^{1}H-detected double-J-modulated INEPT-INADEQUATE for simultaneous determination of one-bond and long-range carbon–carbon connectivities and the measurement of all carbon–carbon coupling constants

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Abstract

We describe a new NMR experiment, ^{1}H-detected double-J-modulated (DJM)-INEPT-INADEQUATE, for tracing out the carbon skeleton of molecules. This experiment allows simultaneous correlation of directly bonded carbon atoms and those separated by multiple bonds, while at the same time also providing the values of all J_{CC} coupling constants. This is achieved by replacing both fixed carbon–carbon coupling evolution intervals of the INEPT-INADEQUATE experiment by variable time intervals, which are incremented in concert with the DQ evolution period (t_1). We show that the analysis of the fine structure of cross-peaks in DJM-INEPT-INADEQUATE spectra leads to accurate values of coupling constants and give guidelines for the proper usage of the method. The proposed experiment is two times less sensitive that the original INEPT-INADEQUATE experiment. We show that, using a 600-MHz cryoprobe and 20 mg of a monosaccharide, spectra that are suitable for the analysis of coupling constants as small as 2 Hz can be obtained within 24 h. Instead of performing multiple experiments, a single DJM-INEPT-INADEQUATE experiment can thus provide a wealth of information for the structural analysis of small molecules.
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1. Introduction

The ultimate NMR experiments for establishing structures of small organic molecules are based on the tracing of their carbon–carbon connectivities. With the introduction of proton detection, pulsed-field gradients (PFGs) and the increasing availability of cryo probes, the application of these, inherently not very sensitive, methods is expected to rise. Several ^{1}H-detected NMR experiments have been proposed to date that can either yield correlations via one-bond ($^1J_{CC}$) or long-range ($^nJ_{CC}$) carbon–carbon coupling constants [1–7]. However, none of the current NMR techniques provides both types of correlations in a single experiment. In addition, the magnitudes of the $^1J_{CC}$ and $^nJ_{CC}$ carbon–carbon coupling constants contain useful information about the stereochemistry and conformation of molecules [8,9]. Once again, separate experiments are needed for the measurement of $^1J_{CC}$ and $^nJ_{CC}$ coupling constants [10–13]. In this contribution we describe a simple modification of the ^{1}H-detected INEPT-INADEQUATE experiment [2] that allows simultaneous correlation of directly bonded carbon atoms and those separated by

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multiple bonds, while at the same time also providing the values of all J_{CC} coupling constants.

2. Results and discussion

Discrimination between the $^1J_{CC}$ and $^nJ_{CC}$ coupling constants that exists in the standard ^{1}H-detected INEPT-INADEQUATE experiment is eliminated when both fixed carbon–carbon coupling evolution intervals ($0.5/J_{CC}$) are made incrementable. Such variable time intervals, which are incremented in concert with the DQ evolution period (t_1), also cause modulation of cross-peaks by J_{CC} coupling constants. We illustrate below that the analysis of such cross-peaks leads to accurate values of coupling constants and give guidelines for the proper usage of the method. The proposed experiment can also be viewed as an extension of J-modulated INADEQUATE/ADEQUATE experiments [12,13] in which only one of the two carbon–carbon coupling evolution intervals is incrementable. Because the latter experiments contain one fixed interval optimized for either $^1J_{CC}$ or $^nJ_{CC}$ coupling constants they cannot yield both types of correlations at the same time. Pointing to the presence of two J-modulated intervals in the new experiment, it is referred to here as a double-J-modulated (DJM)-INEPT-INADEQUATE (Fig. 1).

The basic principles behind the new experiment can also be implemented into ADEQUATE experiments [4,10], displaying single- rather than double-quantum coherences in F_1. Nevertheless, in accord with Meissner et al. [5], we find that when used for compounds containing CH, CH$_2$, and CH$_3$ carbons, such an experiment does not offer a significant sensitivity advantage compared with simple INEPT type methods. Only the DJM-INEPT-INADEQUATE experiment is therefore discussed here.

Although pulsed field gradients provide a very good selection of DQ coherences and suppress, to a large extent, signals originating from both ^{12}C-containing molecules and those with only one ^{13}C, we have initially observed some artifacts in the DJM-INEPT-INADEQUATE spectra. The first kind originated from a coherent magnetization of ^{12}C-containing molecules, which was carried over from one scan to the next. Such magnetization was removed by placing a PFG-90_x90_y-PFG element at the end of the acquisition time, t_2. The second type of artifacts associated with imperfect (i.e., miscalibration, off-resonance effects and spatial B_1 inhomogeneity) 180° ^{13}C pulses originated from ^{13}C-containing molecules. These artifacts were removed by additional phase-cycling (φ_3), PFGs (G_3 and G_4) and the use of adiabatic 180° ^{13}C pulses.

A 2D DJM-INEPT-INADEQUATE spectrum of β-Me-xylopyranoside (**I**) (Fig. 2) shows how the carbon skeleton of organic molecules can be traced out by this method. For compounds containing many quaternary carbons, gaps can occur in the pathways mediated by one-bond coupling constants. This feature is common to all proton-detected INADEQUATE/ADEQUATE experiments. On the other hand, the DJM-INEPT-INADEQUATE spectra also contain the long-range carbon–carbon cross-peaks that involve one quaternary carbon. These long-range connectivities can be used to eliminate ambiguities associated with the absence of one-bond cross-peaks between quaternary carbons in the INEPT-INADEQUATE experiments optimized for $^1J_{CC}$ coupling constants. In addition, all cross-peaks in
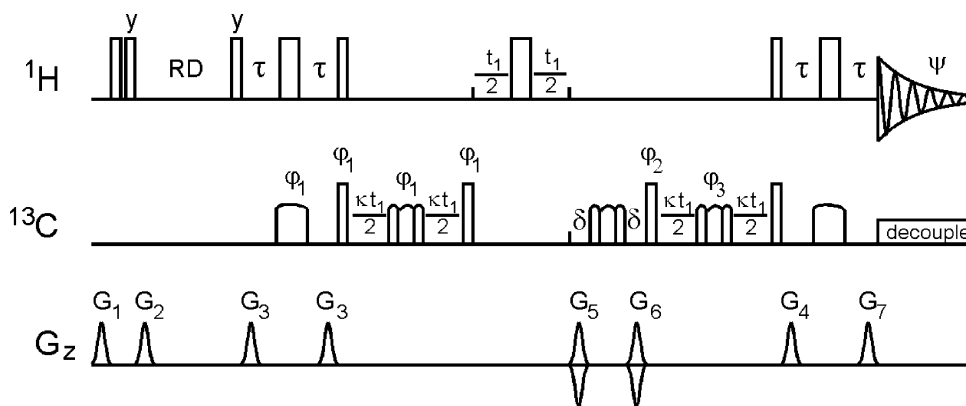

Fig. 1. Pulse sequence of DJM-INEPT-INADEQUATE. Thin and thick open bars represent 90° and 180° rectangular pulses. The first and the last ^{13}C 180° pulses were regular smoothened chirp pulses [14] (500 μs), while the other three ^{13}C 180° pulses were composite smoothened chirp pulses [14] (2 ms). Unless stated otherwise, all pulses were applied from the x direction. The following delays were used: $\tau = 0.25/^1J_{CH}$, $\delta = 1.2$ ms, RD = 1 s. The phases of pulses were cycled as follows: $\varphi_1 = x, y, -x, -y$; $\varphi_2 = 8x, 8(-x)$; $\varphi_3 = 4x, 4(-x)$; $\Psi = 2(x, -x, x, -x)$, $2(-x, x, -x, x)$. PFGs were applied during 1 ms using the following strengths (100% = 75 G/cm): $G_1 = 47\%$, $G_2 = 33\%$, $G_3 = 7\%$. $G_5 = 40\%$, $G_6 = -40\%$, and $G_4 = 3\%$, $G_7 = 43.16\%$ were used for N-type signal selection, while $G_5 = -40\%$, $G_6 = 40\%$, and $G_4 = 3\%$, $G_7 = 43.16\%$ where used for P-type signal selection. Spectra were processed using the echo–antiecho protocol.
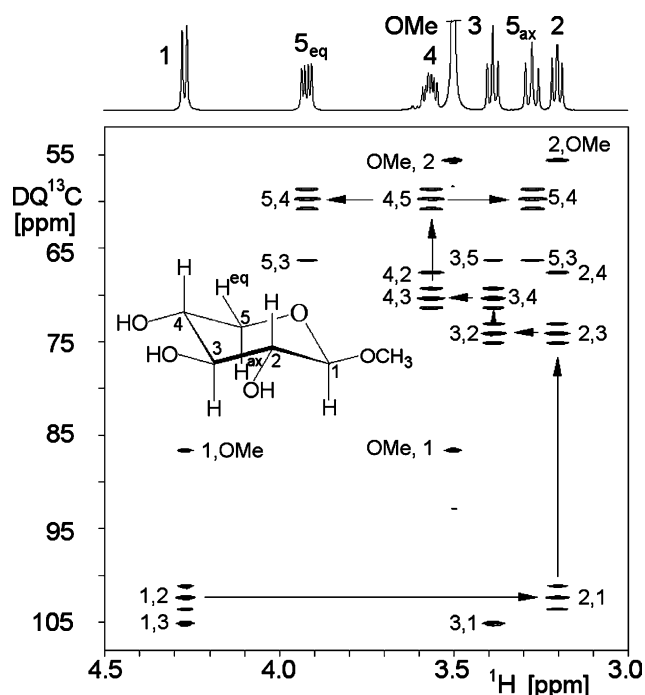
Fig. 2. A 2D DJM-INEPT-INADEQUATE spectrum of β-Me-xylopyranoside (**I**). Cross-peaks are numbered according to the structure of **I** shown in the inset. The first number in the labels of ^{13}C DQ coherences identifies the proton on which the magnetization originated/is detected. Arrows indicate the carbon–carbon connectivities mediated by $^1J_{CC}$ coupling constants. For experimental details see Section. 4.

Fig. 3. F_1 traces from 2D DJM-INEPT-INADEQUATE spectra at ^{1}H chemical shifts of H_3 (A and C) and H_2 (B and D), respectively. The pulse sequence of Fig. 1 with rectangular (A and B) and adiabatic (C and D) 180° ^{13}C pulses were used to acquire the 2D spectra. (E and F) Equivalent traces from a 2D JM-INEPT-INADEQUATE spectrum optimized for $^1J_{CC}$. For details see Section. 4. Cross-peaks are labeled using the numbering shown in Fig. 2.

the DJM-INEPT-INADEQUATE spectra display fine structures (Figs. 2 and 3) from which the values of J_{CC} coupling constants can be extracted as discussed below.

Incrementation of a single interval in *J*-modulated INADEQUATE/ADEQUATE experiments leads to a sine [12] or cosine [13] modulation of the signal, producing anti phase or inphase multiplets. When two such delays are incremented simultaneously, the signal becomes modulated by $\sin^2(\pi J_{CC}\kappa t_1)$, where κ is the scaling-up factor of the *J*-evolution. Using a trigonometric identity, such modulation can also be expressed as $0.5[1 - \cos(\pi 2J_{CC}\kappa t_1)]$. It is now readily seen that the cross-peaks in DJM-INEPT-INADEQUATE spectra consist of three absorption lines in F_1. The two outer lines are separated by $2J_{CC}\kappa$ Hz and have opposite phases relative to the central line that is twice as intense. Bearing in mind that we want to sample coupling constants as small as 2 Hz, we have chosen t_1 acquisition time of 95 ms and $\kappa = 4$. This leads to a maximum *J*-modulation time of 380 ms.

The adiabatic pulses in INADEQUATE/ADE-QUATE-based experiments are essential for compounds with a large spread of carbon resonances [14]. When used in DJM-INEPT-INADEQUATE experiments, these 2 ms composite adiabatic pulses [14] cause some initial ($t_1 = 0$) coupling evolution during the *J*-evolution periods. This is reflected in the shapes of cross-peaks
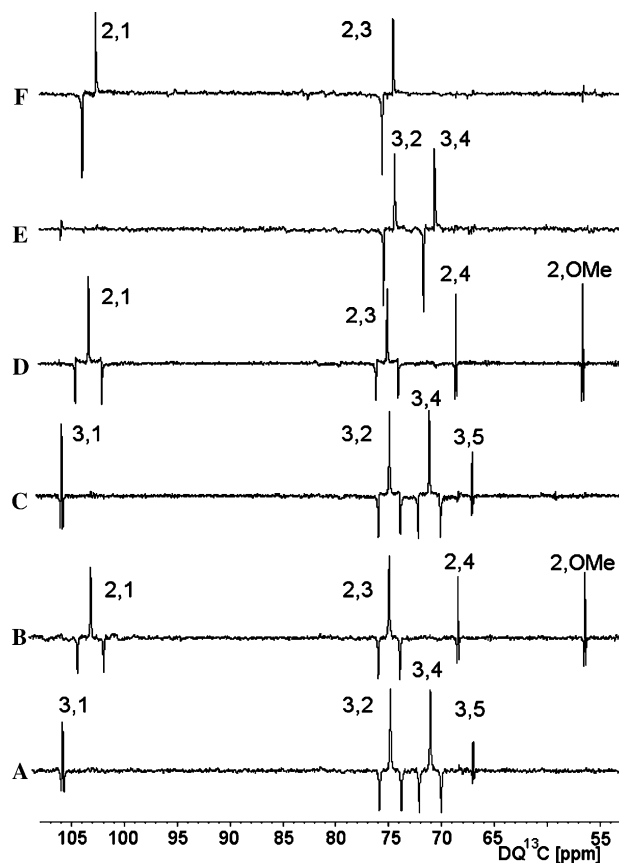
(comp. Figs. 3A, B vs. C, and D), but as shown below, it does not interfere with the analysis of coupling constants.

Sensitivity of the DJM-INEPT-INADEQUATE was compared with that of the JM-INEPT-INADEQUATE [11] optimized for $^1J_{CC}$. In the latter experiment only the initial J_{CC} evolution interval is incremented together with the ^{13}C DQ coherence labeling interval, while the refocusing interval is fixed at 10 ms. This leads to the appearance of antiphase doublets in F_1 separated by $\kappa*^1J_{CC}$. When comparing both spectra, we find that the height of the outer lines of DJM-INEPT-INADE-QUATE multiplets is half that of the antiphase JM-IN-EPT-INADEQUATE doublets (compare Figs. 3C, D vs. E, and F). Therefore, if we consider only the measurement of J_{CC} coupling constants, the new experiment is two times less sensitive. When the DJM-INEPT-INADE-QUATE experiment is used solely for establishing the CC correlations, it is the central line of the multiplets that matters. This line has the same intensity as the antiphase doublets in the single *J*-modulated spectra. By extrapola-

tion we can therefore say that the DJM-INEPT-INADE-QUATE experiment is two times less sensitive then the regular INEPT-INADEQUATE experiment without any J-evolution interval. This gap in sensitivity is reduced for long-range cross-peaks in a J-dependent manner because of the larger spread of $^nJ_{CC}$ coupling constants and the fact that there are no fixed intervals in the DJM-INEPT-INADEQUATE experiment. Thus, one of the merits of the proposed experiment is its ability to provide correlations via both $^1J_{CC}$ and $^nJ_{CC}$ coupling constants at the same time, albeit with halved sensitivity. Other advantages concerning the extractions of coupling constants are discussed later.

The analysis of the coupling constants is performed in the time domain, thus eliminating the effects of a limited digital resolution achievable in the t_1 dimension of 2D experiments. The initial 2D Fourier transformation is followed by extraction of columns containing signals (Fig. 4). Each multiplet excised from these F_1 traces is

then inverse-Fourier transformed. The fast DQ frequency component of the resulting FID is then removed by applying a procedure described by Stonehouse and Keeler [15], which we have modified to include phase optimization by using the Powell minimization algorithm. This leads to a FID that can be described as

$$I_o \times \sin^2(\pi J_{CC}\kappa t_1 + \varphi) \times \exp(-t_1/T_{2eff}), \qquad (1)$$

where I_o is the scaling factor, φ is the phase shift due to non-zero initial coupling evolution, and $T_{2\,eff}$ is the effective transverse relaxation. Although various coherences exist during the J- and DQ-evolution intervals, we find that a single exponential term describes the relaxation adequately. Using the Powell minimization method, a four-parameter fit (I_o, φ, $T_{2\,eff}$ and $J_{CC}\kappa$) yields the values of J_{CC}. When this protocol was applied to the DJM-INEPT-INADEQUATE spectra of **I**, the results were highly reproducible (Table 1). The literature values for $^1J_{CC}$ one-bond coupling constants [16], agreed exactly
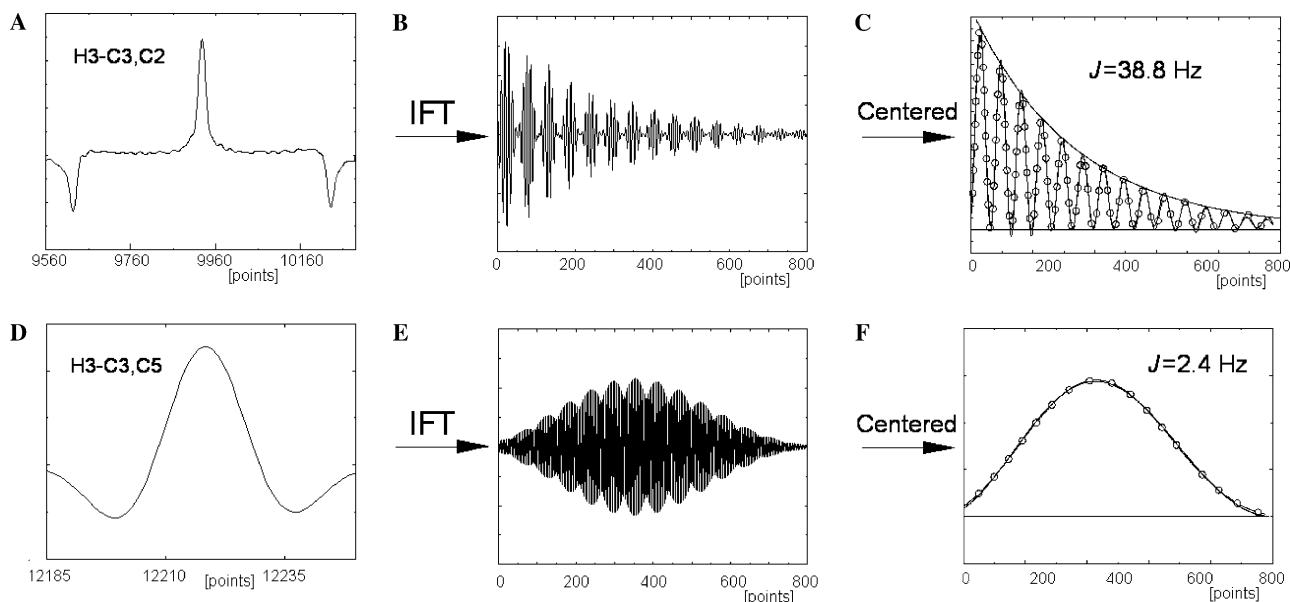

Fig. 4. Two examples of the coupling constant determination from 2D DJM-INEPT-INADEQUATE cross-peaks. (A and D) C3,C2 and C3,C5 cross-peaks, respectively, excised from the H_3 F_1 trace of the spectrum shown in Fig. 2. (B and E) 800 real points of FIDs obtained after inverse Fourier transformation of the multiplets (A and D), respectively. The centered FIDs obtained by deconvolution with DQ frequencies are shown in (C and F). Also shown are the fitted centered FIDs (–○–) obtained using Eq. (1). The exponential decay function in (C) was drawn using the value $T_{2\,eff} = 297$ ms obtained by the fitting.

Table 1
Carbon–carbon coupling constants of **I**[a], in Hz, determined from the spectrum shown in Fig. 2

	C_1C_2	C_2C_3	C_3C_4	C_4C_5	C_1C_3	C_1OMe	C_2C_4	C_2OMe	C_3C_5
$J(C_xC_y)$	46.8	38.8	39.1	40.0	4.1	2.3	2.9	3.2	2.4
	46.8	38.8	39.0	39.9[b]	4.0	2.4	2.9	3.2	2.2[b]
	46.8[d]	38.8[d]	39.1[d]	39.8[c]					2.3[c]
				39.9[d]					

[a] The first row gives values determined from $H_xC_xC_y$ cross-peaks, while the second corresponds to the H_yC_x C_y cross-peaks.
[b] Determined from $H_{5\,eq}$ cross-peak.
[c] Determined from $H_{5\,ax}$ cross-peak.
[d] Values taken from [16].

with our data. Identical values were obtained in experiments using rectangular and adiabatic 180° ^{13}C pulses indicating that the phase of the signal is properly reproduced during the fitting. Encouraged by this result we have set out to investigate the effects of a combination of fast $T_{2\,eff}$ relaxation and poor signal-to-noise ratio (SNR) on the performance of our method.

The effect of relaxation was simulated by applying exponential line broadening prior to the Fourier transformation in t_1 dimension. A series of F_1-traces taken at the chemical shift of H_3 (Fig. 5) illustrates the effects of the exponential broadening of up to 30 Hz, which resulted in approximately four-fold reduction of $T_{2\,eff}$ relaxation times (from approx. 300 to 70 ms). The analysis of one-bond cross-peaks showed that deviations of determined coupling constants from $J_{LB} = 0$ values increased steadily with increasing line broadening to a maximum of ± 0.4 Hz for $J_{LB} = 30$ values (Fig. 6A). Keeping in mind that these results are particular for $^1J_{CC} \sim 40$ Hz and a scaling factor of $\kappa = 4$, we can conclude that the presented method provides $^1J_{CC}$ coupling constants accurate within to 1% for $T_{2\,eff}$ relaxation times as short as 70 ms.

To the contrary, unreliable results were obtained for small $^nJ_{CC}$ values when large line broadening was used causing increased cancellation of negative and positive lines in the long-range correlation cross-peaks (Fig. 6B). We have therefore established criteria that do not depend on $T_{2\,eff}$ or $^nJ_{CC}$ values and which allow to decide whether the obtained long-range coupling constant can be relied upon or not. The first criterion is based on the observation that the partial line cancellation increases the ratio, R_1, between the absolute intensities of the central and the two outer lines of a multiplet. Ideally, when there is no cancellation $R_1 = 2$ and reliable coupling constants are obtained when $R_1 \leqslant 3$. As a secondary criterion, when applicable, we postulate that the ratio of intensities of the central lines of $^1J_{CC}$ and $^nJ_{CC}$ cross-peak (R_2) must be $\leqslant 2$ (Fig. 5). Providing these criteria are met, the values of $^nJ_{CC}$ at larger line broadenings differ $< \pm 0.3$ Hz from $^nJ_{LB} = 0$ values.

These simulations point to another advantage of our method over singly J-modulated techniques [11]. Although the DJM-INEPT-INADEQUATE cross-peaks are more complicated, they provide an inherent check on the reliability of determined $^nJ_{CC}$ coupling constants. This is not the case for singly modulated INEPT-INADEQUATE [11] where antiphase doublets are
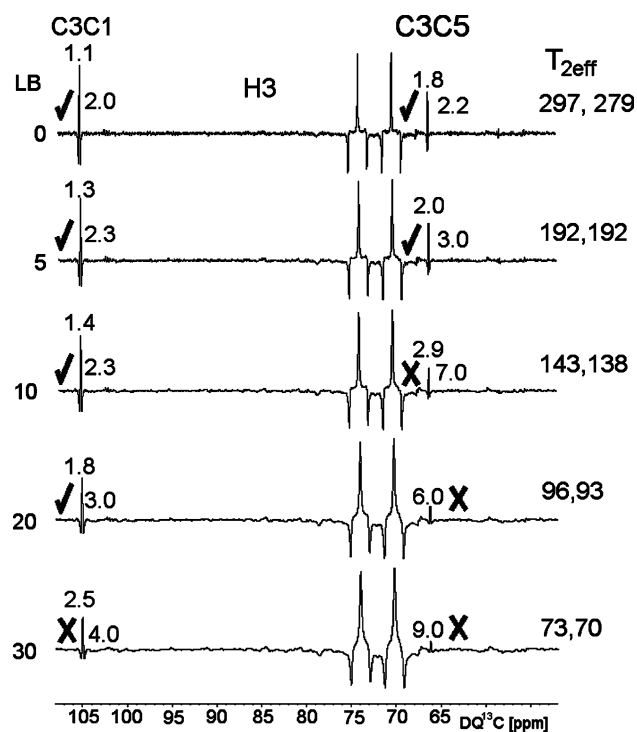

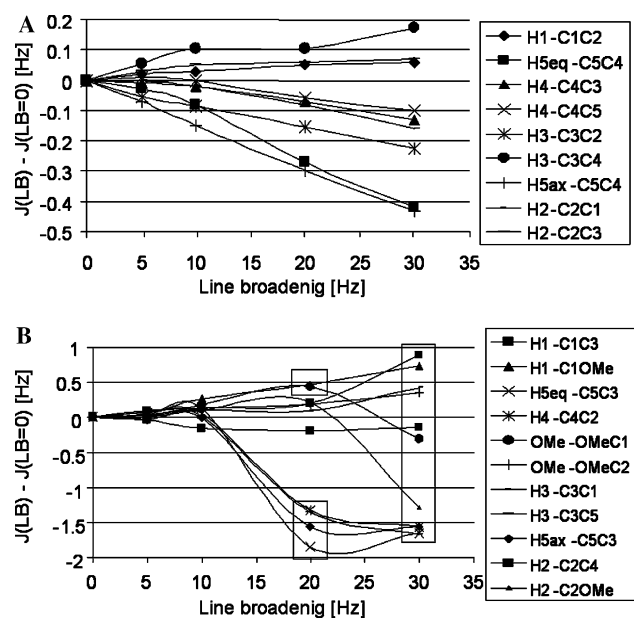Fig. 5. Effects of fast $T_{2\,eff}$ relaxation on the shape of long-range cross-peaks. Shown are H_3 F_1 traces extracted from spectra processed using increasing line broadening (LB). The effective spin–spin relaxation times, $T_{2\,eff}$ in ms, as determined by fitting the one-bond cross-peaks $H_3C_3C_2$ and $H_3C_3C_4$ are given on the right edges of the spectra. Numbers beside and above the long-range correlation cross-peaks give R_1 and R_2 ratios, respectively. For explanation see text. Symbol $\sqrt{}$ is used to indicate that $R_1 \leqslant 3$ and $R_2 \leqslant 2$ and thus the cross-speak is suitable for coupling constant determination; otherwise symbol $\times$ is used.

Fig. 6. Effects of fast $T_{2\,eff}$ relaxation on the determination of carbon–carbon coupling constants. (A) The deviations from $^1J_{CC}$ values determined using LB = 0 increase steadily with increasing line broadening (shorter $T_{2\,eff}$ relaxation times). This is most prominent for the coupling constants determined using H_5 protons due to fastest relaxation of coherences involving this CH_2 group. (B) The same as in (A) but for $^nJ_{CC}$ coupling constants. The values in rectangular boxes are from the analysis of cross-peaks that do not comply with the criteria for reliable determination of coupling constants as defined in the main text.

observed in F_1. It is well known that the peak-to-peak distances of broad antiphase doublets do not represent the true coupling constants, but it is not possible to ascertain the extent of the deviation without knowing the effective relaxation times of the coherences that give rise to these doublets. This problem has been recently addressed by acquiring in phase doublets in F_1 provided by the JM-ADEQUATE experiment [13]. This technique, however, depends on achieving sufficient separation of the two lines of in phase doublets and also lacks an internal indicator of the veracity of the extracted couplings.

Lastly, computer assisted extraction of coupling constants allowed us to use adiabatic ^{13}C pulses that are essential for the use of the INADEQUATE at higher fields [14]. The resulting mixed phase multiplets are easily handled by the computer, but are not compatible with methods that rely on simple peak picking as a means for determining the coupling constants.

We have deliberately used more compound than was necessary when developing this method and subsequently simulated the effects of using a smaller quantity of sample. These simulations, performed as described in Section. 4, also accounted for the faster relaxation that would be found for larger molecules. For $^1J_{CC}$ coupling constants the r.m.s.d., as determined by using five different noise spectra, increased exponentially with decreasing SNR (Fig. 7A), but even for SNR = 10 and

LB = 30 Hz these were <0.25 Hz i.e., always smaller than the systematic errors caused by fast relaxation as discussed above. The r.m.s.d. for the $^nJ_{CC}$ coupling constants followed a similar trend but increased faster with increasing line broadening and decreasing SNR (Fig. 7B). For SNR $\geqslant$ 20 the r.m.s.d. were < 0.25 Hz.

Overall, as expected, the $^1J_{CC}$ coupling constants are less sensitive to faster relaxation and poor SNRs and their values can be determined with high accuracy ($\pm$0.25 Hz) when $T_{2\,eff} \sim 70$ ms and SNR $\geqslant$ 10. When considering small long-range coupling constants ($\sim$2 Hz) a similar absolute accuracy requires $T_{2\,eff} \geqslant 100$ ms and SNR $\geqslant$ 20. This means that the sample quantity (71 mg in our experiments) can be reduced 3–4 times whilst still providing reliable values of coupling constant values. Our simulations take into account the faster relaxation of molecules larger than a monosaccharide and thus provide a good data on which the applicability of our method to large systems can be assessed.

We have seen that, particularly for long-range coupling constants, the reliability of our methods increases with increasing relaxation times. There are several experimental factors that can be controlled aiming at increasing this quantity, such as viscosity, temperature and the gradients strengths. The last point refers to the fact that during the second J-modulation interval molecules diffuse whilst dephased by PFGs. It is therefore advisable to keep the gradient strengths at minimal values which are compatible with sufficient purging of the ^{12}C-attached protons. For organic solvents especially, it is beneficial to decrease the temperature, as at elevated temperatures the diffusion related decrease in $T_{2\,eff}$ relaxation could be significant. As this can increase the viscosity and thus shorten the relaxation times, a compromise needs to be found. This can be conveniently done by recording gradient-selected ^1H–^{13}C HSQC experiments and extracting the $T_{2\,eff}$ from F_1 interferograms. Similarly to the DJM-INEPT-INADEQUATE experiment, the $T_{2\,eff}$ values determined in this way contain the contribution of proton spin-flips and PFGs to the overall relaxation. Another practical comment concerns the pulse sequence repetition rates. These are dictated by T_1 relaxation of ^{13}C-attached protons which can, at least for CH protons, be several times shorter than those of ^{12}C-attached protons. At concentrations used for the INADEQUATE experiments it is trivial to estimate such relaxation times by recording a series of 1D inversion recovery spectra while selecting the one-bond ^1H–^{13}C satellites.

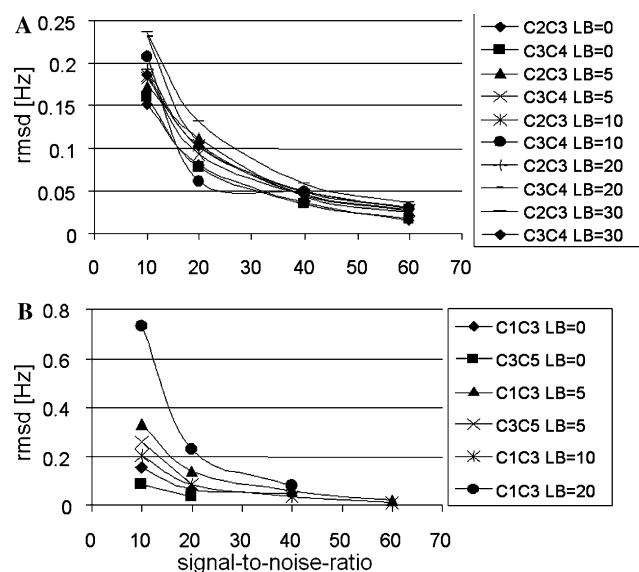Fig. 7. Effects of decreasing signal-to-noise ratios on values of carbon–carbon coupling constants. (A) $^1J_{CC}$ coupling constants. Standard deviations of $^1J_{C3,C2}$ (38.8 Hz) and $^1J_{C3,C4}$ (39.1 Hz) coupling constants obtained by the analysis of spectra processed with different line broadenings as a function of the increasing SNR. For details see Section. 4. (B) The same as (A) but for of $^2J_{C3,C1}$ (4.0 HZ) and $^2J_{C3,C5}$ (2.4 HZ) coupling constants. Only cases which met the criteria for the analysis of $^nJ_{CC}$ coupling constants, as defined in the main text, were considered. Higher SNRs were not available for some of the cross-peaks.

3. Conclusions

In conclusion, we have presented a robust method for the simultaneous detection of carbon–carbon connectivities mediated by $^1J_{CC}$ and $^nJ_{CC}$ coupling constants,

which at the same time also provides values of carbon–carbon coupling constants. This high information content of the DJM-INEPT-INADEQUATE experiment comes at a cost. As only one-half of the signal passes through each of the *J*-modulated intervals, the signal-to-noise ratio (SNR) is reduced by half compared to the standard INEPT-INADEQUATE experiment. This however assumes that both carbon–carbon coupling evolution intervals of the INEPT-INADEQUATE can be matched for either all $^1J_{CC}$ or $^nJ_{CC}$ values, which is not possible because of the large spread of these coupling constants. The relative sensitivity of the DJM-INEPT-INADEQUATE, with no fixed carbon–carbon coupling evolution intervals, therefore automatically increases. This is further improved by the fact that only one spectrum needs to be acquired to obtain both the one-bond and long-range carbon–carbon connectivities. Thus, instead of performing multiple experiments, a single DJM-INEPT-INADEQUATE experiment can provide a wealth of information for the structural analysis of small molecules. We have shown that, for ca. 20 mg of a monosaccharide, acquisition over 24 h on a 600 MHz cryoprobe is sufficient to obtaining data suitable for coupling constant analyses. Our simulations indicate that a similar performance is to be expected for larger molecules, albeit with the caveat that additional material would be required. We have also developed a robust, computer assisted method for extraction of coupling constants from DJM-INEPT-INADEQUATE spectra that contains an inherent check on the reliability of the determined coupling constants. We note that the increased number of cross-peaks in DJM-INEPT-INADEQUATE spectra can lead to spectral overlap for larger molecules and further tests, on establishing the merits of the proposed method using a ∼500 g/mol compound in an organic solvent, are planned and will be reported elsewhere.

4. Materials and methods

All spectra were recorded on an Avance 600 MHz (Bruker) equipped with a 5 mm cryoprobe with inverse geometry and *z*-gradients. β-Me-xylopyranoside, I, was purchased from Sigma–Aldrich; 71 mg were dissolved in 320 μl of D_2O and put to a Shigemi NMR tube. The ^{13}C chemical shifts of I, from C_1 to C_5, are: 105.3, 75.6, 78.5, 71.9, 67.8, and 59.9 ppm (OMe). 2D DJM-INEPT-INADEQUATE spectrum (Fig. 2) was acquired using 32 scans in each of 800 complex t_1 increments. Acquisition times in t_1 and t_2 were 94.7 and 106 ms. The relaxation time was 1 s; the total acquisition time was 23 h. Adiabatic pulses described in [14] were used as 500 μs (inversion) and 2 ms (refocusing) pulses. The 2D JM-INEPT-INADEQUATE spectrum used for the comparison of the sensitivity of the new method was ac-

quired using identical sample and experimental conditions to those described above, with the single difference that the second incrementable period of the DJM-INEPT-INADEQUATE was fixed at 10 ms. The effect of increased noise on the accuracy of the coupling constant determination was investigated using two one-bond ($^1J_{C3, C2}$ and $^1J_{C3, C4}$) and two long-range ($^2J_{C3, C1}$ and $^2J_{C3, C5}$) cross-peaks from H_3 F_1 traces of the spectrum shown in Fig. 2. Artificial 1D traces were created in which SNRs were adjusted to 60, 40, 20, and 10:1 by addition of scaled-up traces with no signal taken from a 2D spectrum processed with zero line broadening. This procedure was performed individually for each cross-peak extracted from spectra processed with LB values of 0, 5, 10, 20, and 30 Hz in F_1 and repeated five times using different noise spectra. An average coupling constant and r.m.s.d. were calculated for each SNR and a given line broadening.

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