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NMR Difference Probe: A Dual-Coil Probe for NMR Difference Spectroscopy

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A unique probe designed to acquire nuclear magnetic resonance difference spectra of two samples is presented. The NMR Difference Probe contains two sample coils in a resonant circuit that switches between parallel excitation and serial acquisition to cancel common signals such as solvent peaks and impurities. Two samples containing a common analyte, acetonitrile, were used to demonstrate signal cancellation in a difference spectrum collected with a single pulse experiment. The cancellation was over 96% effective. The approach described has applications in the areas of solvent subtraction and spectral simplification.

Key Words: **difference spectroscopy; background subtraction; solvent suppression; LC-NMR; microcoils.**

INTRODUCTION

In many spectroscopic techniques undesirable solvent signals are routinely removed from a spectrum using background subtraction. Reference and analyte samples are analyzed, often simultaneously in a dual beam instrument, and their signals are subtracted to give a "background-free" or "difference" spectrum. Such difference techniques are not used in nuclear magnetic resonance (NMR) spectroscopy because it currently operates using a single sample paradigm. In addition, very large solvent signals must be suppressed prior to acquisition due to digital dynamic range limitations (*1*). For ^{1}H NMR experiments this dynamic range must encompass solvent concentrations that often exceed analyte concentrations by a factor of 10^3 or greater. As such, deuterated solvents are commonly used for liquid chromatography LC-NMR (*2, 3*), while sophisticated pulse sequences are used for water suppression in protein NMR (*1*).

Difference spectroscopy is a valuable tool in NMR and is used extensively for nuclear Overhauser effect (NOE) experiments (*4*) and for studying protein-ligand interactions (*5*). In the latter case, difference spectroscopy is used to distinguish between bound and free ligands through changes in the ligand's translational diffusion rate, relaxation rate, or chemical shift. Such methods typically employ sequential NMR experiments using ligand and ligand-protein samples whose spectra are digitally subtracted or simply visually compared. For example, differences in the chemical shifts in ^{1}H and ^{15}N-HSQC spectra are used to screen for potential drug candidates through the investigation of structure activity relationships (SAR by NMR) (*6, 7*). Bound ligands have slower diffusion rates and faster relaxation times compared to free ligands, so relaxation- and diffusion-edited NMR experiments can also be used to identify the binding ligands by using the ligand mixture as a subtraction reference from a ligand-protein mixture (*8–10*). Similar samples are analyzed with transfer techniques such as NOE pumping (*11, 12*) and saturation-transfer difference (STD) NMR spectroscopy (*13, 14*), which utilize pathways available only to the binding ligands for identification.

Solvent signals are similarly reduced or removed using pulse sequences that exploit some difference between the solvent and solute spins such as chemical shift, relaxation time, or diffusion coefficient (*1*). The basic objectives for solvent suppression methods are to remove the entire solvent signal without affecting the solute signal, to not affect the basic operation of the pulse sequence, and to add little time to the experiment. Many problems arise from the first stipulation. For each experiment and sample, a different solvent suppression technique may be best. For example, in protein studies, observation of exchangeable protons and α-protons that resonate close to or under the water resonance is important (*1*). The solvent suppression technique, DRYCLEAN (diffusion-reduced water signals in spectroscopy of molecules moving slower than water) (*15*), is often used in protein studies because it takes advantage of the difference in the diffusion rates of water and the protein. Other solvent suppression techniques such as WATERGATE (water suppression by gradient-tailored excitation) (*16*) and Water-PRESS (water-presequence suppression) (*17, 18*) provide large suppression factors for protein samples, but one must choose between observing exchangeable protons (WATERGATE) and observing resonances close to the water frequency (Water-PRESS). For LC-NMR, solvent suppression is further complicated by the use of multiple solvents and flowing samples (*1–3*). Multiple solvent peaks can be suppressed with techniques that use multiple-frequency-shifted laminar pulses (SLP) (*19*) such as Multigate (a modified version of WATERGATE) (*20*) and WET (water suppression enhanced through T_1 effects) (*21, 22*). Flowing samples cause

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techniques such as presaturation (*23*) and diffusion-based filters to fail because the influenced spins are constantly being replenished with fresh spins (*2, 3*). The WET technique, however, can be used with flowing samples because it is fast (<100 ms), with suppression factors $>10^4$ in a single scan (*22*). Nevertheless, under gradient elution conditions or where impurities are present, the spectra can be less than optimal.

In this article we present preliminary results from our NMR Difference Probe, which represents a new and complementary approach to difference spectroscopy and solvent suppression for NMR. Our technique is based on the traditional idea of dual-beam background subtraction, where the reference and analyte signals are acquired simultaneously and automatically subtracted using the probe hardware before signal processing occurs. As a result, dynamic range problems are avoided, and the difference probe is compatible with many current NMR solvent suppression methods. The NMR Difference Probe is a dual-coil probe with a novel resonant circuit that allows for the simultaneous excitation and detection of two samples. The free induction decay (FID) collected from the NMR difference probe contains sample and reference signals that are 180° out-of-phase with respect to each other. The positive and negative signals destructively interfere in the circuit before digitization to give a single, difference spectrum. By altering the hardware of the NMR and not the pulse sequence, the NMR Difference Probe provides a unique approach to solvent suppression and difference spectroscopy.

THEORY OF OPERATION

The unique resonant circuit of the NMR Difference Probe allows for direct acquisition of a difference spectrum of two samples with a standard $\pi/2$ pulse–acquire sequence. Using crossed-diodes as switches, the resonant circuit shown in Fig. 1a acts as two distinct circuits during transmission and acquisition. During the transmission of the pulse, both crossed-diodes are passively switched on and the sample coils are connected in parallel such that both samples receive the same phase excitation pulse. As depicted in Fig. 1b, the resulting transverse magnetizations point along the same direction after the pulse. Crossed-diode # 2 ensures that both coils are grounded when a radiofrequency pulse is active. The resonant circuit changes during data acquisition because the received signal is small (μvolts) and the crossed-diodes are not activated. As seen from the receiver, the coils are connected in series and crossed-diode # 1 isolates the signal from the transmitter tuning circuit (see Fig. 1c). With respect to the receiver, the transverse magnetizations induce voltages in each coil that are oriented in opposite directions and, therefore, add with opposite phase to give a difference spectrum. The acquired data contain signals from both samples, but due to the effects of parallel excitation and serial acquisition, the signals from the two coils are 180° out-of-phase and, consequently, common signals cancel.

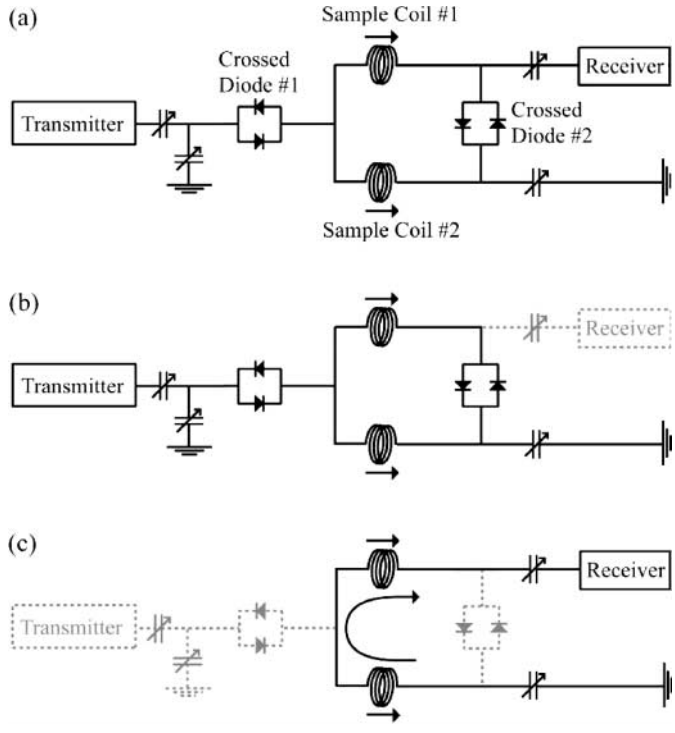

FIG. 1. (a) The schematic diagram of the NMR Difference Probe resonant circuit with two coils and two sets of variable capacitors for tuning and matching each subcircuit. Notice that the transmitter and receiver are connected separately to the circuit. (b) The solid black lines indicate the active part of the circuit during the radiofrequency pulse when the crossed-diodes are on. The arrow beside each coil represents the relative phase of the transverse magnetization after the pulse. Because the coils are connected in parallel with respect to the transmitter, the arrows point in the same direction. (c) During acquisition of the NMR signal, the circuit changes. The crossed-diodes are off because the small voltage induced in the coils by the NMR signal is not great enough to activate the diodes. The solid black lines indicate the active part of the circuit when the signal is collected at the receiver. The arrows beside the coils represent the phase of the voltages induced in each coil by the excited samples. Since the coils are connected in series with respect to the receiver (following the curved arrow), the voltage arrows are opposing and the signals from each coil are 180° out-of-phase.

EXPERIMENTAL

The NMR Difference Probe circuit was constructed using a homebuilt, wide-bore (73 mm) probe body. The probehead, shown in Fig. 2, consists of two solenoid microcoils fabricated by wrapping polyurethane coated high purity (99.99%) 42-gauge (63.5-μm diameter) copper wire (California Fine Wire Co., Grover Beach, CA) around glass capillaries (860-μm o.d., 400-μm i.d.). Microcoils have been used in a variety of NMR experiments such as LC-NMR and capillary electrophoresis CE-NMR because they provide excellent signal-to-noise ratios (S/N) for mass-limited and volume-limited samples (*24*). In our laboratory we have previously used microcoils to analyze multiple samples simultaneously (*25–27*). The microcoils are attached to the capillary tubes using a cyanoacrylate adhesive (Krazy Glue, Borden Inc., Columbus, OH). Each coil consists

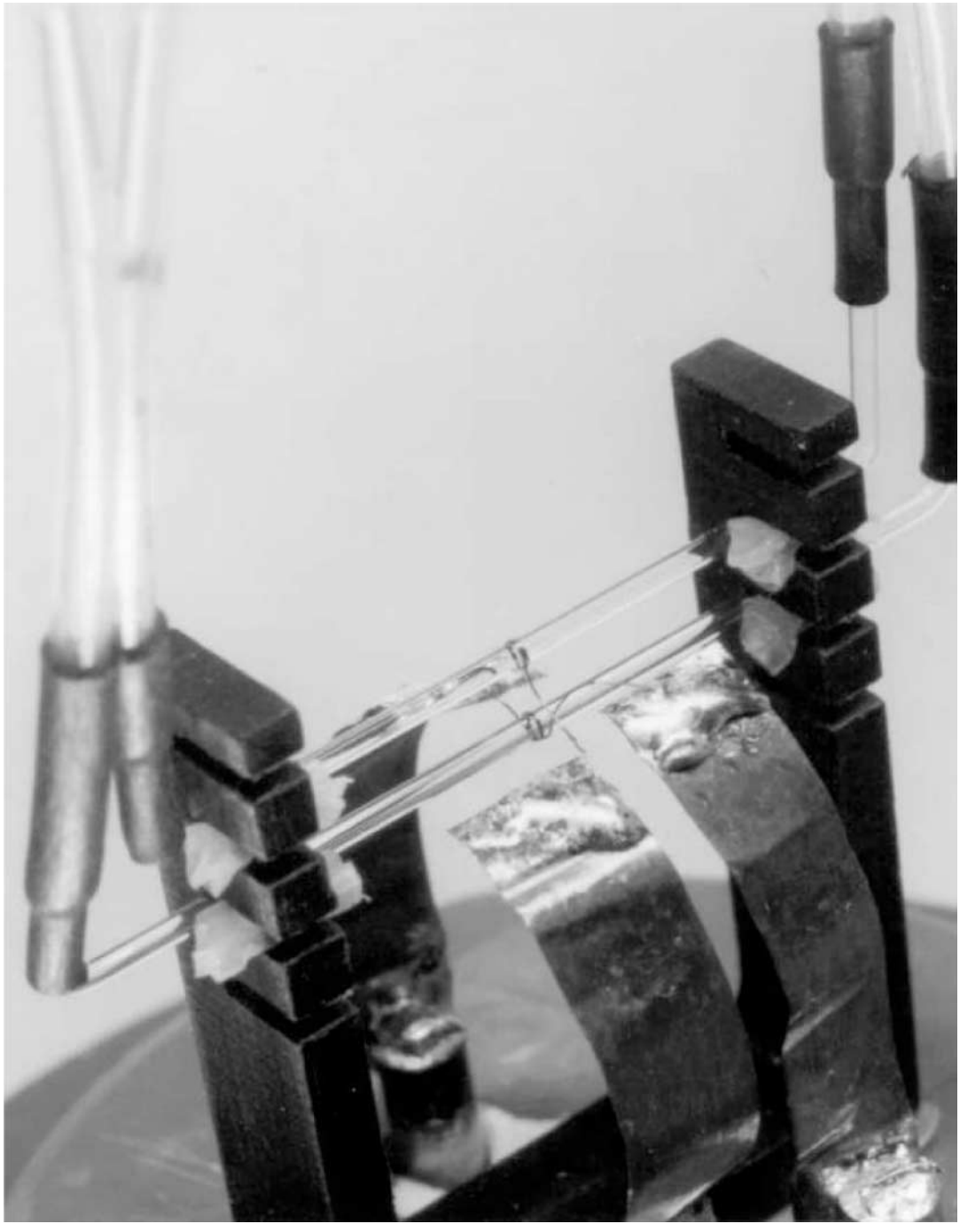

FIG. 2. Photograph of the sample capillaries and coils used in the NMR Difference Probe.

of 3 turns and has an inner diameter of 860 μm and a length of 200 μm. The sample capillaries are mounted on a rectangular support made of black Delrin, which holds the capillary tubes at an intercoil spacing (center to center) of 3 mm. To allow for flow introduction of the samples, Teflon tubing (Small Parts Inc., Miami Shores, FL) is attached to the ends of the capillaries with polyolefin heat-shrink tubing (Small Parts Inc., Miami Shores, FL). The sample coils and holder are housed in a removable PVC container filled with Fluorinert FC-43 (Syn Quest Laboratories, Alachua, FL), a magnetic susceptibility matching fluid shown to improve spectral linewidth (*28*).

The NMR Difference Probe circuit contains the two sample coils, four nonmagnetic tunable capacitors (Voltronics, Denville, NJ), and two sets of crossed-diodes constructed from 1N914

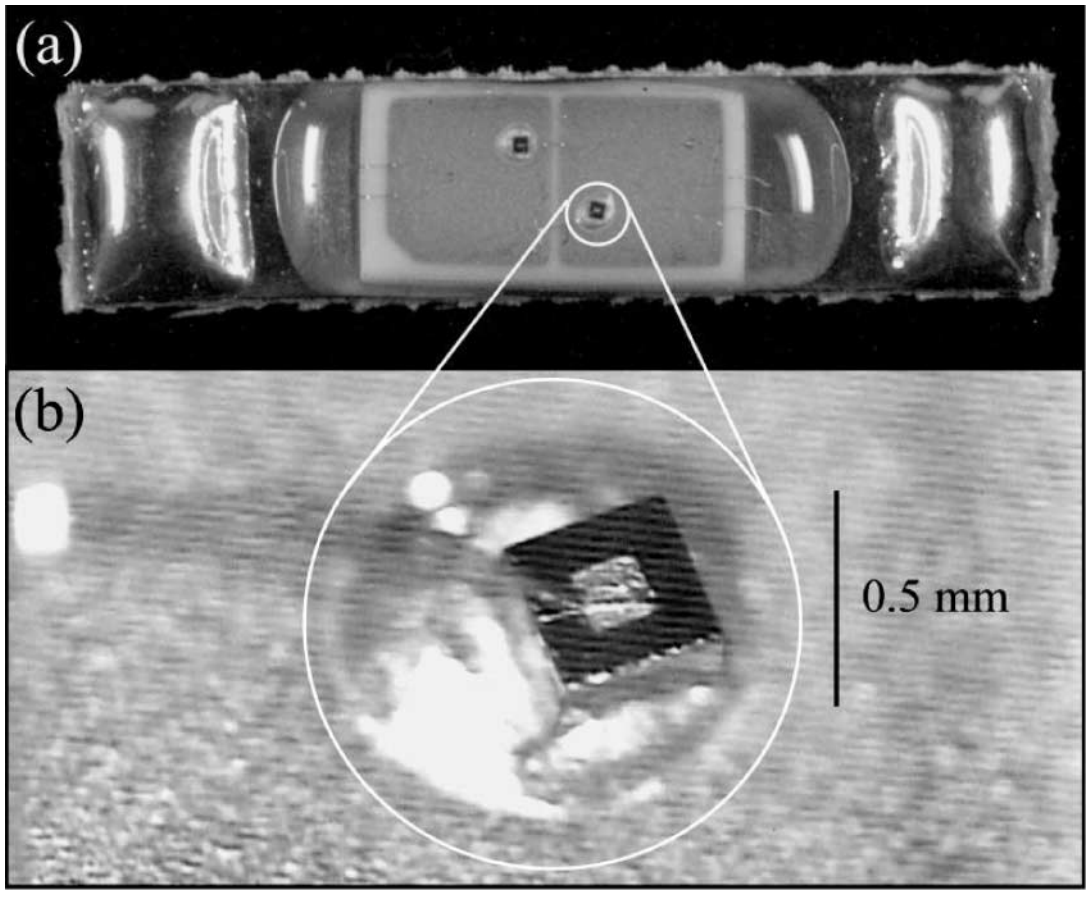

FIG. 3. Photograph of a crossed-diode. (a) A gold-coated ceramic substrate and 1N914 diode dice (small black squares) mounted on a copper-plated circuit board, and (b) enlargement of the diode die, which is adhered to the gold substrate with conductive silver epoxy and has an aluminum wire attached to the top face of the die.

diode dice (Semi Dice, South Easton, MA). The resonant circuit is mounted on top of the probe body with two semi-rigid copper coaxial lines connecting the circuit to two BNC connectors at the base of the probe. The variable capacitors and crossed-diodes were placed below the probehead to minimize magnetic susceptibility mismatching effects. Commercially available diodes could not be used in the resonant circuit because the nickel-coated wires are paramagnetic. The crossed-diodes used were fabricated in the Amy Facility for Chemical Instrumentation at Purdue University by attaching 1N914 diode dice to a gold film on a ceramic substrate (Al_2O_3) with conductive silver epoxy (Epoxy Technology Inc., Billerica, MA) (see Fig. 3). Ultrasonic wire bonding with 0.001-inch diameter aluminum wire was used to make electrical connections between the top of the diode dice and the gold sections. The gold pads were epoxied to FR-4 circuit board with 5-minute epoxy (Devcon, Danvers, MA), and 0.001-inch diameter aluminum wire was used to make electrical connections between the gold film and the copper layer of the circuit board. A protective coating of Syl-Gard (Dow Corning, Midland, MI) was applied over the diode dice and aluminum wires, and 60/40 rosin core solder was applied to the ends of the circuit board for connection into the resonant circuit.

Spectra were collected on a Varian INOVA spectrometer operating at 300 MHz for ^{1}H. Initial shimming of the two coils to a resolution of 2–3 Hz was achieved before the experiments were performed. The single pulse experiment was performed with a single $\pi/2$ pulse followed by a 0.82 second acquisition. A delay of 25 s between each scan was used and 4 scans were collected. Each spectrum was zero-filled twice, but no line broadening was applied.

RESULTS AND DISCUSSION

Two samples were loaded into the NMR Difference Probe with a syringe. One sample contained 1 M acetonitrile (Mallinckrodt Laboratory Chemicals) and 1 M methanol (Mallinckrodt Laboratory Chemicals) in D_2O (99.9% D, Cambridge Isotope Laboratories), and the other contained 1 M acetonitrile and 1 M ethanol (100%, Pharmco) in D_2O. All chemicals were used as received without further purification. Note that both samples contained the same concentration of acetonitrile.

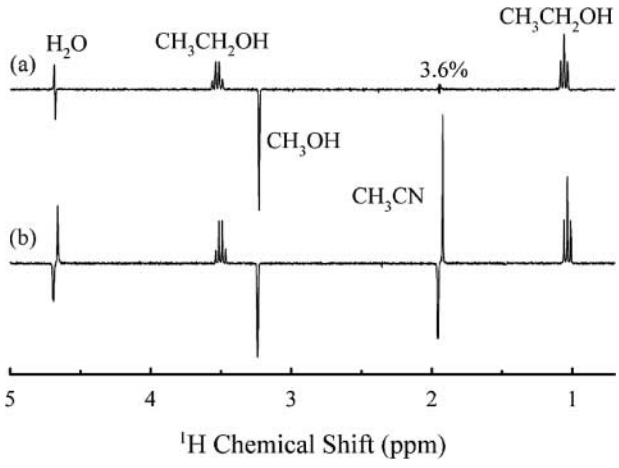

FIG. 4. Spectra acquired with the NMR Difference Probe with (a) no field gradient and (b) a small z1 static magnetic field gradient.

Figure 4a displays the difference spectrum of the two samples evidenced by the ethanol and methanol signals, which have opposite phase and appear as positive and negative peaks, respectively. Little signal is observed at the acetonitrile chemical shift (1.94 ppm) where the oppositely phased acetonitrile peaks from each sample overlap and their signals automatically cancel. For comparison, a second spectrum of the samples was acquired with a small, static z1 magnetic field gradient. With the applied gradient the acetonitrile peaks become visible as the gradient shifts the frequency of the peaks in opposite directions (see Fig. 4b). Compared to the height of the larger acetonitrile peak in the gradient shifted spectrum, the acetonitrile cancellation obtained in Fig. 4a leaves a residual signal of only 3.6%. No solvent suppression pulse sequence was applied to these spectra; the spectra are purely a result of signal cancellation through the circuit hardware. The S/N in Fig. 4a is 120 and the mass sensitivity (S/N per micromole of analyte) is 1600, which is a typical value for solenoid microcoils of this size and at this field strength. The S/N of a conventional difference spectrum computed in software is reduced by a factor of $1/\sqrt{2}$ compared to the individual spectra because the noise increases from the subtraction by a factor of $\sqrt{2}$ while the signals are unaffected (unless they are involved in cancellation). Similarly, the signal from the NMR Difference Probe is a subtraction, albeit through the hardware, and the noise introduced into the spectrum from both coils reduces the S/N by $\sqrt{2}$ compared to a spectrum collected with one coil.

The most critical factor in obtaining sufficient peak cancellation, whether through the hardware of the NMR Difference Probe or by subtraction of two spectra with software, is the lineshape. Thus, shimming is important not only to get the best resolution possible, but also to control the lineshape of the signals from both coils to obtain the best subtraction. Ideally, both coils should produce signals with comparable linewidths and lineshapes with the added stipulation of producing good resolution. In our experiments, the linewidths of the peaks during cancellation were 2.6–2.7 Hz. The lineshapes of the methanol and ethanol triplet peaks in Fig. 4a were simulated with a combination of Lorenztian and Gaussian lineshapes. The best fits for the methanol peak and the center peak of the ethanol triplet had a Gaussian fraction of >0.95 with linewidths in Fig. 4a of 2.62 and 2.69 Hz, respectively. The results indicate a good match between the lineshapes and linewidths of the signals from each coil. The Gaussian lineshape is a result of unoptimized shimming (compared to optimized shimming for each coil separately). Changing the z1 shim as in Fig. 4b alters the lineshape from >95% Gaussian to 50% Gaussian for the ethanol sample and to 100% Gaussian for the methanol sample. These changes are also reflected in the linewidth of the samples in the gradient shifted spectrum with 3.36-Hz linewidth for methanol and 1.57-Hz linewidth for the center peak of the ethanol triplet. Since shimming is a compromise between the best shim values of each coil, the lineshapes depend on how well the coils can be shimmed simultaneously.

Another factor that should be optimized in difference spectroscopy is the frequency match of the peaks being cancelled. With software cancellation of two spectra, a reference is used to match the frequency axes of the spectra before subtraction. In the NMR Difference Probe the coils are separated in space along the z-axis of the magnet. In addition to using the z1 shim to null axial field gradients across the coils, the z1 shim is used to correct for residual frequency shifts such that common peaks coincide for optimum cancellation. The spectra in Figs. 4a and 4b provide an example of the effect of using the z1 gradient to overlap the acetonitrile peaks for cancellation. It should be noted that the water peaks are not overlapped in the difference spectrum because the chemical shifts of water in the two samples differ. For the methanol sample, the chemical shift of water (with respect to acetonitrile) is 4.67 ppm (1402.4 Hz) and for the ethanol sample, it is 4.68 ppm (1403.6 Hz) because of hydrogen bonding between water and the alcohols. Separate experiments were performed to measure the water frequency as a function of the ethanol and methanol concentrations. As the concentrations of the alcohols were decreased, the chemical shifts of the water peaks converged (spectra not shown) indicating that multiple-solvent suppression is more suitable for lower analyte concentrations typical of LC/NMR or protein/ligand studies. Since the z1 shim is so important for obtaining narrow linewidths, using the shim to adjust the frequency may compromise the lineshape. In the future it may be possible to construct customized shim coils for the NMR Difference Probe that are matched to the dual-coil geometry and size in order to shim each coil individually.

CONCLUSION

A new NMR probe has been constructed that can obtain a difference spectrum directly through the hardware. By using crossed-diodes as switches in the resonant circuit of the dual-coil

probe, the parallel excitation of two samples and the serial acquisition of the NMR signals give a spectrum where the signals from the two samples are subtracted. No software manipulation, pulse sequence modification, or spectrometer alteration is necessary to obtain the difference spectrum and suppress common signals. The technique does not lengthen the pulse sequence and reduces experimental time compared to other NMR difference experiments, since a difference spectrum is automatically collected and no postprocessing subtraction is needed. It is anticipated that the NMR Difference Probe could make significant improvements in overcoming dynamic range problems with trace analyte detection, particularly if combined with existing suppression techniques, and in detecting analytes close to the solvent peak. When fully developed the NMR Difference Probe should provide sufficient background subtraction to allow for the use of lower grade solvents with impurities and multiple solvents for LC-NMR. The difference technique also provides suppression without obscuring the solvent frequency region or affecting the exchangeable proton signals, which is not easily accomplished with pulse sequence techniques. The NMR Difference Probe has several advantages over traditional solvent suppression because criteria such as the range of useful signals, pulse sequence duration, flat baseline, uniform excitation, and nonlinear phase corrections are inherently nonissues for the NMR Difference Probe since the solvent signal is subtracted, not suppressed. Although the subtraction currently achieved is modest, there are already a number of applications where a cancellation factor of 30 is useful, and our approach is compatible with a variety of solvent suppression pulse sequences and postprocessing techniques. The NMR Difference Probe is still in the developmental stage and improvements in the resolution and coil geometry of the difference probe are currently being investigated to increase the cancellation factor so that samples with lower concentrations and multiple solvents may be tested. The use of small nonmagnetic diode dice may also prove to be useful in a variety of NMR probe related circuits including radiofrequency switching and Q-spoiling applications.

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