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Triple Resonance Isotope-Edited (TRIED): A Powerful New NMR Technique for Studying Metabolism

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Summary

A powerful new technique for studying metabolism, Triple-Resonance Isotope-Edited (TRIED) NMR, is described and demonstrated experimentally. The experiment uses through-bond (scalar) coupling between spins to select NMR signals from molecules having ^{1}H-^{13}C-^{15}N labeled triples. Natural-abundance background signal from molecules not containing such labeled triples (e.g., signal from ^{1}H-^{12}C and ^{1}H-^{13}C fragments) is effectively suppressed, with a suppression ratio of approximately 10^{4}:1. This effective filtering of signal from labeled molecules allows these species and their metabolites to be detected and characterized without requiring extensive, time-consuming sample purification. Results are presented for ^{13}C,^{15}N-aminomethylphosphonate (AMPA) in soybean and pigweed plant matrices. The potential use of TRIED for studying the pharmacokinetics of drugs and extension of the experiment to include the use of magnetic-field gradients for selecting signal from labeled fragments are also described.

Key Words: Nuclear magnetic resonance, carbon-13, nitrogen-15, stable isotopes, metabolism, pharmacokinetics.

Introduction

Radioactive and stable labels are used in all phases of drug discovery, development, and registration[1]. Stable isotopes are particularly attractive as they require none of the regulatory overhead associated with radiolabels. More generally, radioisotope labeling and detection, combined with chromatographic separation, provide the traditional method for studying metabolism in a wide variety of biological systems[2]. These techniques take advantage of the extremely high sensitivity of radioisotope detection. However, there are a number of significant

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drawbacks to radioisotope methods. Since these methods measure how much radioactivity is present, but cannot provide any product identification, extensive sample purification and preparation are required. In addition, the high cost of disposal, both for radioisotopes themselves and for plant and/or animal material from these studies, is driving the search for alternate methods of studying metabolism. In principle, stable isotope labels and high-resolution nuclear magnetic resonance (NMR) spectroscopy provide such an alternative.

NMR is a powerful method for characterizing the structure and dynamics of molecules, both in solution[3] and in the solid state[4]. One of NMR's great strengths is its ability to provide direct, functional group identification and analysis. As a consequence, NMR is well suited to the study of complex mixtures of materials. However, NMR's relatively low sensitivity and its dependence on sample purity have traditionally limited its usefulness in studying metabolism, where the quantities of material available are generally quite small. Recent progress in a number of areas have combined to make NMR spectroscopy a viable technique for metabolism studies. The recent development of indirect detection methods, which allow ^{1}H detection of heteronuclear (i.e., ^{13}C, ^{15}N) spins, has greatly improved the NMR's sensitivity[5,6,7]. Together with improvements in the sensitivity of NMR probes, improved radio-frequency (rf) electronics, and enhanced computers and computing methods, these indirect methods have lowered NMR detection limits sufficiently to make metabolism studies possible.

We describe here a new NMR method, Triple-Resonance, Isotope-EDited NMR, TRIED, which combines high-sensitivity, proton detection with a technique for suppressing background NMR signals to produce a method capable of use in metabolism studies. In TRIED experiments, small amounts of labeled material can be detected and identified. The presence of large, natural-abundance background signals poses a major challenge. TRIED was specifically developed to allow detection and identification of signals from isotopically-enriched molecular fragments while suppressing these background signals. This effective filtering of signal from labeled molecules allows these species and their metabolites to be detected and characterized without requiring extensive, time-consuming sample purification. Thus, TRIED can provide considerable savings in both the time and cost of performing metabolism studies.

TRIED NMR, patterned after experiments designed to selectively detect proton signals from ^{1}H-^{13}C spin pairs[8], is based on triple-resonance methods developed for studying ^{13}C,^{15}N-labeled peptides and proteins[9,10,11] and nucleic acids[12]. As a one-dimensional filtering experiment, TRIED is actually simpler than many of these multidimensional (e.g., 3-D, 4-D), triple-resonance methods, which generally contain multiple time-evolution periods. The method takes advantage of the through-bond scalar (J) coupling between bonded spins and, specifically, selects signals from labeled ^{1}H-^{13}C-^{15}N triples, while suppressing signals from both ^{1}H-^{12}C and ^{1}H-^{13}C

fragments. Stark, et al. have recently described a method for assigning resonances in isotopically-enriched proteins based on filtering for ^{1}H-^{13}C-^{13}C fragments[13]. Since the natural-abundance levels of ^{13}C and ^{15}N are small (0.011 and 0.0037, respectively), the probability of a ^{13}C-^{15}N labeled spin pair in natural abundance is small (~1:25,000). As we demonstrate below, a suppression of natural-abundance signal of ~10^4 *can* be achieved using TRIED. This level of natural-abundance signal suppression represents an approximately 300-fold improvement over methods which select for ^{1}H-^{13}C pairs alone[8].

Materials and Methods

NMR. All NMR experiments were performed at 30 °C on a Unity-500, triple-channel NMR spectrometer (Varian Associates) equipped with a 3-mm, triple-resonance (H,C,N) probe (Nalorac, Inc.). This 3-mm probe provides high sensitivity and improved rf performance and requires a sample volume of only 125 µl. D_2O was the solvent in all of the NMR experiments.

Samples. [^{13}C,^{15}N]Aminomethylphosphonate (AMPA) (99 atom% ^{13}C; 99 atom% ^{15}N) was supplied by MSD Isotopes and was used as received. The soybean plant matrix was prepared by cryogrinding soybean seeds, hulls, stalks and dried leaves in a high-speed blender. Labeled AMPA was then mixed with this soybean matrix.

For the metabolism work, pigweed seeds were germinated on moist filter paper in the dark at 25 °C. After 3 days, seedlings were treated by topical application of [^{13}C,^{15}N]AMPA (2.5 mg/ml). Treated seedlings were placed back in the dark at 25 °C for 7 days. At harvest, the seedlings were placed in a Buchner funnel and washed with water followed by methanol. Seedlings were homogenized in 50 mM phosphate buffer, pH 2.0 (1/1, w/v) using a Polytron homogenizer. Homogenates were clarified by centrifugation and lyophilized overnight to a dry brown powder.

Results and Discussion

As described in the introduction, TRIED is a triple-resonance experiment which takes advantage of the scalar coupling between directly bonded spins. Figure 1 shows the pulse sequence for the TRIED experiment, which includes several rf pulses on each of 3 rf channels (^{1}H, ^{13}C, and ^{15}N). TRIED involves a total of 4 polarization transfer steps, as summarized in 1:

$$^{1}H \text{ --> } ^{13}C \text{ --> } ^{15}N \text{ --> } ^{13}C \text{ --> } ^{1}H \text{ (Acquire data)} \qquad 1$$

Patterned after multidimensional NMR experiments developed for studying ^{13}C,^{15}N-labeled peptides, proteins, and nucleic acids[9-12], the fixed time delays within the TRIED sequence are selected to maximize these polarization transfers.

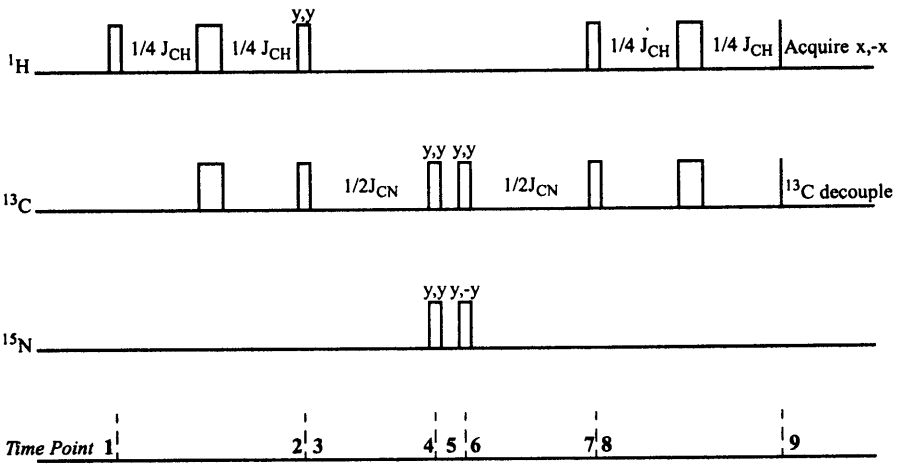

Figure 1. TRIED NMR pulse sequence.

The TRIED experiment begins with a $\pi/2$ pulse applied to the proton spins. The proton magnetization is then sequentially transferred, through an INEPT-like series of polarization transfers[14], from proton to carbon to nitrogen, then back to carbon and finally to proton for signal acquisition. This coherence transfer pathway is only operative in molecules having ^{1}H-^{13}C-^{15}N labeled triples and the phases of the rf pulses and receiver are shifted to select only signal which has followed this particular pathway. The resulting proton data can be collected either ^{13}C-coupled or ^{13}C-decoupled. (The pulse sequence in Figure 1 shows the ^{13}C-decoupled version of the TRIED experiment.)

The spin operators describing the spin system at each stage of the TRIED experiment are listed in Table 1. These operators were calculated assuming only 1-bond ^{1}H-^{13}C, and ^{13}C-^{15}N couplings and ignoring 2-bond ^{1}H-^{15}N and ^{1}H-^{13}C couplings. The column labeled *Time Point* in this table refers to points labeled on the TRIED pulse sequence of Figure 1. Column 2 of Table 1 shows the spin operators during the TRIED sequence for a ^{1}H-^{13}C-^{15}N triple, column 3 the corresponding spin operators for an ^{1}H-^{13}C spin pair. TRIED's ability to select signal from ^{1}H-^{13}C-^{15}N triples *only* is evident from examining the last row in this table, where it is seen that the detected ^{1}H NMR signal for these triples inverts with the phase of the second nitrogen pulse, while signal from ^{1}H-^{13}C spin pairs (and non-^{13}C-coupled protons) is independent of the phase of this pulse. Alternation of the receiver phase leads to cancellation of signal from all but ^{1}H-^{13}C-^{15}N spin triples.

Table 1. TRIED Spin Operators

Time Point	Spin Operator (^1H-^{13}C-^{15}N triple)	Spin Operator (^1H-^{13}C pair)
1	H_z	H_z
2	$-2H_xC_z$	$-2H_xC_z$
3	$-2H_zC_y$	$-2H_zC_y$
4	$-4H_zC_xN_z$	$-2H_zC_y$
5	$4H_zC_zN_x$	$-2H_zC_y$
6	$-4H_zC_xN_z$ / $4H_zC_xN_z$	$-2H_zC_y$ / $-2H_zC_y$
7	$2H_zC_y$ / $-2H_zC_y$	$-2H_zC_y$ / $-2H_zC_y$
8	$2H_xC_z$ / $-2H_xC_z$	$-2H_xC_z$ / $-2H_xC_z$
9	H_y / $-H_y$	H_y / H_y

Figure 2 shows conventional proton and TRIED NMR experiments of a sample of 12 μg of ^{13}C,^{15}N-labeled aminomethylphosphonate (AMPA) [H_2PO_3-$^{13}CH_2$-$^{15}NH_2$] in a matrix of natural-abundance glycine (2331 μg) and ^{13}C-enriched glycine (672 μg). Signals from glycine moieties, which dominate the conventional ^1H NMR spectrum, are effectively suppressed in the TRIED experiment. This result clearly demonstrates the effectiveness of TRIED in selecting signals only from molecules having labeled ^1H-^{13}C-^{15}N triples and suppressing signals from both ^1H-^{12}C and ^1H-^{13}C fragments.

Figure 3 shows spectra of this same labeled AMPA in a soybean plant matrix. Natural-abundance signals from the matrix totally swamp the signal from the labeled AMPA in the conventional ^1H spectrum of this sample (Figure 3, bottom). In fact, the presence of labeled material is not evident in this figure. By contrast, the TRIED spectra of this sample, shown in Figure 3, middle (^{13}C coupled), and Figure 3, top (^{13}C decoupled), are dominated by signal from the methylene protons in labeled AMPA. The effective suppression ratio in these spectra is of the order of 10^4:1, consistent with the calculated value derived from natural abundance levels of ^{13}C and ^{15}N.

Figure 4 directly demonstrates the power of TRIED for studying metabolism in plant systems. Here a solution of labeled ^{13}C,^{15}N-AMPA was applied topically to the leaves of growing pigweed plants. Three days later, the plants were harvested and an extract was prepared as described above. Figure 4 (bottom), the conventional ^1H NMR spectrum of this extract, is dominated by background signals from the plant matrix. The TRIED experiment, shown in Figure 4 (top), shows signals due to ^{13}C,^{15}N-AMPA and a number of metabolites derived from the labeled AMPA. The presence of these additional metabolite signals was confirmed by Bayesian

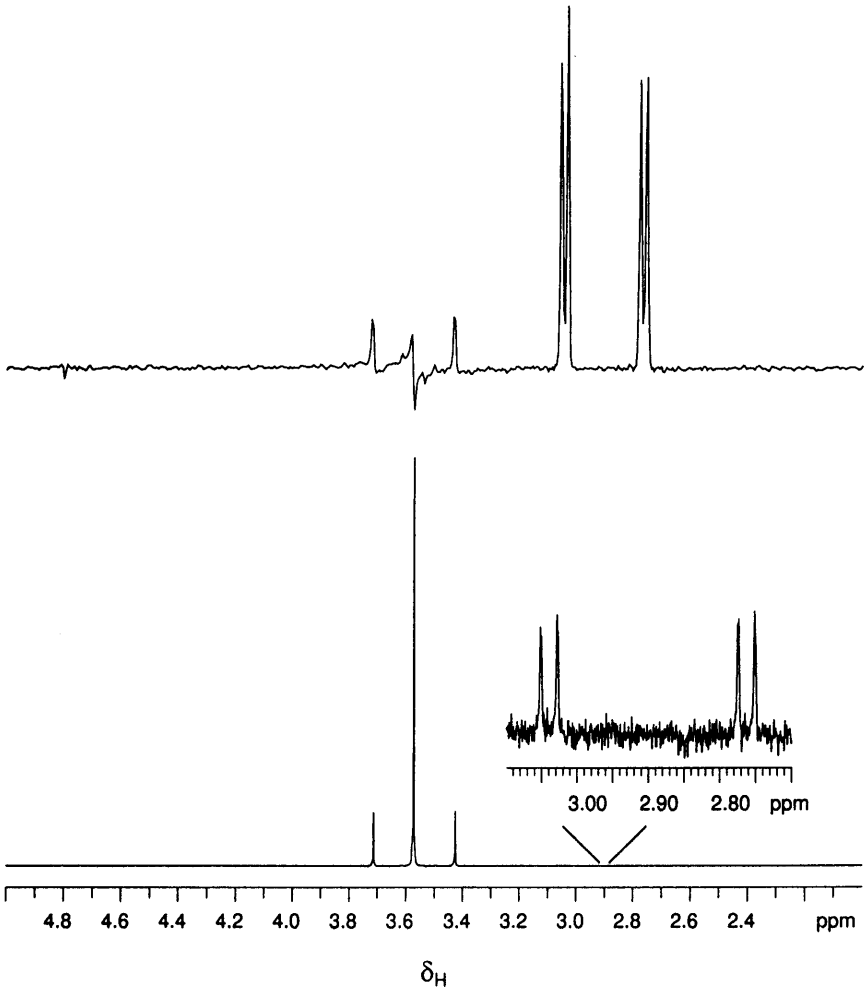

Figure 2. Conventional proton (bottom) and TRIED NMR (top) spectra of a sample of 12 μg of $^{13}C,^{15}N$-labeled aminomethylphosphonate (AMPA) [H_2PO_3-$^{13}CH_2$-$^{15}NH_2$] in a matrix of natural-abundance glycine (2331 μg) and ^{13}C-enriched glycine (672 μg).

Probability theory analysis of these data[15,16], though they remain unidentified. The TRIED sequence illustrated in Figure 1 is a single-quantum sequence, with only 1-quantum coherences present in the density operator during its evolution (Table 1).

In the hope of enhancing the sensitivity of the experiment, two different multiple-quantum versions of TRIED, achieved through simple modification of the pulse sequence in Figure 1, were also examined. However, these sequences caused unwanted complications in the TRIED spectra (e.g., distorted ^1H lineshapes; ^1H signals whose intensities depend upon the ratio J_{CH}/J_{CN}) and did not produce any improvements in signal-to-noise.

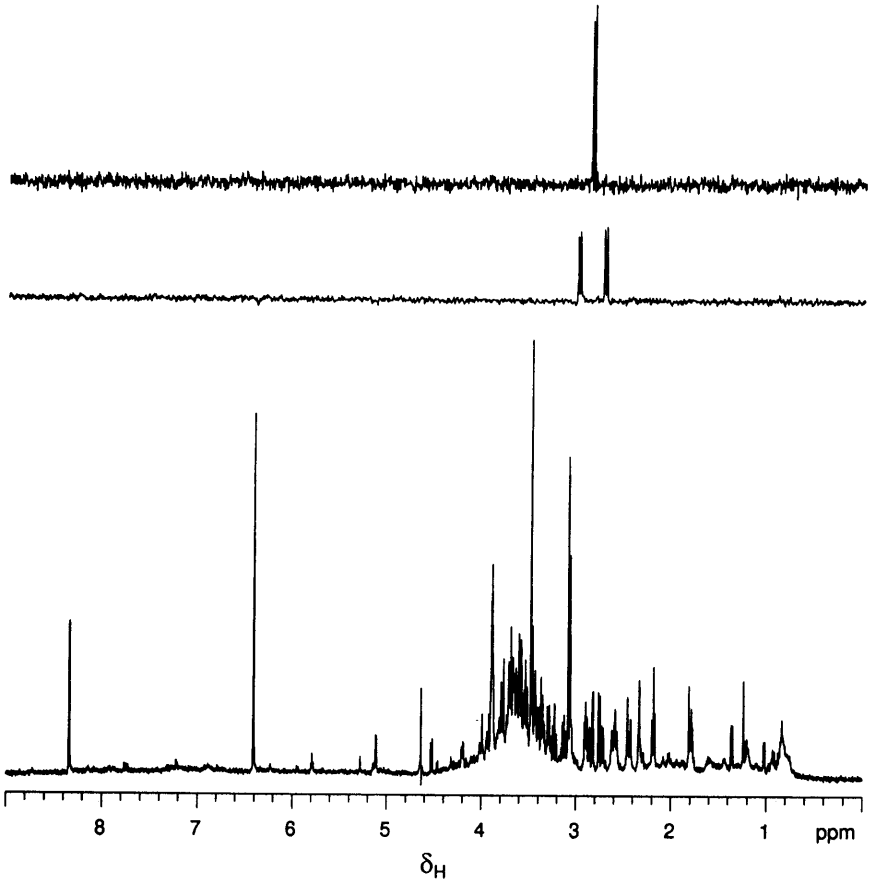

Figure 3. Conventional proton (bottom) and TRIED NMR (middle, top) spectra of $^{13}C,^{15}N$-labeled AMPA in a soybean plant matrix. The conventional ^{1}H spectrum was collected with presaturation to reduce signal from residual HOD solvent protons. The TRIED spectrum shown in the middle panel was collected without ^{13}C decoupling while the spectrum in the top panel was collected with ^{13}C decoupling. In the conventional ^{1}H spectrum, natural-abundance signals from the matrix completely mask the signal from the labeled AMPA. By contrast, the TRIED spectra of this sample are dominated by signal from the methylene protons in labeled AMPA. The effective suppression ratio in these spectra are of the order of 10^4:1.

Conclusion

We have described a new NMR technique, Triple-Resonance, Isotope-Edited NMR, and have demonstrated its effectiveness in detecting signals from molecules containing ^{1}H-^{13}C-^{15}N fragments while suppressing background signals from unlabeled materials. We anticipate this experiment will become an important tool in helping to establish NMR spectroscopy as a technique for studying metabolism. By filtering background signals, TRIED eliminates the need

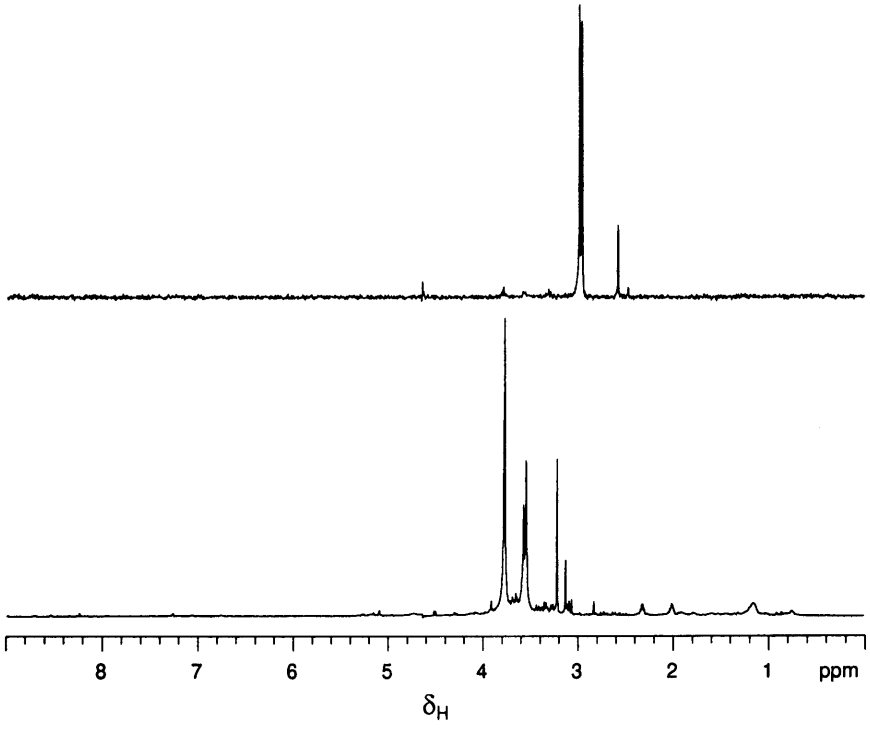

Figure 4. Conventional proton (bottom) and TRIED NMR (top) spectra of pigweed extract from plants treated topically with $^{13}C,^{15}N$-labeled AMPA. The conventional ^{1}H spectrum was collected with presaturation to reduce signal from residual HOD solvent protons. The TRIED spectrum shown in the top panel was collected with ^{13}C decoupling.

for costly, time-consuming sample purification. The TRIED methodology described in this work can clearly be extended to pharmacokinetics study of drug metabolism in animals[1]. We are currently exploring the use of magnetic field gradients, rather than phase cycling, for achieving the necessary discrimination of signal from double (i.e., ^{1}H-^{13}C) and triple (i.e., ^{1}H-^{13}C-^{15}N) labeled metabolites.

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