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Synergy and sensitivity-balance in concatenating experiments in NO Relaxation Delay NMR (NORD)

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The NMR experiment design strategy of NO Relaxation Delay (NORD) introduced mostly as an idealized theoretical approach is extended and put to practical use by considering synergy and sensitivity-balance in concatenation of experiments. It is illustrated by a novel experiment, NORD {HMBC}-{HSQC}-{TOCSY}, where magnetization of non-^{13}C attached protons effectively is channeled from the TOCSY spectrum toward primarily the least sensitive spectrum of HMBC. The experiment is expected to come to serve as a full-package NMR method for metabolomics, carbohydrates, peptides and small-molecules in general.

Since NMR is an inherently low-sensitivity method it is a matter of constant attention to find ways of improving sensitivity or reducing measurement time. On a background of on-going generally applicable technological advances in hardware and new data processing methods development of numerous specialized spin engineering approaches to manipulate spin systems toward a desired outcome have kept the field vibrant. Some of these have been the result of systematic research, others have been serendipitous whilst yet others have come about by bright ideas to combine existing spin engineering elements.[1]

For example, ways of manipulating proton magnetization simultaneously and independently according to a ^{13}C atom being directly attached or not had been around for decades without applications exploiting their full capabilities. That changed following the idea that if an experiment using the first pool is concatenated with an experiment using the second pool they can share a single relaxation delay because the two orthogonal pools of magnetization relax simultaneously.[2] This saves spectrometer time without significant reduction in signal-to-noise ratio (S/N) per scan compared to separate running of two such experiments.

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More recently, it was demonstrated that in a complex experiment it was possible to dispense with a relaxation delay altogether using the NORD (NO Relaxation Delay) strategy.[3] Such experiments are laid out not only to operate with two orthogonal pools of magnetization but also to have one of the pools relax while the other is being used and vice versa, or to save magnetization in individual modules to be used in succeeding scans in the style of the Ernst angle concept.[4]

In small-molecule NMR, a typical set of experiments is one-bond ^{1}H-^{13}C correlation (HSQC), long-range ^{1}H-^{13}C correlation (HMBC), and ^{1}H-^{1}H correlation (TOCSY), and it will be shown that these three experiments are ideally suited for concatenation according to the extended NORD strategy. The result is a significant gain to the least sensitive of the three, HMBC, and thus a corresponding welcome saving in instrument time.

For a fair comparison, the new NORD {HMBC}-{HSQC}-{TOCSY} experiment outlined in Fig. 1 is held up against the corresponding individual optimized experiments of NORD {HMBC}, NORD {HSQC}, and NORD {TOCSY} shown in ESI (Fig. S1).

The most sensitive way to perform NORD {HMBC} is to apply an excitation flip angle between $\pi/2$ and π (Fig. S2), so that part of the available magnetization is saved for succeeding scans.[4,5] Then the new idea is that if the magnetization pertinent for the HMBC experiment can relax during other modules in a concatenation, an HMBC excitation flip angle closer to $\pi/2$ can be applied to enhance the HMBC S/N per scan. Exactly that type of synergy is realized in NORD {HMBC}-{HSQC}-{TOCSY}.

In concatenation of experiments, one typically operates with the pool of protons attached (the IS pool) and the pool of protons not-attached (the I pool) to ^{13}C. Both HMBC and TOCSY rely on the I pool, so in order to obtain the gain for HMBC only a small share of that pool is to be used for the TOCSY experiment and interference with ongoing I pool longitudinal relaxation during TOCSY is to be kept low. A consequence of this is an expected S/N reduction in the TOCSY spectrum, but that kind of sensitivity balancing is without practical relevance because TOCSY inherently is about two orders of magnitude more sensitive than HMBC. All that is needed to convert regular TOCSY to NORD {TOCSY} is to reduce the flip angle of the excitation pulse (Fig. S1).

The HSQC spectrum relies on the IS pool and in order to manipulate that pool independent of the I pool a BANGO[6] pulse sequence element is employed for HMBC excitation like in earlier similar applications.[3,7,8] This ensures that the HSQC sensitivity is not compromised by the optimization of the HMBC sensitivity. The HSQC module in NORD {HMBC}-{HSQC}-{TOCSY} is upgraded from an earlier implementation[9] by TIG-BIRD[10] excitation, odd/even editing, and the heteronuclear coherence transfer element applicable for all multiplicities.[11]

Like INEPT excitation in conventional HSQC the employed TIG-BIRD element inverts one of the ^{1}H doublet resonances in the IS pool but in addition also inverts the I pool. The BANGO element excites the I pool by a $\pi/2$ (or slightly larger) rotation and inverts both resonances of the IS pool.

It is useful to summarize the actions of building blocks and modules in the new NORD experiment. In the first module (HMBC), the I pool is for $\beta^I = \pi/2$ exhausted in full and the IS pool is kept longitudinal outside the BANGO element. In the second module (HSQC), the IS pool is exhausted in full and the I pool is kept longitudinal outside the TIG-BIRD and mixing elements. In the TOCSY module, both pools are "cut" by a factor $1 - |cos(\alpha)|$ in the preparation sequence and kept longitudinal outside the mixing sequence whilst the TOCSY spectrum is excited according to $sin(\alpha)$.

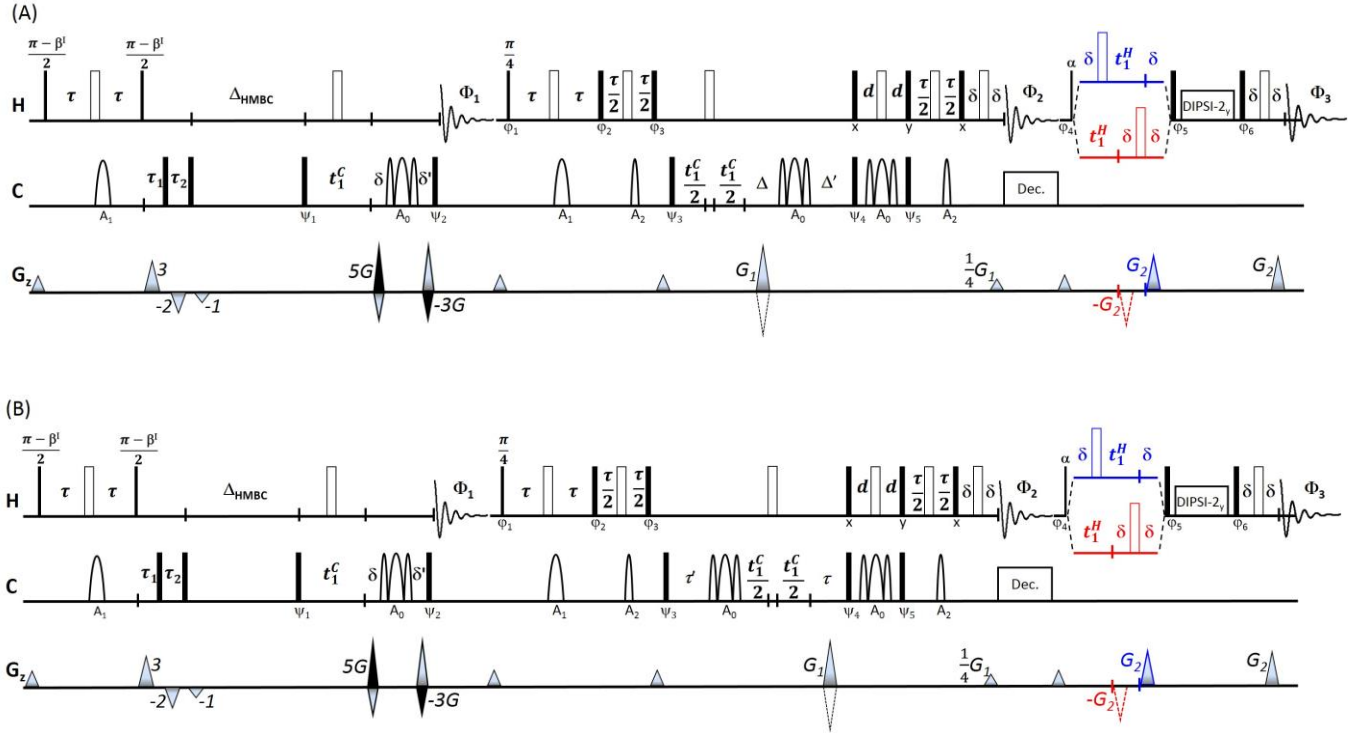


Figure 1. NORD {HMBC}-{HSQC}-{TOCSY} pulse sequences, (A) standard and (B) updown (i.e. opposite phases of odd and even ^{13}C-multiplicities) in the HSQC module. Narrow filled, wider filled and open bars refer to $\pi/4$, $\pi/2$ and π pulses, respectively, whilst α represents a 10-30° angle and the BANGO excitation pulse β^I in the first module is $\pi/2$ (or slightly larger). A_1 and A_2 are *J*-compensated adiabatic ^{13}C CAWURST-20 inversion pulses (240 ppm (or 121 ppm for sugars), 1.92 and 0.97 ms, respectively) with low-to-high frequency sweep. A_0 is a composite 20% smoothed CHIRP (2 ms, 80 kHz sweep; Crp80comp.4). The delay $\tau = 0.5(^1J)^{-1}$ is set according to a ^1J of 113 Hz. A 2nd order low-pass *J* filter is employed with $\tau_1 = 0.5[^1J_{min} + 0.146(^1J_{max} - {}^1J_{min})]^{-1}$ and $\tau_2 = 0.5[^1J_{max} - 0.146(^1J_{max} - {}^1J_{min})]^{-1}$. δ is a gradient delay and $\delta' = \delta + t_{180(H)}$. d is set to 1.86 ms as a compromise for all carbon multiplicities. In (A) $\Delta = \delta$ and $\Delta' = \Delta + t_{180(H)}$, in (B) $\tau' = \tau + t_{180(H)}$. The delay Δ_{HMBC} is for evolution under heteronuclear long-range couplings. The DIPSI-2 sequences used for mixing in TOCSY. The amplitude of the purging gradients (triangles without labelling) and the low-pass *J* filter gradients in the HMBC module can be set an order of magnitude lower than the amplitudes of the other ones selecting coherence transfer echo and antiecho. The receiver phases in all three modules always alternate by π, i.e. $\Phi_1/\Phi_2/\Phi_3 = \{x, -x\}$ and for the spectra presented only 2 scans were recorded in each module with the first two steps of the following phase cycles. HMBC: $\psi_1 = \{x, -x, -x, x\}$, $\psi_2 = \{x, x, -x, -x\}$; echo/antiecho selection occurs with the gradients (5G,-3G)/(-3G,5G). For antiecho selection in HSQC: $\varphi_1 = \left\{\frac{\pi}{4}, \frac{\pi}{4}, \frac{\pi}{4}, \frac{\pi}{4}, -\frac{\pi}{4}, -\frac{\pi}{4}, -\frac{\pi}{4}, -\frac{\pi}{4}\right\}$, $\varphi_2 = \left\{\frac{5\pi}{4}, \frac{5\pi}{4}, \frac{5\pi}{4}, \frac{5\pi}{4}, \frac{3\pi}{4}, \frac{3\pi}{4}, \frac{3\pi}{4}, \frac{3\pi}{4}\right\}$, $\varphi_3 = \{y, y, y, y, -y, -y, -y, -y\}$, $\psi_3 = \{x, -x, -x, x\}$, $\psi_4 = \{-x, -x, x, x, x, x, -x, -x\}$, $\psi_5 = \{y, y, -y, -y, -y, -y, y, y\}$; for echo selection phase ψ_5 is inverted. TOCSY: $\varphi_4 = \{x, -x\}$, $\varphi_5 = \{x, x, -x, -x\}$, $\varphi_6 = \{-x, -x, x, x\}$ and the upper and lower panels applicable for echo and antiecho, respectively. In HSQC and TOCSY the echo is selected with the positive gradients G_1 and G_2, respectively, prior to mixing and the antiecho with the corresponding negative dashed gradients. Before standard processing the obtained combined dataset is separated into three blocks, corresponding to HMBC, HSQC and TOCSY data, using the Bruker au-program *splitx*. The HSQC data sets from (A) and (B) are added or subtracted from each other to yield even and odd ^{13}C multiplicity spectra, respectively. The F_1 chemical shift scale in TOCSY is corrected with au-program *fixF1*. Then the data are processed as in their stand-alone experiments. The pulse sequence code for Bruker spectrometers can be found in ESI.

The NORD {HMBC}-{HSQC}-{TOCSY} pulse sequence has been applied to a monosaccharide (4,6-benzylidene-1-metoxy-α-D-glucose, **1**), and the crucial S/N comparison with individual NORD experiments is illustrated with selected 1D sections in Fig. 2. The complete set of comparisons can be found in ESI (Figs. S4-S5). The two HMBC spectra were recorded with the same number of scans and the optimum excitation angle in NORD {HMBC} was determined experimentally to 130° (Fig. S2) whilst $\beta^I = 90°$ was employed in NORD {HMBC}-{HSQC}-{TOCSY}. The TOCSY excitation angle in NORD {TOCSY} and NORD {HMBC}-{HSQC}-{TOCSY} was 60° and 30°, respectively. The average relative HMBC S/N gain in NORD {HMBC}-{HSQC}-{TOCSY} amounts to 94% in an interval of 70-140% (Fig. 2 and Fig. S4).

Concurrent with the HMBC S/N gain is the expected loss in the TOCSY spectrum averaging 37%. In the HSQC spectrum there is an average S/N gain of 38% (Fig. 2 and Fig. S6). The gain in HSQC S/N reflects the homonuclear couplings of the proton attached to ^{13}C. Protons with small or no such couplings show the largest improvement since they do not benefit from polarization transfer from the I pool in NORD {HSQC}, but they have extra time to relax toward equilibrium during the TOCSY and HMBC part in NORD {HMBC}-{HSQC}-{TOCSY}. However, given that both HSQC and TOCSY are much more sensitive than HMBC the fluctuations in HSQC and TOCSY S/N are of no practical implications. The focus is solely on optimizing S/N in the least sensitive spectrum, i.e. HMBC, and even a TOCSY excitation angle around 10° will do fine.

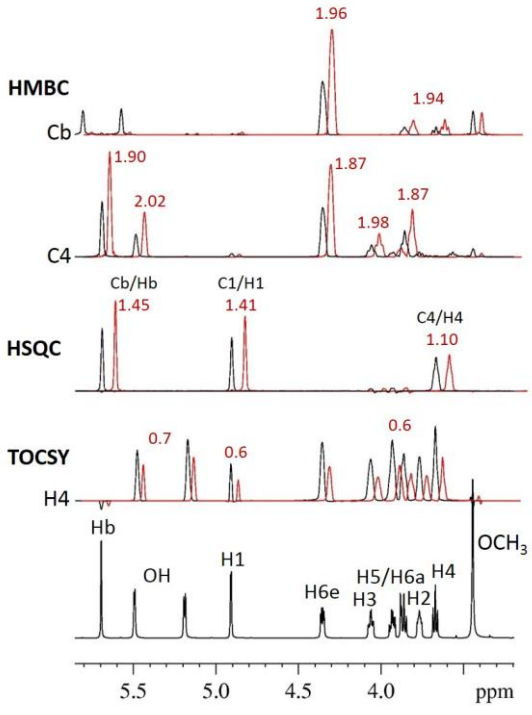
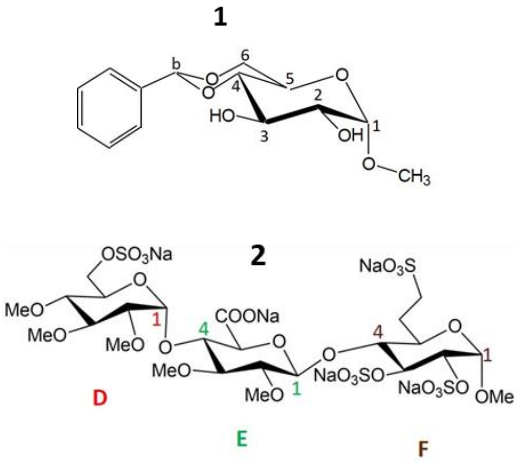


Figure 2. Sensitivity comparison between the NORD {HMBC}-{HSQC}-{TOCSY} (red, shifted for better visualization) and the corresponding individual NORD experiments (black). All experiments were run without relaxation delay on the monosaccharide **1** (60 mg in 550 µl DMSO-d_6/C_6D_6). The measured relative S/N ratios are given above the extracted F_2-sections and the ^1H spectrum at the bottom shows the resonance assignments of the sugar ring protons. 2 scans were recorded per increment with 1024 points acquired in t_1 and t_2 in all modules. The spectral width was 8.4 ppm in the ^1H dimension and 100 ppm in the ^{13}C dimension. To compensate for the spread in $^1J_{CH}$ CAWURST-20 (121 ppm, 1.94 ms; H2L) and CAWURST-20 (121 ppm, 0.97 ms; H2L) adiabatic ^{13}C inversion pulses were used. HMBC was run with a $\beta^I = \pi/2$ BANGO excitation angle and the magnetization of HSQC was excited by TIG-BIRD as described in the text. For the TOCSY module, an $\alpha = \pi/6$ excitation pulse and 80 ms mixing time was used. The individual NORD experiments were optimized seperately: 130° excitation pulse for NORD {HMBC} recorded in 8 min 44 sec, 40 ms DIPSI-2 prepolarization for NORD {HSQC} recorded in 8 min 14 sec and 60° excitation pulse for NORD {TOCSY} recorded in 10 min 41 sec (Fig. S2). Excerpts of 2D HMBC, HSQC and TOCSY spectra recorded in 26 min 28 sec as well as further F_2 sections of HMBC spectra to demonstrate the resulting sensitivity improvement are shown in Figs. S3-S4.



Another demonstration of the NORD {HMBC}-{HSQC}-{TOCSY} experiment shown on a heparin-analogue trisaccharide (**2**). Fig. 3 shows excerpts from the three spectra recorded in 37 minutes, allowing unambiguous assignment of all ^1H and ^{13}C resonances. The sequential order of sugar residues is established by the respective interglycosidic long-range correlations in the HMBC spectrum (framed in dotted boxes).

A truncation of NORD {HMBC}-{HSQC}-{TOCSY} to the two-module NORD {HSQC}-{TOCSY} experiment (Fig. S1F) is illustrated by application to a decapeptide in Fig. S7. The odd/even ^{13}C-multiplicity editing of HSQC peaks expedites the assignment of characteristic spin systems of different amino acid residues.

In conclusion, we have extended the NORD NMR strategy by exploiting synergy and sensitivity balancing of the modules in a concatenated experiment to accomplish a significant gain in S/N in the least sensitive experiment. The new NORD {HMBC}-{HSQC}-{TOCSY} experiment serves most of the needs across a wide range of NMR applications and it is possible to exchange a module by another relying on the same pool of magnetization. Clearly, there are a large number of possible concatenations of experiments, but there needs to be synergy between the individual modules for concatenation to be worthwhile. Finally, various truncated versions of NORD {HMBC}-{HSQC}-{TOCSY} might suffice for applications (see ESI) not requiring all three spectra.

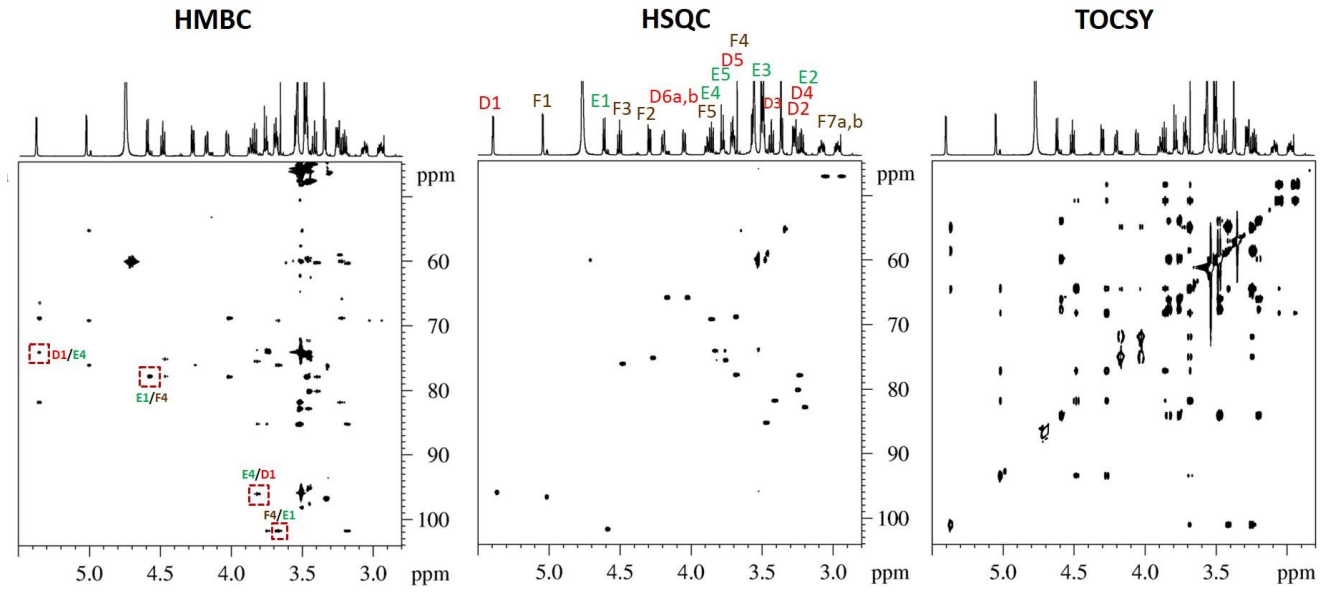


Figure 3. Excerpts of HMBC, HSQC and TOCSY spectra of a heparin-analogue trisaccharide[12] (60 mg of **2** dissolved in 550 µl D_2O, structure shown above) recorded on a Bruker 700 MHz Avance NEO spectrometer equipped with a TCI z-gradient prodigy probe in 37 minutes using the NORD {HMBC}-{HSQC}-{TOCSY} pulse sequence in Fig. 1A. The ^1H resonance assignment of sugar residues is shown on the top of the HSQC spectrum. The sequential connectivities of D-E and E-F residues were established and the corresponding HMBC peaks are highlighted by dashed red box frames. The spectra were acquired with the parameters: Δ_{HMBC} = 83 ms, DIPSI-2 mixing time 80 ms, $^1J_{min}$ = 125 Hz, $^1J_{max}$ = 165 Hz, ^1H excitation pulses β^I and α of $\pi/2$ and $\pi/6$, respectively, spectral widths of 8.4 ppm (^1H) and 100 ppm (^{13}C), using 1024 points in t_1 with 2 scans per increment and 2048 data points in t_2. CAWURST-20 (121 ppm, 1.94 ms; H2L) and CAWURST-20 (121 ppm, 0.97 ms; H2L) adiabatic ^{13}C inversion pulses were used. The delay τ was set according to a ^1J of 113 Hz.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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