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Communication

Resolving ambiguities in two-dimensional NMR spectra: the 'TILT' experiment

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Abstract

Ambiguities in two-dimensional nuclear magnetic resonance spectra due to overlap are usually resolved by recording a three-dimensional version of the experiment. It is shown that a simpler solution is to record a tilted projection of the three-dimensional spectrum, derived by Fourier transformation of the time-domain signal acquired while the two evolution parameters are varied simultaneously at the appropriate rates. By avoiding the need to record the full three-dimensional spectrum, this saves an order of magnitude in measurement time. The tilt technique is illustrated by reference to degenerate responses in the TOCSY and NOESY spectra of a small protein, agitoxin, where the ^{1}H and ^{15}N frequencies are incremented in tandem.

Keywords: Resolving ambiguities; Tilted projections; Two-dimensional NMR

1. Introduction

Two-dimensional NMR spectroscopy [1,2] has been applied to a wide range of molecular structure problems by identifying interacting chemical sites, displaying the results as correlation peaks in two frequency dimensions. Occasionally ambiguities arise, caused by overlap of two or more responses. The traditional remedy has been to introduce a third frequency dimension where the degenerate frequencies are separated. The penalty is a longer measurement time—between one and two orders of magnitude, depending on the fineness of digitization in the new time dimension.

This communication suggests a simpler and much quicker solution to the overlap problem. We can think of the two-dimensional spectrum as the projection of a hypothetical three-dimensional spectrum $F_1F_2F_3$ onto the F_1F_3 plane, obtained by setting the evolution parameter $t_2 = 0$. Any overlap of responses in this plane can be separated by tilting the projection, achieved by incrementing t_2 in tandem with t_1 [3–8]. In real life, this is analogous to viewing a scene where two objects of interest happen to be eclipsed; we naturally move a short distance laterally to get a better point of view. In situations where providence has made an inconvenient choice of NMR frequencies, a tilted projection allows us to rectify her mistakes.

2. Tilted projections

There is a well-established theorem that relates the Fourier transform of a section through a time-domain signal to the corresponding projection in the frequency domain [9,10]. Suppose the evolution parameters t_1 and t_2 are incremented jointly, and the time-domain data collected in a plane tilted at an angle α defined by $\tan\alpha = \Delta t_2/\Delta t_1$ (Fig. 1A). The theorem states that the Fourier transform of this data set is the projection of

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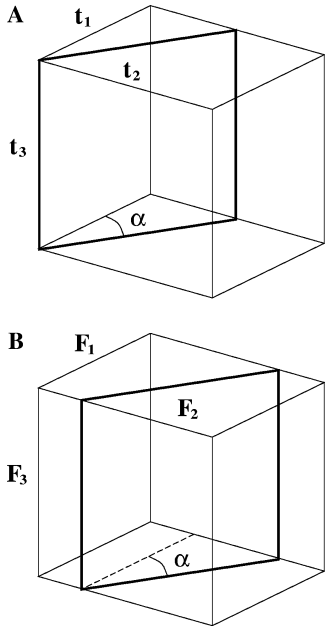

Fig. 1. Fourier transformation of time-domain data recorded in the tilted plane (A) generates a projection of the frequency-domain spectrum onto a similarly tilted plane (B). The tilt angle α is given by $\tan\alpha = \Delta t_2/\Delta t_1$ where Δt_1 and Δt_2 are the time increments in the linked evolution dimensions.

the three-dimensional spectrum onto a plane inclined at the same angle α in the frequency domain (Fig. 1B). An NMR frequency in this tilted plane is displaced by an amount determined by the tilt angle and the corresponding F_2 frequency (normally that of a heteronuclear species, for example ^{13}C or ^{15}N). Suppose two resonances are degenerate in a two-dimensional proton spectrum. By exploiting the difference between their ^{15}N frequencies, the TILT spectrum resolves the ambiguity. If Ω_H is the common proton frequency and Ω_N and Ω_N^* the corresponding resolved ^{15}N frequencies, then the peak positions in the tilted projection are:

$$\Omega = \Omega_H \cos\alpha + \Omega_N \sin\alpha, \tag{1}$$

$$\Omega^* = \Omega_H \cos\alpha + \Omega_N^* \sin\alpha. \tag{2}$$

Consequently, the choice of a suitable tilt angle depends on how well Ω_N and Ω_N^* are separated. (Note that because peak positions in the tilted projection are 'contaminated' with frequencies from the heteronuclear species, it is no longer possible to express the corresponding chemical shift scale in parts per million.)

In this manner, peaks that were originally overlapping can be separated by a single additional measurement, rather than by recording the entire three-dimensional

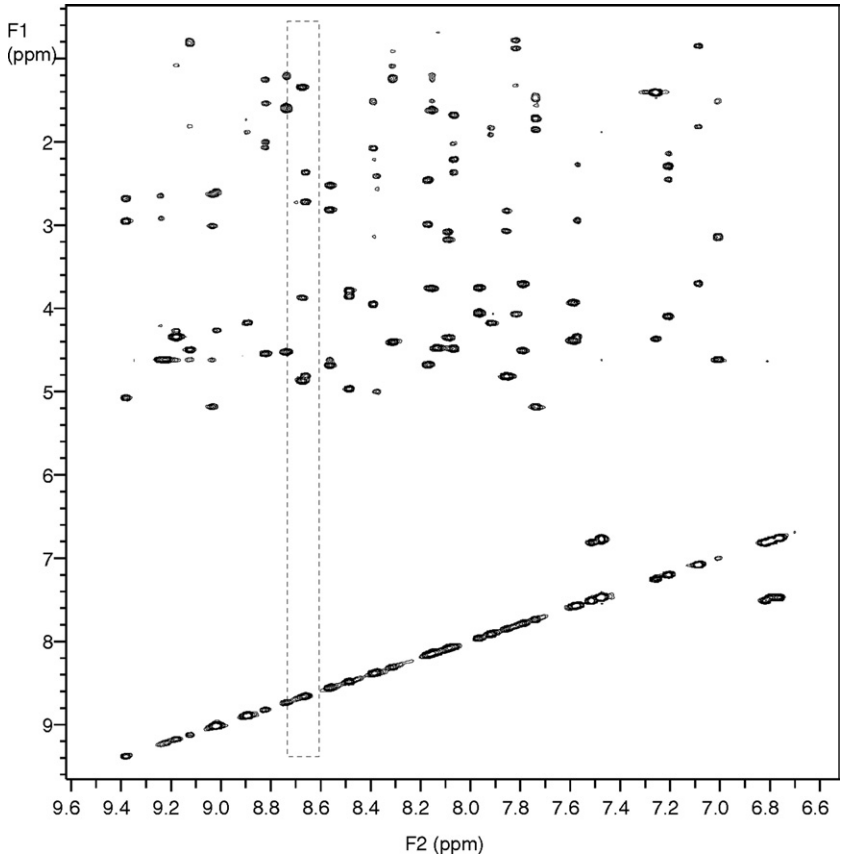

Fig. 2. The 900 MHz proton–proton plane of the ^{15}N-TOCSY–HSQC spectrum of the small protein agitoxin. In this spectrum, which is analogous to the NH region of the standard two-dimensional TOCSY spectrum, the correlation peaks from residues Cys-28 and Thre-36 are degenerate in frequency. The strip bounded by dashed lines is re-examined in Fig. 3.

spectrum. The TILT technique (time-domain increments linked together) offers an improvement in speed of data acquisition by an order of magnitude. Two promising applications are the determination of relaxation times [11] or nuclear Overhauser effects for the newly separated NMR responses, measurements that are difficult or impossible for overlapping signals.

The TILT concept is readily extended to resolving ambiguities in higher-order k-dimensional spectra by simultaneous incrementation of the additional evolution parameter of the corresponding $(k+1)$-dimensional experiment. No significant modification of the standard $(k+1)$-dimensional pulse sequence is required, only the establishment of a suitably scaled linkage between the rates of incrementation of two evolution parameters. There are obvious similarities in the 'Accordion' technique [12,13] and reduced-dimensionality experiments [14–18].

3. Applications of the TILT method

Multi-dimensional spectra of proteins involve many instances of ambiguity. The effectiveness of the TILT technique has been demonstrated by measuring individual ^{15}N spin–lattice relaxation times for several previously overlapping responses [11]. For small molecules in samples with good sensitivity, isotopic enrichment should not be necessary, provided that the ^{1}H responses from ^{12}C or ^{14}N molecules are suitably suppressed.

Illustrative examples of the TILT method are provided by the spectra of a 0.3 mM aqueous solution (10% D_2O) of a small 39-residue protein agitoxin [19], isotopically labelled with ^{15}N and ^{13}C. The first application involves the 900 MHz two-dimensional TOCSY spectrum recorded at 30 °C. For the purpose of illustration, we show the analogous partial spectrum of the NH sites obtained by projecting the ^{15}N-TOCSY–HSQC [20] spectrum on the H–H plane (Fig. 2). The TOCSY mixing time was 80 ms, four scans were collected and the experimental duration was 47 min. The diagonal responses from Cys-28 and Thr-36 overlap, as highlighted in the strip center at a proton frequency of 8.65 ppm. This leads to ambiguities in the assignment of the corresponding correlation peaks.

The ambiguities are resolved by repeating the experiment with ^{15}N evolving in tandem with ^{1}H, giving a projection tilted at $+30°$ with respect to the H–H plane. With the usual quadrature detection scheme, a corresponding plane tilted at $-30°$ is also generated, offering

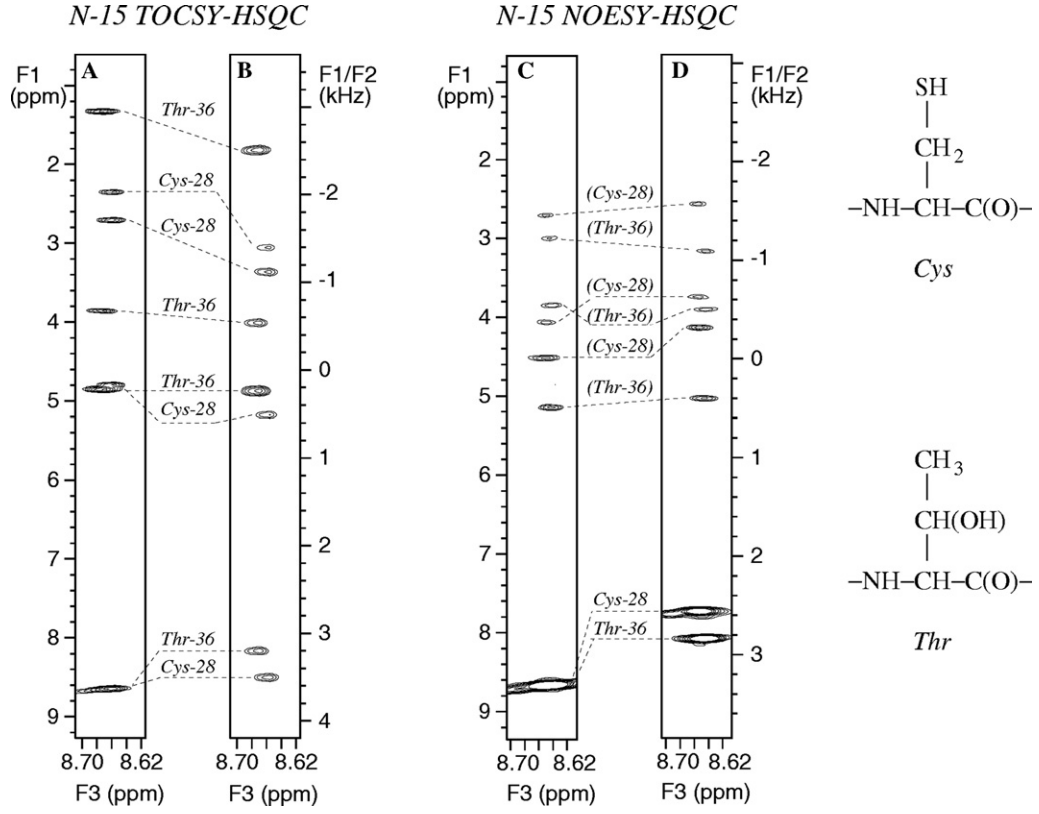

Fig. 3. Strip plots of the ^{15}N-TOCSY–HSQC spectrum of agitoxin centered at a proton frequency of 8.65 ppm, showing the degenerate Cys-28 and Thre-36 responses. (A) The proton–proton plane ($\alpha = 0$). (B) The tilted plane ($\alpha = +30°$) obtained by simultaneous evolution of the ^{15}N and ^{1}H frequencies. The corresponding ^{15}N-NOESY–HSQC spectra recorded with (C) $\alpha = 0$, (D) $\alpha = -30°$. The separation of peaks allows the individual nuclear Overhauser enhancements to be measured.

an independent view of the spectrum. If Ω_H is the proton frequency and Ω_N the corresponding ^{15}N frequency, then the positions of the peaks in these two tilted projection are:

$$\Omega(+) = \Omega_H \cos\alpha + \Omega_N \sin\alpha, \quad (3)$$

$$\Omega(-) = \Omega_H \cos\alpha - \Omega_N \sin\alpha. \quad (4)$$

By predicting the frequencies in the tilted plane, these equations determine the assignments, indicated by dashed lines in Fig. 3. The calculated peak frequencies agree with those observed experimentally within limit set by the digital resolution (±10 Hz), except for two peaks that overlap in the H–H projection, resulting in less accurate measurements. The correlation peaks in the tilted plane are shown in Fig. 3B, where the Cys-28 and Thr-36 peaks are now well separated.

A second illustration focuses on the ^{15}N-NOESY–HSQC [21] spectrum (not shown) of the same sample recorded at the lower frequency of 800 MHz and a temperature of 25 °C. This is analogous to the NH region of the conventional two-dimensional NOESY spectrum. The mixing time was 150 ms, four scans were collected and the total experimental duration was 49 min. Because this spectrum was recorded at the lower temperature (25 °C) the positions of Thr-36 and Cys-28 responses are interchanged. When the ^{15}N spins evolve jointly with the protons, generating a projection tilted at −30°, differential shifts separate the overlapping responses (Fig. 3D). In this case, all the experimental frequencies agree with those predicted within the ±10 Hz digitization steps.

Normally peak positions would be measured in the conventional TOCSY or NOESY spectra, but when there is overlap, frequencies derived from projections tilted at ±α can be used to calculate the positions of the individual responses:

$$\Omega_H = [\Omega(+) + \Omega(-)]/2\cos\alpha, \quad (5)$$

$$\Omega_X = [\Omega(+) - \Omega(-)]/2\sin\alpha, \quad (6)$$

where X represents the heteronuclear species. Tilted projections of this kind should prove useful for measuring the nuclear Overhauser enhancements or relaxation times of formerly degenerate resonances. The results are achieved significantly more quickly than in the complete three-dimensional version of the experiment.

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