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Communication

Sensitive, quantitative carbon-13 NMR spectra by mechanical sample translation

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ABSTRACT

Collecting a truly quantitative carbon-13 spectrum is a time-consuming chore. Very long relaxation delays, required between transients to allow the *z*-magnetization, M_z, of the spin with the *longest* T_1 to return to the equilibrium value, M_0, must precede each transient. These long delays also reduce sensitivity, as fewer transients per unit time can be acquired. In addition, sometimes T_1 is not known to within even a factor of two: a conservative guess for the relaxation delay then leads to very low sensitivity. We demonstrate a fresh method to bypass these problems and collect quantitative carbon-13 spectra by swapping the sample volume after each acquisition with a different portion where the magnetization is already equilibrated to M_0. Loading larger sample volumes of 10–20 mL into an unusually long (1520 mm) 5 mm OD. NMR tube and vertically sliding the tube between acquisitions accomplishes the swap. The relaxation delay can then be skipped altogether. The spectra are thus both quantitative, and far more sensitive. We demonstrate the moving tube technique on two small molecules (thymol and butylhydroxytoluene) and show good carbon-13 quantification. The gain in sensitivity can be as much as 10-fold for slowly-relaxing ^{13}C resonances. These experiments show that quantitative, sensitive carbon-13 spectra are possible whenever sufficient sample volumes are available. The method is applicable to any slow-relaxing nuclear spin species, such as ^{29}Si, ^{15}N and other low-γ nuclei.

1. Introduction

In a quantitative NMR spectrum, integrals of individual peaks (or spin multiplets) show the relative number of nuclei in the sample. Peak integrals add telltale information, and are useful in assignment or analysis. Quantitative NMR is common in proton NMR, but not for most other nuclei. Waiting for nearly full recovery of *z*-magnetization, M_z, to equilibrium magnetization, M_0, before each transient [1], is very insensitive with slowly-relaxing nuclei. For example, natural abundance ^{13}C has much lower sensitivity, and longer and more variable T_1 values than ^{1}H, so quantitative ^{13}C NMR requires much more experiment time than a quantitative ^{1}H NMR. Other impediments include the large and variable heteronuclear nuclear Overhauser enhancement (NOE) that, though it improves ^{13}C intensities of some peaks significantly, simultaneously removes the possibility of quantitative integrals. Quenching the NOE and reducing T_1 has been achieved by the addition of paramagnetic relaxation agents like chromium tris(acetylacetonate), $Cr(acac)_3$ [2] but such addition is undesirable when sample contamination or re-isolation are issues: the ^{13}C line widths are also increased somewhat. In addition, speeding up T_1 relaxation may be easier than completely quenching the NOE [3], yet both are required for quantitative NMR.

The inverse gated decoupling experiment [4], in which the protons are decoupled during acquisition (but not irradiated during the relaxation delay), gives a decoupled ^{13}C spectrum that can be quantitative if a sufficiently long relaxation delay is used. This sequence was accelerated somewhat by including an additional delay with proton decoupling and selective pulses at the beginning of the sequence to accelerate the establishment of equilibrium [5].

In this study we collect truly quantitative carbon-13 spectra using different means. A standard probe was modified to accommodate a very long 5 mm OD NMR tube. With this hardware, the long tube can be slid vertically once the data acquisition is complete, removing the sample portion containing residual non-equilibrium magnetization, and replacing it with a nearly fully equilibrated aliquot (see below). The result is a sensitive, quantitative spectrum in a fraction of the instrument time.

Mechanical sample translation has been implemented in other situations for other purposes. In the case of zero-field NMR, a polycrystalline sample was moved during an experiment from the magnet bore to a point outside of the bore where it was allowed to evolve under a zero-field Hamiltonian [6]. A similar principle inspired a shuttling device for liquid samples to allow the sample to relax or evolve at low magnetic field [7]. In the present case, the sample is not shuttled, but is instead used to mimic a stopped-flow setup, without all the problems that would accompany a flowing system.

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Optimizing precious magnet time has become a focus of research over the last few years. Previous work in high-throughput liquids NMR used a multiplex probe with multiple coils for simultaneous data acquisition of several different samples [8]. In solid-state NMR, a magic-angle spinning (MAS) probe with several stators was developed that can acquire several spectra in rapid succession [9]. Our approach shares elements from the second design, which involved moving the stators vertically to acquire MAS spectra with better sensitivity. Using it, quantitative spectra for two organic molecules (thymol and butylhydroxytoluene, or BHT) were collected using the moving tube technique. When the T_1 times are long, as for quaternary carbon-13 spins, the moving tube technique enhances carbon-13 sensitivity by a large factor in addition to making the spectrum quantitative.

The moving tube technique is suitable in situations where there is enough sample quantity to make approximately 10–20 mL of solution, and quantification is required to characterize the relative amounts of various sample components. Such situations include metabolite quantification [10], food analysis [11–13], and petrochemical applications [14,15], to name a few. Furthermore, the moving tube technique could be applied to improve experiments other than simple quantitative 1D carbon-13 NMR. Examples include a faster inversion recovery experiment [16] or a larger sample volume to study an insoluble compound that would otherwise be impossible in the sample volume afforded by a conventional tube within instrument time constraints. Many 2D and 3D experiments can, of course, benefit from application of this idea.

2. Materials and methods

The moving tube experiments were performed with an outdated 4-channel (^{1}H, ^{13}C, ^{31}P, and ^{2}H) surplus NMR probe designed for ^{13}C/^{31}P observation, proton decoupling, and deuterium lock. The ^{1}H (192) and ^{13}C (21) sensitivity were determined using the appropriate ASTM sensitivity standards. To modify the probe, the variable temperature dewar was removed, creating a hollow passage down its center that allows the tube to slide unimpeded along the axis of the magnet. Fig. 1 shows a simple two-part Delrin sleeve used to hold the tube in the probe. The sleeve was secured in the bottom plate of the probe where the dewar formerly resided. It provided just enough friction to hold the tube in place under its own weight, while still allowing it to move freely enough under a light applied force, so that it could be moved between transients.

Thymol and BHT (Sigma–Aldrich, St. Louis, MO) were used as test samples. Each was dissolved in deutero-chloroform ($CDCl_3$) and then loaded into a 1520 mm long tube (New Era Enterprises, Vineland, NJ) of approximately 20 mL volume. The tubes were fitted with vortex plugs to minimize any liquid motion within the tube. Sample spinning was not employed in any of the experiments, and all data were obtained at ambient lab temperature without the usual temperature control.

Spectra were collected using the ^{13}C inverse gated decoupling pulse sequence [4] using low-power WALTZ-16 decoupling [17] during acquisition to minimize unwanted dielectric heating. The ^{13}C transmitter frequency was set to approximately 87 ppm and the ^{1}H decoupler frequency was set to approximately 4.9 ppm.

To isolate the effect of moving the tube, pairs of experiments were run in tandem. In the control, "stationary tube" experiment, the same long tube and large sample were used, but held stationary, to simulate a conventional tube. Aside from minor translational diffusion of spins from volume elements outside the receiver coil, or unwanted fluid convection by decoupler dielectric heating, the results should match those of a conventional, short, stationary tube. Where they may differ, the long stationary tube should show an advantage compared to the traditional arrangement, making it a valid control. The relaxation delay was 1.0 s and the acquisition time with decoupling was 0.512 s. These were chosen to be 'typical' parameters that one may use for a carbon-13 experiment, and to circumvent any fluid convection that might occur in the absence of active VT control of the sample temperature. In moving tube experiments, the tube was slid vertically between scans after each data acquisition was complete. In every case at least 16 dummy scans were executed, to mimic steady-state acquisition conditions. Data from the stationary and moving tube experiments were compared to illustrate the result of moving the tube.

For the moving tube experiments, the tube was displaced manually by ~3 in. between each scan. This should remove all non-equilibrium samples from the coil, replacing it with the next sample aliquot. As the decoupler coil is somewhat larger than the receiver coil in direct-detection probes, it is important to avoid inadvertent NOE build-up in the next sample volume by the decoupling during acquisition of the previous one.

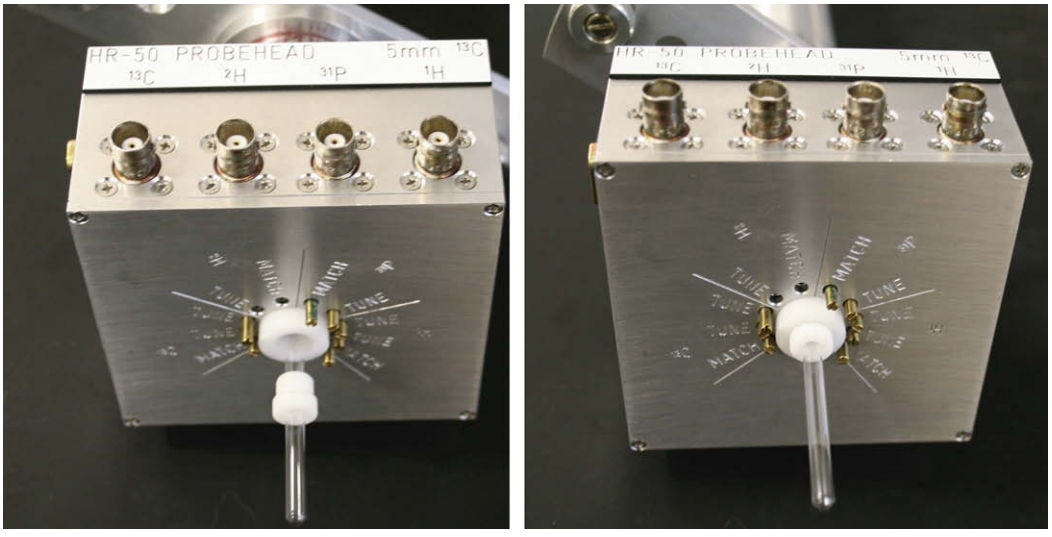

Fig. 1. Tube sleeve protruding from the bottom plate of the four-channel probe. On the left, the tube sleeve is unscrewed from the larger piece that is used to secure the sleeve in the probe. On the right, the sleeve is screwed into place. The sleeve was designed so that the tube could be inserted through the larger piece before it was placed on the tube and secured. When the parts are screwed together, the tube remains in place in the probe under its own weight.

All experiments were conducted on a 500 MHz Oxford 500/51 narrow bore unshielded magnet. Fig. 2 shows a plot of the magnetic field of this magnet as a function of axial distance from center line. An unshielded magnet is advantageous for moving tube experiments, as its larger fringe field provides a greater spatial region in which to equilibrate the incoming sample. An aliquot equilibrated in the fringe magnetic field within 3 in. of the center field experiences a B_0 strength that is 99.5% of that in the spatial region interrogated by the probe ^{13}C coil. This ensures that each subsequent sample aliquot will arrive at the receiver coil with nearly full equilibrium magnetization.

Moving the sample tube too far can result in a sensitivity loss, as polarization has equilibrated in a somewhat weaker field, meaning that M_z at the time of the read pulse will be less than if it were allowed to equilibrate completely in the strongest part of the field. This sensitivity loss affects all carbons equally (regardless of their T_1). For example, 5.12 in. from the magnet center, the fringe field is 10.56 T, versus 11.74 T at the receiver coil position and M_z is reduced 10%.

Additionally, moving the tube too slowly can result in a quantification loss if the sample is equilibrated in a significantly weaker part of the fringe field, and allowed to relax further (but not completely equilibrate) in the nominal field. Carbon spins with different T_1 values will relax at different rates, leading to different values of M_z. For example, if the tube is instantaneously moved 5.12 in., and then the transported liquid resides in the coil for 1 s before the read pulse, a simple calculation predicts an approximately 5% loss of quantification between two carbon sites with 1 s and 10 s relaxation times. A 5% loss of quantification is of the order of the accuracy of the measured integrals in most cases. Transporting the tube rapidly and then applying the pulse without further delay, lowers this figure further. Moving the tube more slowly also does the same thing, although it of course leads to no improvement in sensitivity and hence is not an option.

3. Results

Figs. 3 and 4 demonstrate how moving the sample tube impacts relative peak integrals by comparing thymol and BHT spectra from stationary, and moving, tube experiments. Table 1 lays out the results by comparing the expected and observed peak integrals for each of these molecules in stationary and moving tube spectra. The raw signal-to-noise ratio (SNR) should be sufficient to allow integrals to be evaluated to better than 1%. Slight errors in baseline correction, phasing, choices in the upper and lower cutoffs for the integration region, and finite digitization errors will all lead to larger error bars than the SNR-limited estimate. Yet, except for a couple exceptions, the moving tube experiments enjoy a match between expected and observed peak intensities to within 5%. By contrast, intensity distortions in the stationary tube experiments were as large as 50%.

The potential for sensitivity enhancement was explored by increasing the relaxation delay (0.25, 1.0, and 4.0 s) in stationary tube experiments, and comparing the results to a moving tube experiment with the same number of transients. The SNR of each spectrum was determined for the $CDCl_3$ triplet, which has a long T_1. Fig. 5 illustrates the improvement. Stationary tube spectra show

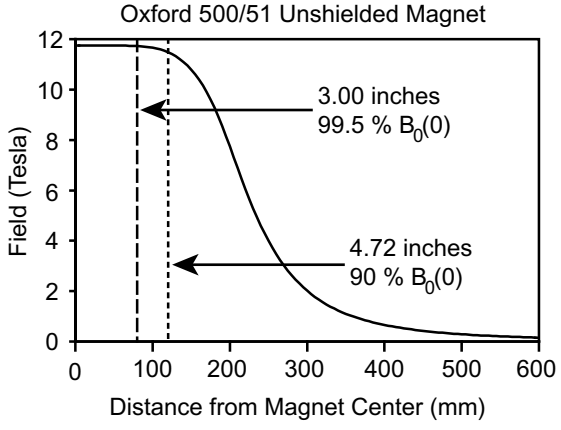

Fig. 2. Magnetic field plot for the unshielded Oxford 500/51 magnet used to conduct the moving tube experiments. The field is symmetrical with respect to the vertical ($z = 0$) axis. The plot shows two positions from center (3.00 and 4.72 in.) with fringe fields of 99.5% B_0 and 90% B_0, respectively. The tube can be moved more than ±3 in. and the incoming sample portion will still be at nearly full equilibrium magnetization. This would require a relaxation $>5T_1$ (for the most slowly-relaxing site) in the stationary tube case. At ±4.72 in., there may be a slight reduction in the sensitivity, although the quantitative nature of the experiment should still be excellent.

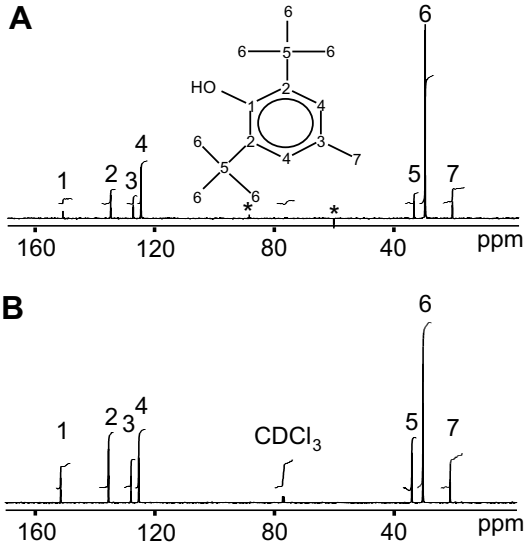

Fig. 3. (A) Stationary tube BHT spectrum. (B) Moving tube BHT spectrum. The assignment of the carbons is indicated. Integrals are superimposed on each peak. The relative integrals are laid out in Table 1.

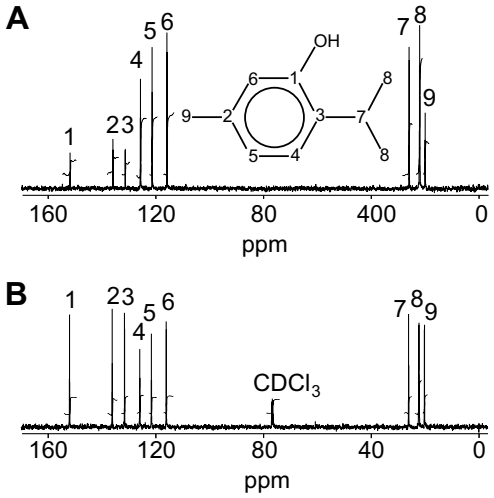

Fig. 4. (A) Stationary tube thymol spectrum. (B) Moving tube thymol spectrum. The assignment of the carbons is indicated. Integrals are superimposed on each peak. The relative integrals are laid out in Table 1.

Table 1
Expected and observed ^{13}C peak integrals.

Thymol/^{13}C peak	Expected	Stationary	Moving
C1	1.0	0.30	0.98
C2	1.0	0.40	0.87
C3	1.0	0.30	0.98
C4	1.0	1.30	1.10
C5	1.0	1.40	0.98
C6	1.0	1.50	0.98
C7	1.0	1.20	0.98
C8	2.0	2.80	2.10
C9	1.0	0.80	1.10
BHT/^{13}C peak	Expected	Stationary	Moving
C1	1.0	0.30	0.96
C2	2.0	0.91	2.10
C3	1.0	0.45	0.96
C4	2.0	2.90	2.10
C5	2.0	0.76	1.80
C6	6.0	8.60	6.00
C7	1.0	1.30	1.10

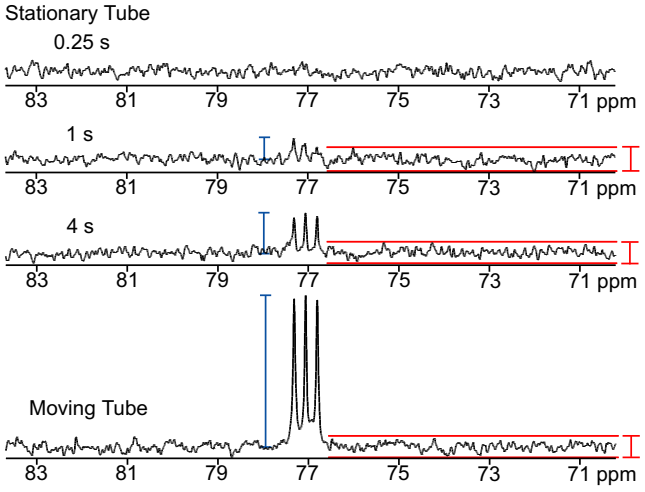

Fig. 5. Comparison of $CDCl_3$ SNR in three stationary tube experiments (with relaxation delay values of 0.25 s, 0.1 s, and 4 s, respectively) and a moving tube experiment. A relaxation delay of 0.25 s yields a signal-to-noise ratio lower than unity (a peak cannot be visualized from the noise) while the moving tube experiment gives the highest SNR, approximately four times that achieved using the 4 s relaxation delay.

increasing raw SNR with increasing relaxation delay. The moving tube spectrum shows an approximately fourfold increase from the control with the longest relaxation delay (4 s). The improvement per unit time is, of course, potentially much larger than four, because many additional transients can be acquired (rather than an identical number) depending on the rapidity with which the tube can be moved and stopped.

We should remark that irreproducible shimming might seem like a potential roadblock. The shimming was examined by measuring the line widths of various peaks along the length of the tube in a series of stationary tube experiments. The change in full width at half maximum was less than 0.2 Hz over the entire tube, showing that the shimming is preserved when the tube is moved through the guides. This issue would be more problematical in multidimensional applications or difference spectroscopy, where changes in intensity might introduce excessive "t_1–noise" [18] or other artifacts. (of course, irreproducible z-magnetization from transient to transient, depending on the order in which the phase cycle is executed and how fast the repetition rate is pushed, also influence the amount of t_1–noise and/or the artifact intensity [19].) The consistent shimming reflects well on the reproducible camber and eccentricity of the tube itself. The 1520 mm tubes seem to maintain a reasonably consistent magnetic profile throughout their entire length.

4. Discussion

The moving tube technique was shown to give sensitive, quantitative small molecule carbon-13 NMR spectra that would normally require very long relaxation delays to achieve reliable integrals. Although a flow NMR cell could also potentially deliver a virtuously endless supply of new sample, there are a different set of technical hurdles to overcome in the flow case. In particular, the moving tube method is well-suited to modern pulsed field gradient experiments, as there is little concern that convection or turbulent flow can arise. Even the slightest eddy current in the fluid sample could destroy the delicate spatial helices of magnetization that are wound by the application of strong linear pulsed field gradients. In multidimensional NMR it may be possible to complete each step in a phase cycle over an identical part of the tube, improving the potential for reproducible cancellation of unwanted signals. Many different scenarios can be envisioned, and we will report on a number of them in the near future. When magnet time is more limited than sample size, the moving tube method offers an attractive way to improve NMR throughput.

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