] J. P. C. Vissers, *J. Chromatogr. A* **1999**, *856*, 117.
[32] R. A. Kautz, M. E. Lacey, A. M. Wolters, F. Foret, A. G. Webb, B. L. Karger, J. V. Sweedler, *J. Am. Chem. Soc.* **2001**, *123*, 3159.
[33] E. Rapp, A. Jakob, A. B. Schefer, E. Bayer, K. Albert, *Anal. Bioanal. Chem.* **2003**, *376*, 1053.
[34] V. Exarchou, M. Krucker, T. A. van Beek, J. Vervoort, I. P. Gerothanassis, K. Albert, *Magn. Reson. Chem.* **2005**, *43*, 681.
[35] S. H. Hansen, A. G. Jensen, C. Cornett, I. Bjornsdottir, S. Taylor, B. Wright, I. D. Wilson, *Anal. Chem.* **1999**, *71*, 5235.
[36] A. G. Webb, *Magn. Reson. Chem.* **2005**, *43*, 688.
[37] M. Sandvoss in *Online NMR and Related Techniques* (Ed.: K. Albert), Wiley, Chichester, **2002**, p. 111.
[38] M. Krucker, A. Lienau, K. Putzbach, M. D. Grynbaum, P. Schuler, K. Albert, *Anal. Chem.* **2004**, *76*, 2623.
[39] A. Lienau, T. Glaser, M. Krucker, D. Zeeb, F. Ley, F. Curro, K. Albert, *Anal. Chem.* **2002**, *74*, 5192.
[40] H. B. Xiao, M. Krucker, K. Putzbach, K. Albert, *J. Chromatogr. A* **2005**, *1067*, 135.
[41] P. Hentschel, M. D. Grynbaum, P. Molnar, K. Putzbach, J. Rehbein, J. Deli, K. Albert, *J. Chromatogr. A* **2006**, *1112*, 285.
[42] R. J. Lewis, M. A. Bernstein, S. J. Duncan, C. J. Sleigh, *Magn. Reson. Chem.* **2005**, *43*, 783.
[43] M. Sandvoss, A. D. Roberts, I. M. Ismail, S. E. North, *J. Chromatogr. A* **2004**, *1028*, 259.
[44] P. Hentschel, M. Krucker, M. D. Grynbaum, K. Putzbach, R. Bischoff, K. Albert, *Magn. Reson. Chem.* **2005**, *43*, 747.
[45] http://www.bruker-biospin.de/NMR/hyphenation/caplc.html.
[46] L. DeFrancesco, *Anal. Chem.* **2001**, *73*, 497A.
[47] A. K. Korir, V. K. Almeida, D. S. Malkin, C. K. Larive, *Anal. Chem.* **2005**, *77*, 5998.
[48] A. M. Wolters, D. A. Jayawickrama, J. V. Sweedler, *J. Nat. Prod.* **2005**, *68*, 162.
[49] V. K. Almeida, C. K. Larive, *Magn. Reson. Chem.* **2005**, *43*, 755.
[50] D. Raftery, *Anal. Bioanal. Chem.* **2004**, *378*, 1403.
[51] S. B. Shuker, P. J. Hajduk, R. P. Meadows, S. W. Fesik, *Science* **1996**, *274*, 1531.
[52] A. D. Chen, M. J. Shapiro, *J. Am. Chem. Soc.* **2000**, *122*, 414.
[53] A. H. Siriwardena, F. Tian, S. Noble, J. H. Prestegard, *Angew. Chem.* **2002**, *114*, 3604; *Angew. Chem. Int. Ed.* **2002**, *41*, 3454.
[54] P. A. Keifer, *Curr. Opin. Chem. Biol.* **2003**, *7*, 388.
[55] N. J. C. Bailey, I. R. Marshall, *Anal. Chem.* **2005**, *77*, 3947.
[56] R. A. Kautz, W. K. Goetzinger, B. L. Karger, *J. Comb. Chem.* **2005**, *7*, 14.
[57] M. A. Macnaughtan, T. Hou, J. Xu, D. Raftery, *Anal. Chem.* **2003**, *75*, 5116.

