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More than ADEQUATE: doubling the sensitivity of ^{13}CH–^{13}CH correlations in double-quantum NMR experiments†

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We present modifications of the ADEQUATE experiment which more than double the sensitivity of carbon–carbon correlations of ^{13}CH–^{13}CH moieties. Additionally, these improvements can be applied without a sensitivity penalty to obtain spectra with a ^{13}C chemical shift axis in the indirectly detected dimension, instead of a double-quantum frequency, allowing simpler interpretation of spectra. The modified experiments, which use refocussing of $^{1}J_{CH}$ couplings and ^{1}H decoupling during J_{CC} evolution intervals, were tested on several molecules, including a pentasaccharide (20 mg, 19 mM), where on average a 2.6-fold signal-to-noise improvement was achieved and the number of observable correlations increased. Doubling sensitivity results in a 4-fold reduction of the experimental time, allowing ADEQUATE spectra to be recorded overnight instead of over multiple days.

The INADEQUATE (incredible natural abundance double-quantum transfer experiment) NMR experiment[1,2] is of substantial interest to chemists as it allows tracing out the carbon skeleton of a molecule.[3] It relies on the detection of double-quantum (DQ) coherences between two coupled ^{13}C spins and therefore has inherently inadequate sensitivity, as the probability of a molecule containing a pair of ^{13}C atoms is approximately 1-in-8300. Historically, this has limited its use, as long experimental times and/or high sample concentrations were typically required. However, the development of cryogenically cooled NMR probes and the associated sensitivity gains enabled INADEQUATE and its variants to become routine experiments, which have been reviewed extensively.[4–9]

For protonated ^{13}C atoms, sensitivity of INADEQUATE can be increased by using ^{1}H detection as demonstrated by the INEPT-INADEQUATE experiment.[10] The addition of the INEPT (insensitive nuclei enhancement by polarisation transfer) step offers increased sensitivity due to the higher gyromagnetic ratio (γ) of ^{1}H compared to ^{13}C. Considering proton singlets and neglecting the sensitivity enhancement of ^{13}C-detected INADEQUATE through heteronuclear NOE, the maximum theoretical signal-to-noise ratio (SNR) increase associated with ^{1}H detection is $(\gamma_H/\gamma_C)^{5/2} \approx 32$.[11] However, such a SNR increase is not achieved for various reasons.[12] A factor of $\sqrt{2}$ is lost due to pulsed-field gradient (PFG) selection,[13] and a loss of a further factor of 2 occurs because in the ^{1}H-detected experiment the DQ coherences start and end on the same proton, whereas in ^{13}C-detected INADEQUATE the coherences are generated from two nuclei. Additional sensitivity loses occur due to relaxation effects: the INEPT transfer of the ^{1}H-detected experiments generates mixed proton–carbon coherences that in medium size molecules relax faster than pure carbon coherences. While sharp ^{1}H-decoupled antiphase ^{13}C–^{13}C doublets are acquired in ^{13}C-detected INADEQUATE, complex ^{1}H multiplets degrade sensitivity of ^{1}H-detected methods.

As a result, approaches have been developed to improve the sensitivity of ^{1}H detection. It has been demonstrated that removing the proton term from mixed CH coherences prolongs their relaxation times and increases the overall sensitivity of experiments.[12] This approach is especially beneficial for long-range ($^{n}J_{CC}$, $n \geq 2$) optimised ^{1}H-detected experiments containing longer $^{n}J_{CC}$ evolution delays. The sensitivity of ^{1}H-detected INADEQUATE was substantially increased by ^{1}H-detected ADEQUATE (adequate sensitivity double-quantum spectroscopy).[14,15] Sensitivity can be further improved by homonuclear decoupling that at least partially simplifies the structure of ^{1}H multiplets.[17,18]

In order to avoid complications associated with the setup and interpretation of experiments that produce DQ frequencies in the indirectly detected dimension (F_1), a version of the ADEQUATE experiment was reported that samples ^{13}C single-quantum (SQ) coherences in F_1.[14,15] This modification, originally termed ω_1-refocussed ADEQUATE, is referred to as SQ ADEQUATE herein. For an H_n-C_n-C_m-H_m spin system this amounts to H_n being modulated by the C_m frequency (and H_m by C_n), and thus the $^{1}J_{CC}$-optimised SQ ADEQUATE experiment produces a spectrum containing *pseudo*-2-bond C–H correlations at the (F_2, F_1) chemical shifts of

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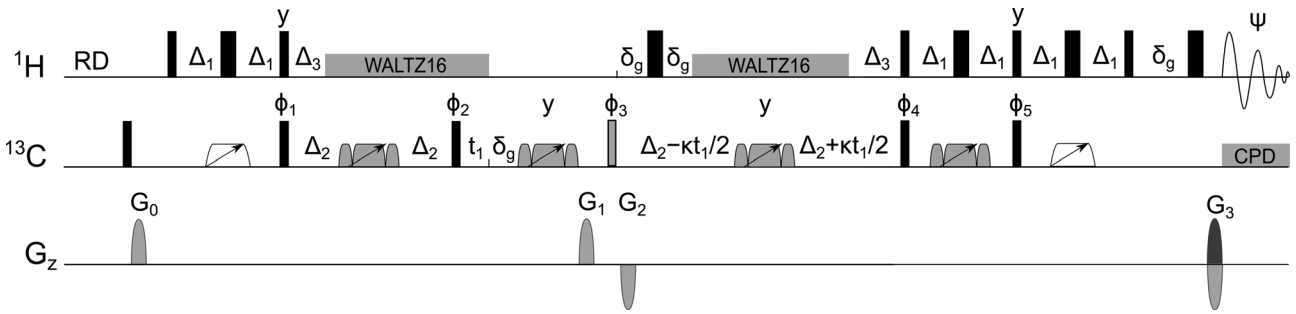


Fig. 1 $^{1}J_{CH}$-refocussed ADEQUATE pulse sequence. If $\kappa = 0$, double-quantum chemical shift is obtained in F_1. If $\kappa = 1$, single-quantum ^{13}C chemical shift is obtained in F_1. For full description of parameters, see ESI.†

(H_n, C_m) and (H_m, C_n). A comparison of SQ ADEQUATE and 2D ^{1}H, ^{13}C HSQC spectra (the latter acquired in a fraction of time) allows for a straightforward interpretation of ^{13}C–^{13}C correlations observed in SQ ADEQUATE experiments.

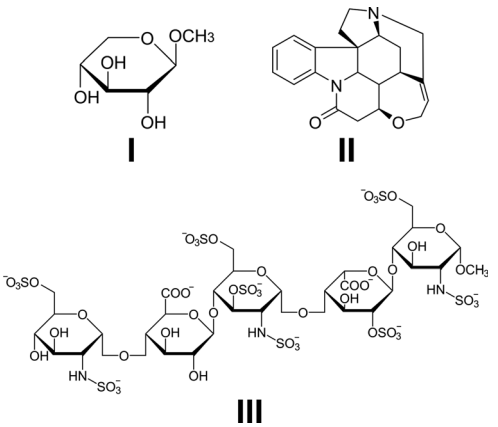
In this work, we present modifications of the ADEQUATE experiments that significantly increase the sensitivity of ^{13}C–^{13}C correlations for molecular moieties containing CH–CH fragments. These modifications can be applied to experiments that sample either DQ or SQ frequencies in F_1 and are herein referred to collectively as $^{1}J_{CH}$-refocussed ADEQUATE (Fig. 1). The full pulse sequence parameters and experimental details are presented in Fig. S1 (ESI†).

The first minor modification of the original experiments is the addition of a 90° ^{13}C pulse and a PFG purge element at the beginning of the pulse sequence, which defocusses any ^{13}C magnetisation, thus eliminating polarisation transfer pathways starting on ^{13}C. Although the ADEQUATE experiment employs phase cycling and PFG selection to remove the strong signals coming from ^{12}C-bonded protons, isolated ^{13}C-bonded protons or ^{13}C atoms, imperfect defocussing of these signals can obscure correlations of interest. The addition of this purge element reduced cancellation artefacts and slightly increased the SNR as shown in Fig. S2 (ESI†).

A major sensitivity increase was achieved by refocussing the $^{1}J_{CH}$ coupling at the beginning of the $1/(2^{1,n}J_{CC})$ evolution interval following the initial $^{1}H \rightarrow {}^{13}C$ INEPT transfer. This conversion of carbon-proton antiphase coherences into pure ^{13}C coherences prior to excitation of DQ coherence removes the leakage of signal into undetectable zero-quantum coherences and generates a 2-fold sensitivity increase.[19] Application of ^{1}H decoupling while SQ or DQ ^{13}C coherences are present, reduces relaxation and further increases the sensitivity of experiments.[12] To regenerate the antiphase proton–carbon coherences required for the reverse $^{13}C \rightarrow {}^{1}H$ INEPT step, the decoupling is turned off for a period of $1/(2^{1}J_{CH})$ at the end of the $1/(2^{1,n}J_{CC})$ refocussing interval.

The increased SNR of the modified ADEQUATE experiments was evaluated using a concentrated sample of methyl β-D-xylopyranoside (**I**, 1.1 M, Scheme 1). This model compound was chosen because it contains CH, CH_2 and CH_3 carbons; its high concentration enabled fast and accurate comparison of related spectra. Relative SNR changes for CH groups are presented in Table 1 and Fig. 2; full spectra are shown in Fig. S3 (ESI†). SNR improvement for selected correlations is highlighted in Fig. S4 (ESI†). Not reported previously in the literature, these results indicated that the original $^{1}J_{CC}$-optimised SQ (ω_1-refocussed) ADEQUATE experiment shows an up to 32% sensitivity loss compared to the DQ ADEQUATE experiment. This loss, which can be tolerated in exchange for a more straightforward analysis of spectra when sample concentration is not limiting, is associated with the appearance of HSQC-like artefacts at frequencies of (H_n, C_n) as shown in Fig. S3c (ESI†). These extra correlations, which can be much more efficiently obtained from ^{1}H, ^{13}C HSQC spectra, can hinder analysis of complicated spectra by introducing peak overlap. The proposed $^{1}J_{CH}$-refocussed SQ ADEQUATE eliminates this leakage (*cf.* Fig. S3c and d, ESI†), which explains larger SNR increases observed for the $^{1}J_{CC}$-optimised SQ compared to the DQ experiment (2.1× *vs* 1.7×). Long-range SQ ADEQUATE spectra do not show significant HSQC-like artefacts, hence the increase in sensitivity is comparable for the DQ and SQ experiment (2.3× for both).

Overall, the data summarised in Table 1 indicate that $^{1}J_{CH}$-refocussed SQ ADEQUATE experiment performs best for both one-bond and long-range ^{13}CH–^{13}CH correlations. Therefore, simpler spectra, displaying SQ ^{13}C chemical shift along F_1 can now be obtained without a sensitivity penalty and with a 1.8–2.0-fold enhancement compared to original DQ ADEQUATE. As the $^{1}J_{CH}$-refocussed ADEQUATE experiments are designed to refocus



Scheme 1 Compounds used for ADEQUATE experiments: methyl β-D-xylopyranoside (**I**), strychnine (**II**) and fondaparinux (**III**).

Table 1 Average (mean ± SD) signal-to-noise ratio (SNR) gain obtained for CH–CH correlations of **I** in DQ and SQ ADEQUATE experiments[14,15] with and without refocussing of $^1J_{CH}$ couplings

	SNR fold change	
Experiment	One-bond[a]	Long-range[b]
DQ ADEQUATE	1.0	1.0
SQ ADEQUATE[c]	0.9 ± 0.1	0.9 ± 0.1
$^1J_{CH}$-refocussed DQ[c]	1.7 ± 0.3	2.3 ± 0.3
$^1J_{CH}$-refocussed SQ[c]	1.8 ± 0.3	2.0 ± 0.4
$^1J_{CH}$-refocussed SQ[d]	2.1 ± 0.2	2.3 ± 0.4

[a] n (number of correlations) = 6. [b] n = 4. [c] With respect to original DQ ADEQUATE. [d] With respect to original SQ ADEQUATE.

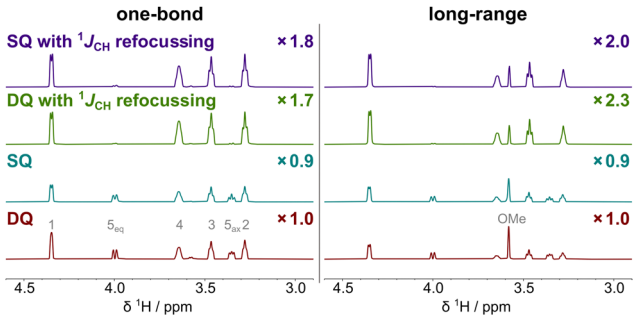


Fig. 2 Positive projections of ADEQUATE spectra of **I** optimised for one-bond (45 Hz) and long-range (6 Hz) J_{CC} couplings. The factors indicate the average SNR improvement for CH groups compared to DQ ADEQUATE. Full one-bond 2D spectra are shown in Fig. S3 (ESI†).

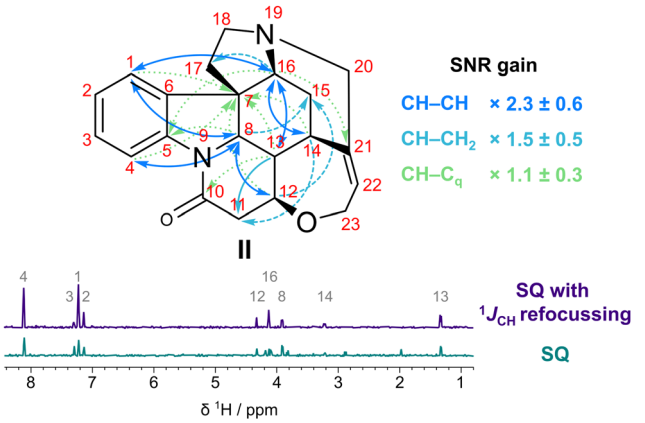


Fig. 3 Correlations observed and the SNR improvement achieved by $^1J_{CH}$ refocussing in $^nJ_{CC}$-optimised SQ ADEQUATE experiments for **II**. F_2 projection with and without $^1J_{CH}$ refocussing are shown. Full spectra are presented in Fig. S7 (ESI†). Note that correlations observed for the aromatic system have been omitted for clarity.

couplings of CH groups only, the sensitivity of CH_3-detected correlations is reduced and CH_2-detected correlations are absent. The CH-detected CH–CH_x (x = 0, 2, 3) connectivities are present with improved sensitivity (1.3–2.0×) compared to the original ADEQUATE spectra as evaluated on the spectra of L-isoleucine (Fig. S5, ESI†).

In order to investigate the SNR improvements on a compound with a more diverse structure, one-bond and long-range optimised SQ ADEQUATE spectra were acquired for strychnine (**II**, 32 mM). Despite the wide range of coupling constants reported for **II** ($^1J_{CH}$ = 124–168 Hz, $^1J_{CC}$ = 32–71 Hz, $^3J_{CC}$ = 3–7 Hz),[20,21] experiments optimised for $^1J_{CH}$ (150 Hz) and $^{1,n}J_{CC}$ (50 Hz or 6 Hz) yielded sensitivity improvements similar to those observed for **I**. A SNR gain of (1.9 ± 0.4)× was observed for CH–CH correlations in $^1J_{CC}$-optimised ADEQUATE (Fig. S6 and Table S1, ESI†). A more significant increase in sensitivity was observed in the long-range spectra (Fig. S7 and Table S2, ESI†), with an average SNR increase of 2.3× for CH–CH correlations (Fig. 3). The sensitivity is also improved for CH–CH_x (x = 0, 2) moieties, although by not as much, especially for quaternary carbons. The lower increase can be attributed to quaternary carbons of **II** having relaxation times of ≥20 s,[22] therefore not benefiting much from reducing relaxation losses.

To illustrate the benefits of the improved ADEQUATE experiment on a weaker sample of a larger molecule, one-bond and long-range ^{13}CH–^{13}CH correlations of a sulfated pentasaccharide, fondaparinux Na (**III**, Scheme 1, M_w = 1728 g mol^{-1}, 19 mM), were obtained on an 800 MHz spectrometer equipped with a TCI cryoprobe in 7 and 21 hours, respectively (Fig. 4 and Fig. S8–S10, ESI†). SNR improvements in the range of 1.4- to 4.4-fold were obtained for the $^1J_{CH}$-refocussed SQ ADEQUATE experiment optimised for $^1J_{CC}$ coupling constants (Table S3, ESI†). Low SNR of the spectra is responsible for such large variations, yielding an average (2.6 ± 0.7)-fold sensitivity increase. Increased SNRs were also seen in the $^nJ_{CC}$-optimised $^1J_{CH}$-refocussed SQ ADEQUATE spectra (2.2 ± 0.5-fold, Table S4, ESI†). The increase in sensitivity is larger than for **I** or **II**, which illustrates that larger molecules benefit more from reduced relaxation losses due to their shorter ^{1}H relaxation times.

The sensitivity increase in both one-bond and long-range optimised $^1J_{CH}$-refocussed experiments is significant, however the values only refer to SNR increases for signals present in both the original and improved spectra. In fact, $^1J_{CH}$-refocussing revealed extra correlations not present in the spectra of the original experiments, therefore the sensitivity gains are even larger than the calculated factors. This is particularly true for the $^nJ_{CC}$-optimised experiments.

Correlations between adjacent monomer units in the pentasaccharide, providing valuable ring connectivity information, were observed. These experiments demonstrate that with <4 mg per monosaccharide residue, $^1J_{CH}$-refocussed SQ ADEQUATE is an efficient experiment for structure determination of oligosaccharides.

It is worth noting that due to fast evolution of $^1J_{CC}$ compared to $^nJ_{CC}$ coupling constants, one-bond correlations can appear in long-range optimised spectra. In this work, these correlations were identified by comparison to $^1J_{CC}$-optimised spectra, however methods exist to allow discrimination between $^1J_{CC}$ and $^nJ_{CC}$ correlations in $^nJ_{CC}$-optimised experiments.[23]

In conclusion, the pulse sequences presented in this work provide significant improvements for the detection of ^{13}CH–^{13}CH correlation by double-quantum NMR experiments. Larger than 2-fold increases have been achieved, which translate to a 4-times reduction in spectrometer time. Double quantum ^{13}C–^{13}C experiments are regularly recorded over multiple days,

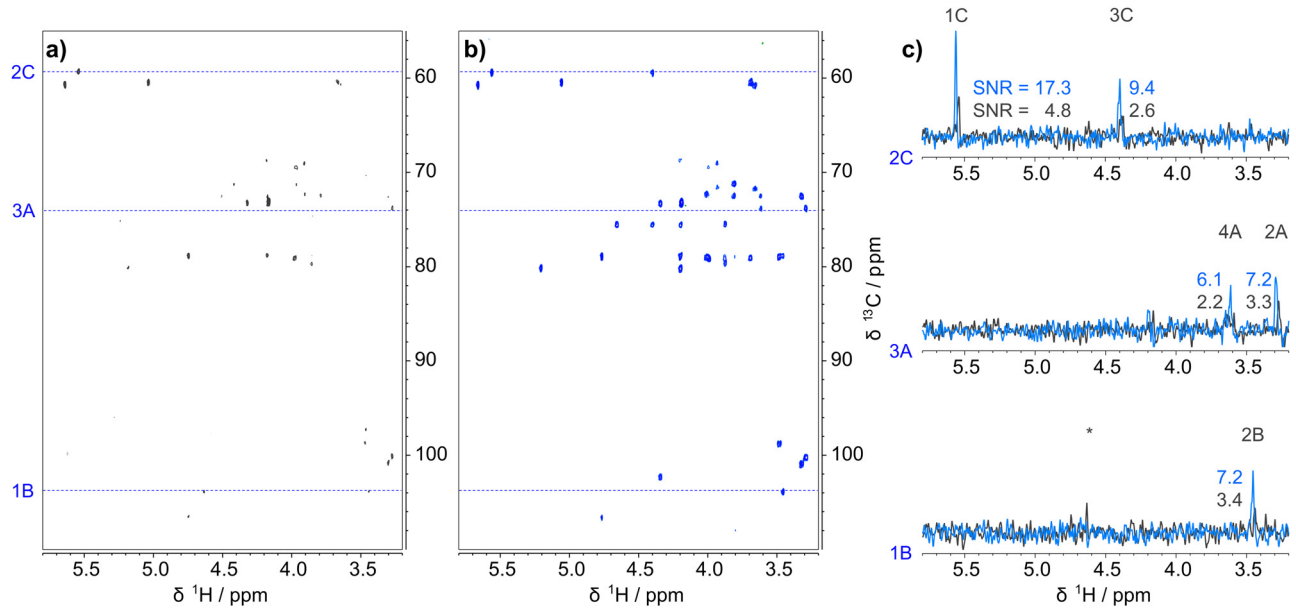


Fig. 4 $^{1}J_{CC}$-optimised SQ ADEQUATE spectra of **III** (a) without (black) and (b) with $^{1}J_{CH}$ refocussing (blue). The spectra were recorded for the same length of time and are plotted at the same intensity level. Comparison of marked traces (c) shows the sensitivity gains obtained by refocussing. Note that the 1B trace for spectrum (a) contains a weak HSQC-like artefact (*) which could be confused with a genuine correlation. For full peak assignment, see Fig. S8 (ESI†).

yet the sensitivity-improved experiments will be able to provide the same information overnight. The new method is compatible with other schemes, such as homonuclear decoupling and non-uniform sampling, that have already been successfully applied to ADEQUATE experiments.[17,18,24,25] Additionally, the proposed modifications allow observation of SQ ^{13}C frequency in the F_1 dimension without a sensitivity penalty. These sensitivity enhancements increase the potential of using ^{13}C–^{13}C correlations for structure elucidation and will benefit areas such as carbohydrates, natural products or mixture analysis, as well as general NMR applications when sample quantities are limited.

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Conflicts of interest

There are no conflicts to declare.

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