

Fast measurement of heteronuclear relaxation: frequency-domain analysis of NMR accordion spectroscopy[†]

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NMR relaxation parameters are usually derived from series of 2D experiments. The whole procedure can be very time consuming, especially for the study of the relaxation of nuclei at natural abundance. Palmer and Mandel have proposed the use of accordion spectroscopy to determine one relaxation parameter using two experiments only. In this paper, we show that the experimental time can be further reduced, by recording only three experiments for the determination of both the longitudinal and transverse relaxation rates. The analysis of the experiments is performed in the frequency domain, the relaxation rates being deduced from the linewidth of the peaks of interest. A detailed statistical analysis of errors introduced by the line fitting procedure on derived relaxation parameters was used to derive guidelines for the choice of experimental parameters. This procedure was applied to the study of the C α relaxation parameters of a six-residue unlabeled peptide. The results were compared with those obtained by classical accordion spectroscopy. Copyright © 2001 John Wiley & Sons, Ltd.

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INTRODUCTION

NMR relaxation data provide valuable insights into protein dynamics. Heteronuclear experiments that measure the relaxation rates of amide ¹⁵N nuclei have been widely used to identify functionally important motions in proteins,¹ to detect dynamic changes upon ligand binding (e.g. metal ion binding to calbindin^{2–6}) or to derive structural information such as the N—H vector orientation in the molecular frame.⁷ The description of dynamics changes that arise upon ligand binding provides detailed information about the relationship between protein stability, dynamics and function.^{8,9} More recently, there has been a growing interest in probing changes in protein dynamics induced by variations of physical parameters such as temperature^{10–12} or pressure,¹³ providing valuable insights into the thermodynamic aspects of protein motions. However, one of the major bottleneck of these studies is the duration of the relaxation experiments. Despite many improvements both in the technology of the NMR spectrometers (such as the use of pulse field gradients) and in experimental procedures (such as indirect detection of low-abundance X nuclei through protons), relaxation measurements remain very time consuming and further developments are needed.

The spin–lattice [$R_X(X_z)$ or R_1] and spin–spin [$R_X(X_{xy})$ or R_2] relaxation parameters of the heteronuclei X are usually derived from a series of 2D experiments recorded with different relaxation delays t_x .^{14,15} In these protocols, the relaxation rate constant is computed from the exponential decay of the intensity of the magnetization as a function of the time t_x . In effect, these experiments can be seen as pseudo-3D relaxation experiments, for which the first dimension is described by the acquisition time t_2 , the second dimension corresponds to the frequency sampling time t_1 and the third dimension is labeled by t_x , the relaxation delay. Accordion spectroscopy, which was initially proposed by Bodenhausen and Ernst¹⁶ and developed by Mandel and Palmer,¹⁷ appeared as a viable and efficient alternative. Basically, an accordion experiment reduces the dimensionality of the pseudo-3D relaxation experiment mentioned above by co-varying t_x and t_1 within a single 2D experiment. The relaxation parameters [$R_X(X_z)$ or $R_X(X_{xy})$] are embedded in the linewidth of the corresponding individual peaks in the indirect dimension of the heteronuclear correlation spectrum. Two types of experiments have been proposed, namely ‘forward’ and ‘reverse,’ in which the two times t_x and t_1 are incremented synchronously, or in opposite direction, respectively. In the procedure described by Mandel and Palmer, the measurement of each relaxation parameter [$R_X(X_z)$ or $R_X(X_{xy})$] requires the acquisition of one ‘forward’ and one ‘reverse’ experiments, resulting in the need to record four experiments to derive the two relaxation parameters.

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[†]This work is dedicated to the memory of Jean-François Lefèvre.

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In this paper, we suggest that only three experiments (two 'forward' or two 'reverse,' and one reference) are needed to measure the two relaxation rates $R_X(X_z)$ and $R_X(X_{xy})$. The method is described in detail after a brief overview of accordion spectroscopy. We also describe a new method for extracting spectral parameters from a 1D NMR experiment, which we refer to as REFCOL. A statistical analysis of the inherent errors of this line fitting procedure is provided, and the results of this study are used to derive guidelines for the choice of accordion experiment parameters. Finally, we apply our method to the study of the ^{13}C relaxation parameters at natural abundance of a short linear peptide containing six residues (KQAGDV), and the corresponding results are compared with those obtained from the 'classical' accordion spectroscopy protocol proposed by Mandel and Palmer.

THEORY

Background: principles of accordion spectroscopy

The typical scheme of pulse sequences used for heteronuclear spin-lattice [$R_X(X_z)$] and spin-spin [$R_X(X_{xy})$] relaxation rate constants measurements is^{14,15}:

Magnetization transfer from H to X INEPT	Preparation of X magnetization state	X relaxation time t_x	X frequency sampling time t_1	Magnetization back transfer from H to X INEPT	Acquisition t_2
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The concept of accordion spectroscopy is to co-vary the frequency sampling time t_1 and the relaxation time t_x during the same experiment. The t_x time is thus projected on to the t_1 axis of the 2D experiment. The magnetization evolves during t_x under $R_X(X_z)$ or $R_X(X_{xy})$ and during t_1 under transverse relaxation, including inhomogeneity in the magnetic field, described by R_2^* .

In an accordion experiment, the signal at the resonance frequency $f = \omega/2\pi$ is given by:

$$S(k) = S_0 \exp[-R_X(D)k\Delta t_x] \exp(-R_2^*k\Delta t_1) \exp(-i\omega k\Delta t_1) \quad (1)$$

in which S_0 is the amplitude of the magnetization at the beginning of the relaxation period, k ranges from 1 to N , where N is the total number of complex points sampled in the t_1 dimension and $R_X(D)$ is the relaxation rate constant to be determined, with D describing the magnetization state which is considered (X_z or X_{xy}).

The ratio of the two increments Δt_1 and Δt_x defines the scaling factor κ :

$$\kappa = \frac{\Delta t_x}{\Delta t_1} \quad (2)$$

A 'forward' experiment is characterized by the synchronous increments of the two variable delays t_1 and t_x whereas in a 'reverse' experiment the t_x delay is decreased while t_1 is incremented. The signal of a 'forward' experiment is defined by

$$S^{\text{fw}}(k) = S_0 \exp[-(\kappa^{\text{fw}}R_X(D) + R_2^*)k\Delta t_1] \exp(-i\omega k\Delta t_1) \quad (3)$$

The apparent damping factor of this signal R^{fw} is defined by

$$R^{\text{fw}}(D) = \kappa^{\text{fw}}R_X(D) + R_2^* \quad (4)$$

Similarly, the signal of a 'reverse' experiment is defined by

$$S^{\text{rev}}(k) = S_0 \exp[-(N+1)R_X(D)\Delta t_x] \exp\{-[R_2^* - \kappa^{\text{rev}}R_X(D)] \times k\Delta t_1\} \exp(-i\omega k\Delta t_1) \quad (5)$$

The apparent damping factor R^{rev} of the signal is defined by

$$R^{\text{rev}}(D) = -\kappa^{\text{rev}}R_X(D) + R_2^* \quad (6)$$

Usually $\kappa R_X(D)$ is chosen to be greater than R_2^* , thus the signal increases as a function of time in the 'reverse' experiment. Rather than adapting the signal processing to the analysis of a signal containing increasing exponential functions, the time axis of the FID is reversed, which generates a pseudo-FID whose components have apparent damping factors given by

$$R^{\text{rev}}(D) = \kappa^{\text{rev}}R_X(D) - R_2^* \quad (7)$$

Combining Eqns (4) and (7), we obtain

$$R_X(D) = \frac{\pi}{(\kappa^{\text{fw}} + \kappa^{\text{rev}})} [lw^{\text{fw}}(D) + lw^{\text{rev}}(D)] \quad (8)$$

where $lw^{\text{fw}}(D)$ and $lw^{\text{rev}}(D)$ are the linewidths of the cross peaks corresponding to the nucleus X in a 2D 'forward' and a 2D 'reverse' accordion experiment, respectively. The linewidths lw are related to the apparent damping factors by

$$R(D) = \pi lw(D) \quad (9)$$

Determination of $R_X(D)$ through Eqn (8) therefore requires that two accordion experiments have to be acquired. Each of these experiments is specific to the choice of D (i.e. spin-lattice relaxation for $D = X_z$ and spin-spin relaxation for $D = X_{xy}$) and the total number of experiments required to derive both $R_X(X_z)$ and $R_X(X_{xy})$ is therefore four.

A simplified scheme for accordion spectroscopy

If the total relaxation time t_x in one of the experiments described above is set to zero, each spin of the molecule under study relaxes only with the relaxation rate R_2^* . Such a reference experiment can be combined with one of the accordion experiment (forward or reverse), and this combination provides a new method to derive the relaxation rates $R_X(D)$:

$$R_X(D) = \frac{\pi}{\kappa^{\text{fw}}} [lw^{\text{fw}}(D) - lw^{\text{ref}}] = \frac{\pi}{\kappa^{\text{rev}}} [lw^{\text{rev}}(D) + lw^{\text{ref}}] \quad (10)$$

where ref denotes reference.

It is worth noting that lw^{ref} is independent of D , and can therefore be used for the determination of both $R_X(X_z)$ and $R_X(X_{xy})$. As a result, only two 'forward' (or two 'reverse') experiments and one 'reference' experiments are required to derive $R_X(X_z)$ and $R_X(X_{xy})$. This can provide a theoretical reduction in experimental time of 25% compared with classical accordion spectroscopy, a significant gain in the case of natural abundance spectroscopy.

METHODS

Extracting spectral information from an NMR accordion experiment

A 2D accordion experiment is designed such that the relaxation rate $R_X(X_z)$ or $R_X(X_{xy})$ is encoded in the linewidth of the cross peak observed for the nucleus X in a 2D relaxation experiment. Success of the method therefore relies on accurate measurements of peak linewidths in a 2D experiment.

A direct estimate of the linewidth of a peak by counting the number of points it covers is usually inaccurate, owing to peak overlap, phase and baseline problems, as well as resolution issues. The same factors make non-linear least-squares fits in the spectral domain equally difficult. In the latter case, it should be noted also that an analytical model for the shape of the peak is usually not known when the FID has been apodized prior to Fourier transformation. Apodization is usually required in the reconstructed dimension of the spectrum, to avoid artifacts due to truncation of the FID. A non-linear least-squares fit of the FID in the time domain is

not easier, since the latter usually contains a large number of components. An alternative approach would be to analyze the FID by means of quantitative linear prediction. This method, however, has been shown to yield poor estimates of peak linewidth.¹⁸

In order to address these issues, we propose the use of a hybrid non-linear least-squares method for spectral parameter determination, in which the model function is defined in the time domain, and the residual (or χ^2) that is minimized is computed in the spectral domain. An outline of the method is given in Fig. 1. It proceeds as follow:

- (i) The region $R(\text{exp})$ of the complex experimental spectrum containing the P peaks of interest is extracted and stored.
- (ii) A pseudo-complex FID $s(t)$ containing P peaks is built, based on

$$s(k\Delta t) = \sum_{i=1}^P A_i \exp(j\varphi_i) \exp(-j\omega_i k\Delta t) \exp(-R_i k\Delta t) \quad (1 \leq k \leq N) \quad (11)$$

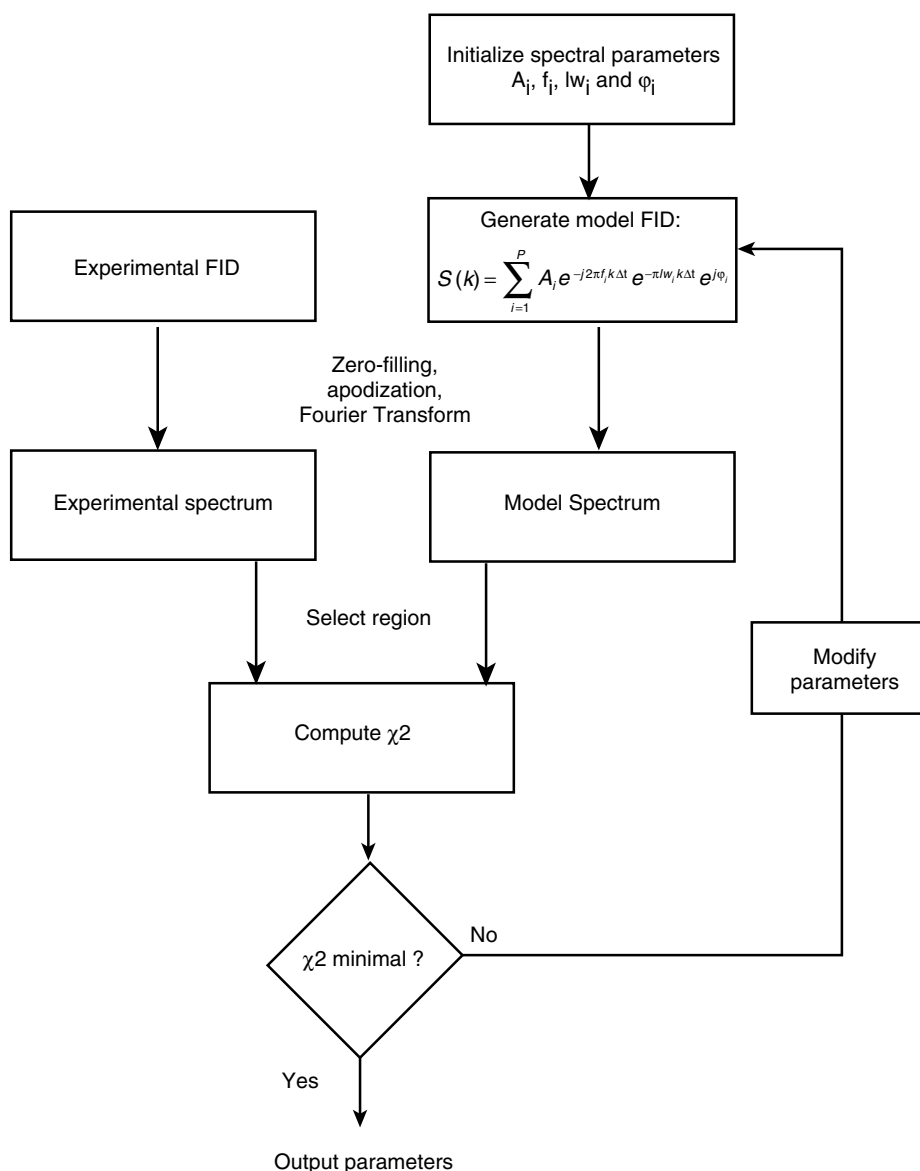


Figure 1. Outline of the program REFCOL. The parameters A_i , f_i , lw_i and φ_i are described in the text. Δt is the dwell time.

where A is the amplitude, φ the phase, $f = \omega/2\pi$ the frequency and R the damping factor. Δt , the dwell time, and N , the total number of points of this computed FID, are set equal to their corresponding values in the experimental FID.

- (iii) The pseudo-FID is padded with zeros, apodized and Fourier transformed based on the exact parameters that were used to process the experimental FID.
- (iv) The corresponding region $R(\text{model})$ of the model spectrum is extracted, and compared with $R(\text{exp})$. If they are statistically similar, the procedure is stopped, and the spectral parameters of the N peaks, including the linewidth, are given as output. If these regions differ, new values are given for the spectral parameters in Eqn (11), and the procedure is iterated at step (ii). The Powell minimization procedure¹⁹ is used for parameter update.

The complete procedure has been implemented in a FORTRAN program, REFCOL. Figure 2 shows an illustration of its application on an experimental FID.

Statistics on linewidth measurements

The precision and accuracy of the linewidth estimates provided by REFCOL were assessed on synthetic data. One hundred independent REFCOL calculations were performed on synthetic FIDs, which usually contained a single peak and differed only in their noise sequence. The mean, lw_{mean} , and standard deviation, σ , of the corresponding distribution of linewidth estimates were computed. We consider a global measure of the accuracy and precision of these estimates:

$$\sigma_{\text{tot}}^2 = \frac{1}{K} \sum_{i=1}^K [lw(i) - lw_{\text{true}}]^2 \quad (12)$$

where lw_{true} is the 'true' value of the linewidth, given as input to generate the FID, and K the number of calculations. σ_{tot} differs from σ as follows:

$$\sigma_{\text{tot}}^2 = \sigma^2 + (lw_{\text{mean}} - lw_{\text{true}})^2 \quad (13)$$

The first term of the right-hand side of Eqn (13) is the true variance, which measures the precision, and the second term is the bias, which measures the accuracy of the estimate of the linewidth.

In the following, all references to standard deviation will in fact correspond to σ_{tot} .

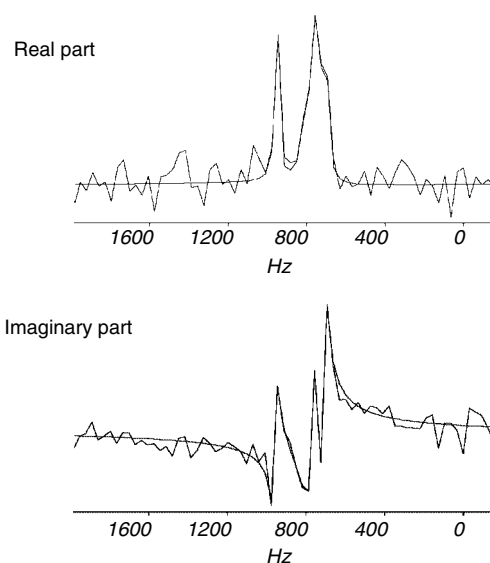
Computing synthetic FIDs

Noise-free FIDs were generated based on Eqn (11). A noise-corrupted signal was generated by adding independent white Gaussian sequences to the real and imaginary parts of these interferograms. The level of noise is characterized by the signal-to-noise ratio (SNR), defined as

$$\text{SNR} = 10 \log \left(\frac{C_{\text{max}}^2}{\sigma^2} \right) \quad (14)$$

where C_{max} is the maximum amplitude of the peak.

'Forward' relaxation experiment



'Reference' experiment

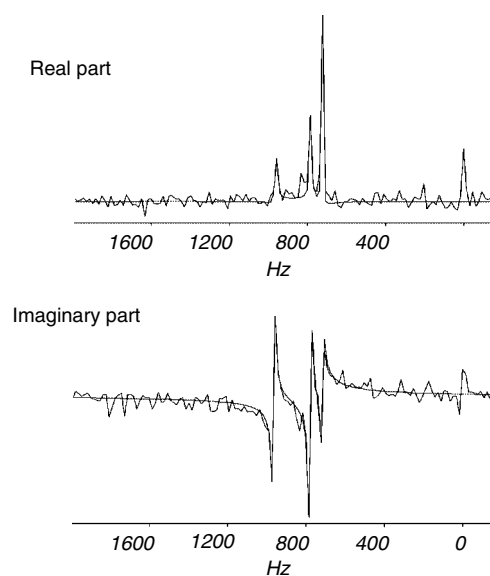


Figure 2. Fit of an experimental FID containing three ^{13}C resonances using the program REFCOL. The spectra were obtained from two experiments ('forward' accordion experiment with a scaling factor $\kappa = 12$ and 'reference' experiment) recorded at 30°C .

NMR experiments

All experiments were recorded on a Bruker AMX 500 instrument operating at 500.13 MHz for the proton.

The pulse sequences used to measure relaxation rates are shown in Fig. 3. They are derived from the framework published by Dayie and Wagner,¹⁴ with the following differences:

- (i) In all experiments, the total proton decoupling time (relaxation time t_x plus pre-relaxation time d_0) was maintained constant throughout the experiments by adding

an appropriate irradiation time during the recovery delay, in order to avoid temperature fluctuations.

- (ii) A single spin-echo sequence rather than a CPMG sequence was used in the $R_C(C_{xy})$ experiment. The CPMG sequence should however be preferred for the measurement of ^{15}N relaxation parameters for which the cross-correlation between dipolar and chemical shift anisotropy relaxation mechanisms is no longer negligible.^{20–22}

Unless indicated otherwise, FIDs in the t_2 dimension were defined with 2048 complex points. In the t_1 dimension, 128 and 256 complex points were acquired in the STATES mode²³ for the accordion and 'reference' experiments, respectively. Data were zero filled and processed by a 90° -shifted

sine-squared bell function prior to Fourier transformation in the acquisition dimension only. The final size of matrices were 2048×256 and 2048×512 points for the accordion and 'reference' experiments, respectively.

A test case: the six-residue peptide KQAGDV

To assess the validity of the method at natural abundance of carbon, we used a six-residue peptide, which is available to us at high concentration. The ^{13}C -H correlation spectrum contains no overlap. This peptide (KQAGDV) is the C-terminal sequence of the γ chain of fibrinogen, implicated in the binding of this protein to blood platelets during the process of aggregation. It represents the minimal sequence capable of inhibiting this binding.^{24,25}

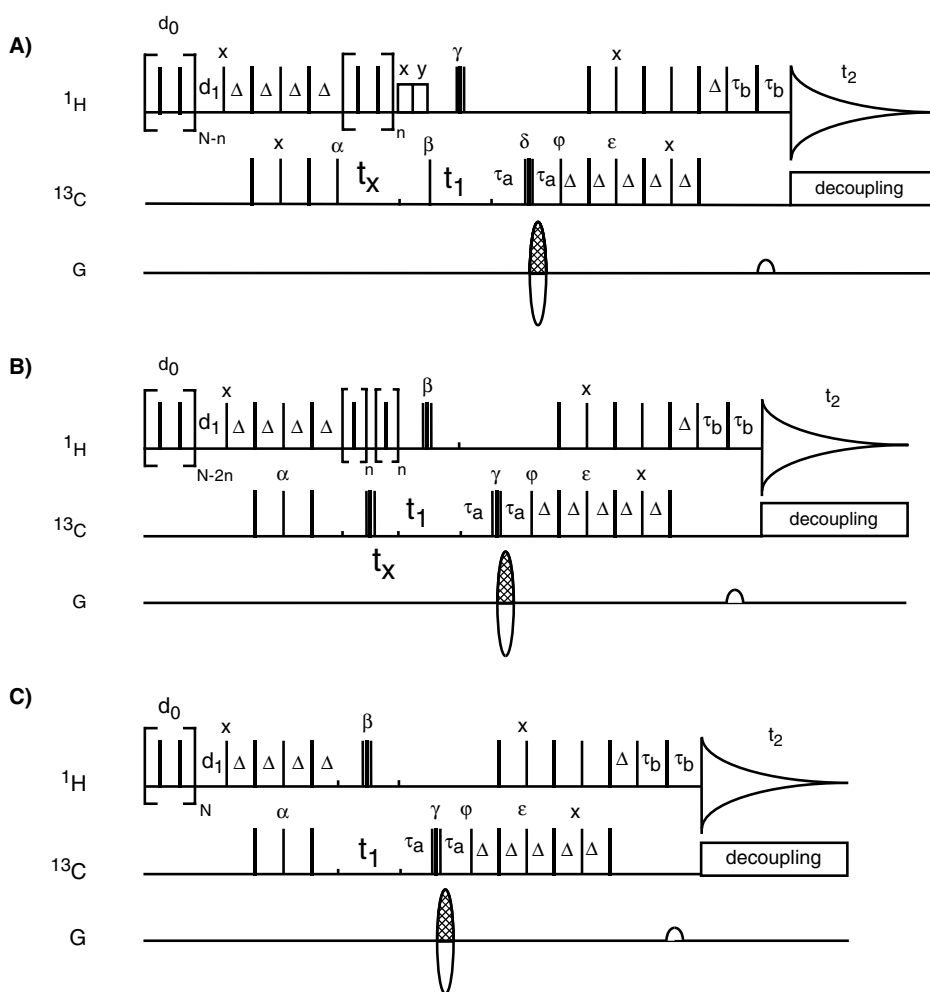


Figure 3. Pulse sequences used for ^{13}C relaxation rate constant measurement. (A) $R_C(C_z)$ measurement; (B) $R_C(C_{xy})$ measurement; (C) reference experiment. The phase cycling for sequence (A) is $\alpha(y-y)$, $\beta(xx-x-x)$, $\gamma(y)$, $\delta(x)$, $\epsilon(yy-y-y)$, $\phi(y)$ and $\text{rec}(x-x-x)$; for the sequences (B) and (C) it is $\alpha(x-x)$, $\beta(y)$, $\gamma(x)$, $\epsilon(yy-y-y)$, $\phi(y)$ and $\text{rec}(x-x-x)$; if not indicated, the phase is y . Δ is set to $1/4J$, where J is the H- ^{13}C coupling constant. The phase ϕ is inverted to $-y$ for even FIDs together with the first gradient. The gradient pulses were applied on the z-axis for a duration of $800\ \mu\text{s}$ with a recovery delay of $200\ \mu\text{s}$. In the $R_C(C_z)$ experiment, two trim pulses were applied after the relaxation delay for $1\ \text{ms}$ each. Typically, the proton decoupling pulses were applied at a power attenuation of $14\text{--}18\ \text{dB}$, which correspond to 90° proton pulses of 23 and $35\ \mu\text{s}$, respectively, and the delay between two 180° pulses was $200\ \mu\text{s}$; a relaxation delay d_1 after the decoupling sequence was set to $500\ \text{ms}$. The decoupling of carbon during acquisition was accomplished by a GARP sequence (90° carbon pulse $80\ \mu\text{s}$); the 180° composite refocusing pulse was obtained by the sequence $90_x^\circ\ 180^\circ\ 90_x^\circ$.

Experiments were recorded at 30 °C in D₂O, with a peptide concentration of about 15 mM. The 'forward' and 'reverse' experiments were acquired with 16 transients per FID. The spectral widths were set to 5000 and 4000 Hz in the ¹H and ¹³C dimensions, respectively. The scaling factor κ was set to 24 and 16 in the $R_C(C_z)$ and $R_C(C_{xy})$ experiments respectively. Total proton decoupling times were 774 ms and 516 ms for the $R_C(C_z)$ and $R_C(C_{xy})$ experiments, respectively. The reference experiment was recorded with eight transients and a proton decoupling time of 774 ms. A second 'reference' experiment with a proton decoupling time of 516 ms was recorded to ensure that the different proton decoupling times did not introduce significant deviation of linewidth values (data not shown).

RESULTS AND DISCUSSION

Statistical analysis of REFCOL line-fitting procedure

Linewidth estimates for an isolated peak

A peak P of an NMR spectrum is characterized by its frequency, $f(P)$, amplitude, $A(P)$, linewidth, $lw(P)$, and phase, $\varphi(P)$. Since the spectrum is discrete, this information can only be derived from the $N(P)$ points that describe P. $N(P)$ depends both on the digital resolution, DR , of the spectrum, and on the physical linewidth, $lw(P)$:

$$N(P) = \frac{lw(P)}{DR} = N_{\text{tot}} \frac{lw(P)}{SW} \quad (15)$$

where SW is the spectral width of the signal and N_{tot} the total number of points. Any method designed for extracting

the parameters that describe P is therefore dependent on $N(P)$. For example, it is expected that both small and large values of $N(P)$ will lead to large errors in the estimates of the linewidth: in the first case, the number of points is too small to provide enough information, whereas in the latter, a large number of points increases the proportion of noise included in the spectral region defined by P.

We first study the effect of both the exact value of the linewidth and the spectral width of the signal on the accuracy and precision of the linewidth estimates provided by REFCOL, for a fixed number of points N_{tot} . For this purpose, a simulated FID containing a single exponential function was generated. The amplitude of the peak was set to 100, its frequency to 40 points and its phase to 0°. Noise with an SNR of 15 dB was added to this simulated FID, as described in Methods. Series of 100 FIDs were generated for values of SW varying between 2000 and 10000 Hz and linewidth values varying between 15 and 80 Hz. Each FID, containing 128 points, was analyzed using REFCOL, and the total variance σ_{tot}^2 (see Methods) of the measured linewidth was computed. Results are shown in Fig. 4. A small value for SW defines a discrete spectrum with small, therefore good, digital resolution. In such cases, the smaller the linewidth of the peak P, the better it is estimated by REFCOL. Inversely, a large value for SW defines a spectrum with poor digital resolution. Peaks with small linewidth are then defined by a too small number of points, in which case REFCOL does not perform well. For a spectral width around 4000 Hz, the best results are obtained for peaks with linewidths around 20 Hz.

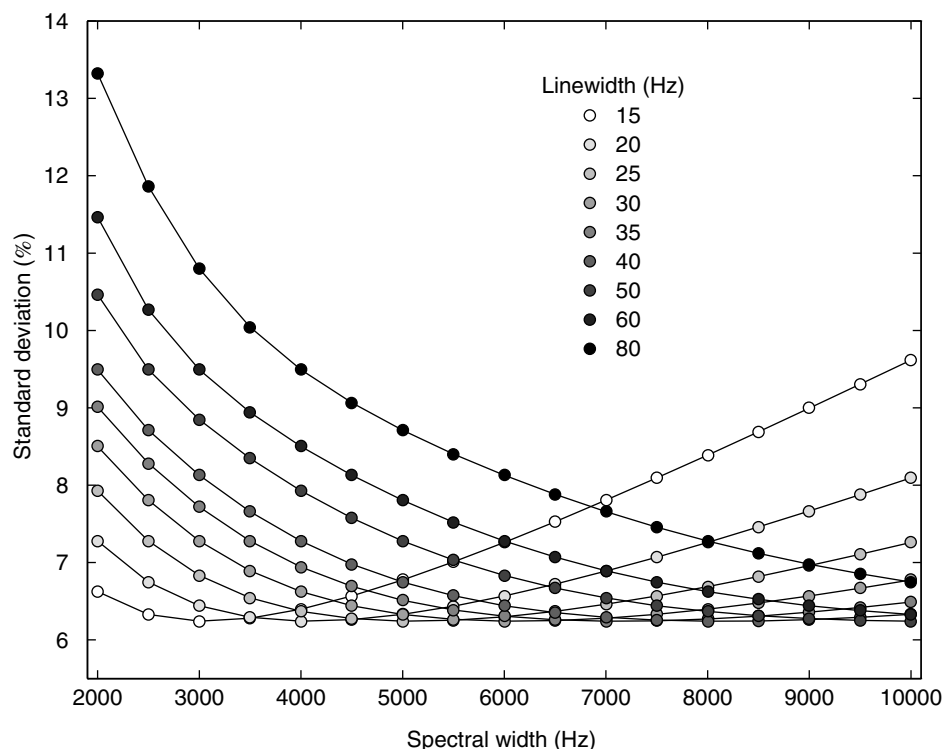


Figure 4. Accuracy and precision of the linewidth estimates provided by REFCOL versus the spectral width of the signal. Calculations were performed on series of 100 FIDs containing a single peak at the frequency $F = 40$ points. Each FID contained 128 complex points and random noise at 15 dB. The accuracy and precision were measured by means of a 'total' standard deviation σ_{tot} (see Methods), given as percentage of the actual linewidth.

Acquisition of a large number of points to describe an FID in the reconstructed dimension of a 2D experiment is time-consuming. In the case of heteronuclear NMR relaxation experiments at natural abundance, N_{tot} is usually set to 128 or 256. Such an FID can be artificially extended by simple zero-filling or by linear prediction. We report here the effect of the latter method on the precision and accuracy of the linewidth measured by REFCOL. Five series of 100 FIDs containing a single resonance ($f = 40$ points, $A = 100$, $lw = 25$ Hz, $\varphi = 0^\circ$) were generated containing 150, 165, 180,

210 and 256 'experimental' data points. Noise was added to each signal, with $SNR = 20$ dB. All FIDs containing N points, with $128 < N < 256$ were extended to 256 points using an in-house linear prediction program based on LPSVD.¹⁸ The linear prediction procedure used the last 128 data points of the synthetic FIDs and 64 poles. All FIDs were then analyzed using REFCOL; a statistical analysis of the distribution of measured linewidth is illustrated in Fig. 5(A). linewidths estimated from signals containing 256 'experimental' complex points are more accurate and

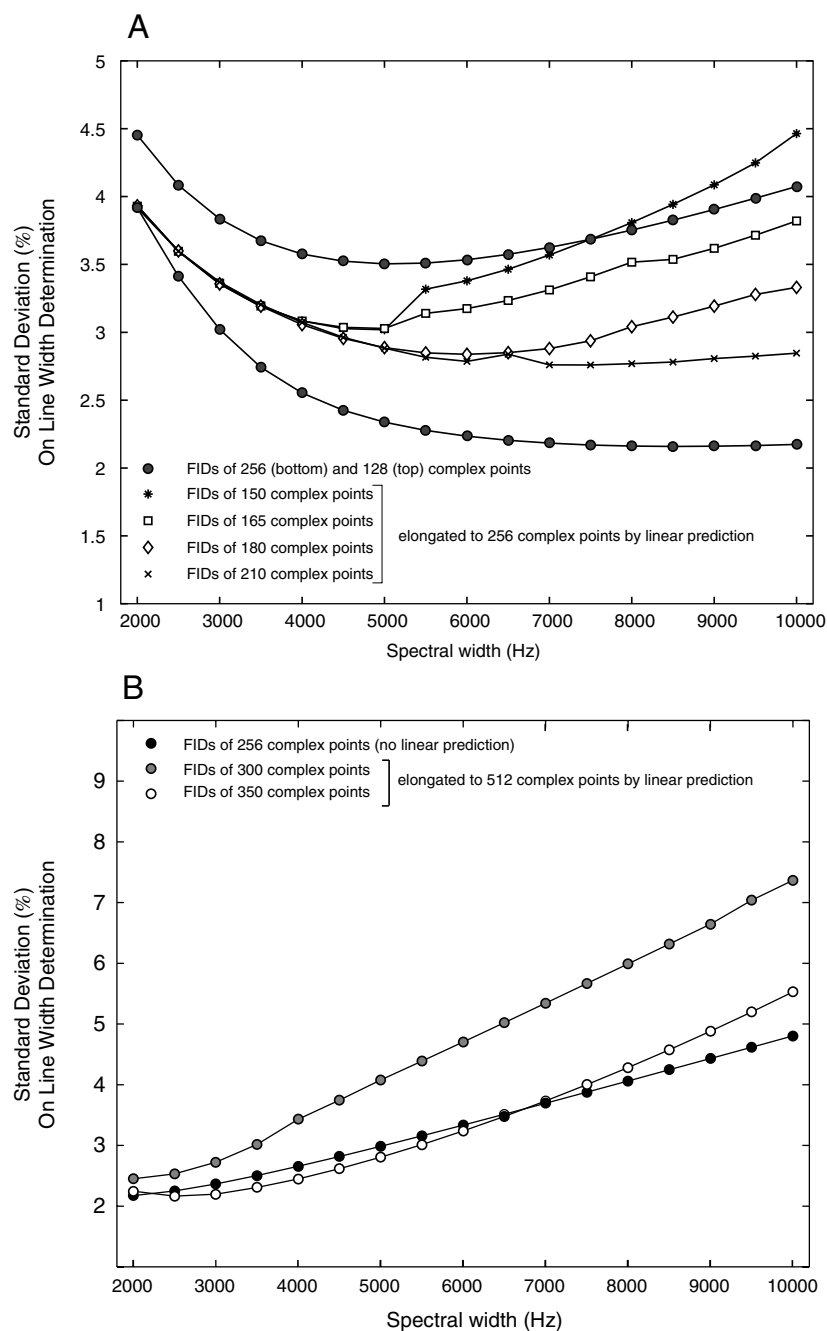


Figure 5. (A) Effect of increasing the digital resolution after acquisition. Series of FIDs of 150, 165, 180 and 210 complex points were elongated by linear prediction to 256 complex points. Each FID contained a single peak, defined by frequency (40 points), amplitude (100), phase (0°), linewidth (25 Hz) and a signal-to-noise ratio of 20 dB. All FIDs were analyzed using REFCOL, and the accuracy and precision of the estimated linewidth were plotted versus the spectral width of the signal. The results are compared with those obtained for FIDs of 128 and 256 complex points, which were not elongated. (B) Same analysis as described above, but for FIDs containing a single narrow peak ($lw = 5$ Hz).

precise than those derived from the same signals truncated at 128 points. When the latter signals are extended by linear prediction to 256 points, the estimates improve, but not to the level observed for the fully experimental signal.

The same procedure was repeated for peaks with a linewidth of 5 Hz and a number of 'experimental' data points set to 256, 300 and 350 [Fig. 5(B)]. In the case of narrow peaks, no improvement could be obtained by using linear prediction and its use could even lead to increased uncertainty on the linewidth values (see results obtained for 300 points). It has been shown that the damping factor is usually poorly estimated by LPSVD algorithms.¹⁸ When the signal is characterized by a small damping factor, the truncation of the FID leads to an increased weight of the predicted points in the linewidth estimation by REFCOL, which leads to increasing uncertainty. Altogether, these simulations show that linear predicted points could not substitute for experimental points. Similar computer experiments in which the FIDs were extended by zero-filling instead of linear prediction showed worse results (data not shown).

Recovering information from overlapping peaks

Increasing the linewidth in a 'forward' accordion experiment may lead to the problem of overlapping in crowded regions of the correlation spectrum. It is therefore of interest to test the ability of the program to extract the linewidth of peaks that are close or even that are overlapping in the spectrum. We considered a series of FIDs of 128 complex points, computed with a spectral width of 4000 Hz. Each FID contains three resonances. The peak corresponding to the first component is fixed (point 20), the position of the second peak varied

from points 21 to 36 and that of the third peak from 22 to 52 points (the distance between two points in the spectrum corresponds to 31.25 Hz). Determination of the parameters of peaks separated by only one point is highly imprecise (data not shown). When the peaks are separated from each other by at least four points, the estimates of the spectral parameters found for one of these peaks compare well to those obtained when the corresponding peak is isolated in the spectrum (see Fig. 6). Even with a separation of just two points, corresponding to a difference in frequency of 62.5 Hz between the peaks, the results remain reasonable.

Designing an accordion experiment: application to a short peptide

'Forward' or 'reverse' accordion spectroscopy?

Both 'forward' and 'reverse' relaxation experiments are required to measure each relaxation parameter in the method proposed by Mandel and Palmer¹⁷ for accordion spectroscopy. Our method differs in that we have the choice between these two experiments. There is an important difference in the amplitude of the signal between a 'forward' and a 'reverse' accordion experiment. The amplitude of a peak is related to the intensity $I^{\text{fw}}(1)$ of the first point of the corresponding FID in a 'forward' spectrum, and to the intensity $I^{\text{rev}}(N)$ of the N th point in a 'reverse' experiment, because of the time reversion (see above). The ratio of the amplitudes is calculated from Eqns (3) and (5):

$$S^{\text{rev}}(N) = S_0 \exp[-(\kappa R_x + NR_2^*)\Delta t_1] \exp(-i\omega N\Delta t_1) \quad (16)$$

and

$$S^{\text{fw}}(1) = S_0 \exp[-(\kappa R_x + R_2^*)\Delta t_1] \exp(-i\omega\Delta t_1) \quad (17)$$

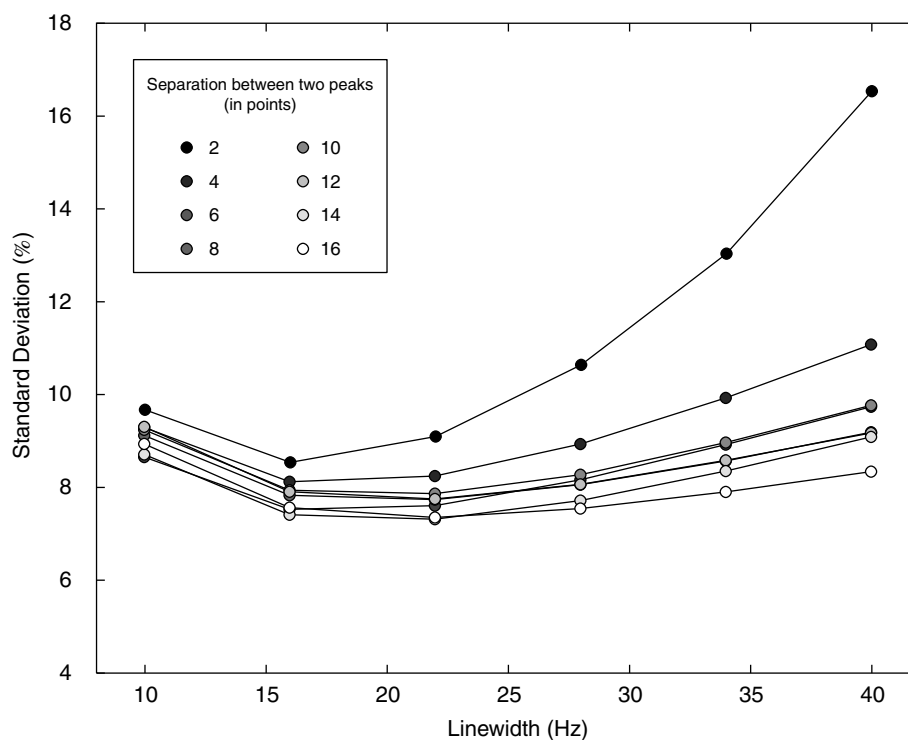


Figure 6. Calculation using REFCOL over a series of 100 FIDs containing three peaks having the same amplitude and linewidth. The FIDs have a signal-to-noise ratio of 10 dB and 128 complex points for a spectral width of 4000 Hz. The position of the first peak is 20 points, the position of the second peak varies from point 22 to 36 and the position of the third peak varies from point 24 to 52. The standard deviation (as a percentage of the linewidth) is an average over the three peaks.

Therefore,

$$\frac{I^{\text{rev}}(N)}{I^{\text{fw}}(1)} = \left| \frac{S^{\text{rev}}(N)}{S^{\text{fw}}(1)} \right| = \exp \left[-\frac{(N-1)R_2^*}{SW} \right] \quad (18)$$

The exponential on the right-hand side of Eqn (18) is always <1 . Consequently, the amplitude of a peak will always be greater in a 'forward' than in a 'reverse' experiment. Since the amplitude determines the signal-to-noise ratio and the precision of the determination of linewidth by REFCOL, a 'forward' experiment appears to be preferable in most cases.

Parameters

Three parameters that define an accordion relaxation experiment affect the estimation of the relaxation parameters using REFCOL, namely the spectral width of the signal in the reconstructed dimension F_1 , the number of data points in F_1 and the apparent linewidths of the peaks of interest. The spectral width SW is optimized such as to provide the best coverage of the spectral region of interest, without spectrum folding. As described above, the best results of the signal processing using REFCOL are obtained when the number of available experimental data points is large. Since the analysis is performed in the reconstructed dimension (i.e. the dimension corresponding to the X nucleus), acquiring a large number of points is time consuming. The only parameter that can be freely adjusted is therefore the actual linewidth of the peaks. In an accordion experiment, the damping factors of the signals are scaled by a factor κ [see Eqns (4) and (6)]. This factor is set such as to provide the best combination (SW , lw) for REFCOL, as described by Fig. 4.

A small test molecule: KQAGDV

The spectral width for KQAGDV is determined through a ^1H - ^{13}C correlation spectrum to be optimal with 4000 Hz. An estimate of R_2^* is obtained from the same correlation spectrum (by measuring the linewidth of the peaks): $R_2^* \approx 10$ Hz. We expect $R_C(C_z)$ to be approximately 2 Hz. Based on Fig. 4, it can be seen that the linewidths that are best determined by REFCOL for a spectral width of 4000 Hz and 128 complex points are in the range 15–25 Hz. If we choose 20 Hz, $R^{\text{fw}} \approx 60$ Hz and

$$\kappa = \frac{(R^{\text{fw}} - R_2^*)}{R_C(C_z)} = \frac{60 - 10}{2} = 25 \quad (19)$$

The relaxation parameters $R_C(C_{xy})$ of the $^{13}\text{C}\alpha$ carbons of the KQAGDV peptide are expected to be 1.5–2 times greater than $R_C(C_z)$. To maintain similar R^{fw} , κ should then be a factor 1.5–2 times lower. κ^{fw} was consequently chosen to be 24 and 16 for the measurements of $R_C(C_z)$ and $R_C(C_{xy})$. These values will not be optimal for all residues since they do not have the same relaxation rate constants.

A total of five experiments were performed: a 'reverse' and 'forward' 2D accordion experiments for both spin–lattice and spin–spin relaxation, and one 'reference' experiment (see Methods). For each α -carbon, the relaxation parameters $R_C(C_z)$ and $R_C(C_{xy})$ were derived from three different combinations: 'reverse' and 'reference', 'forward' and 'reference' and 'reverse' and 'forward.' Results are shown in Fig. 7. All three combinations gave very similar results.

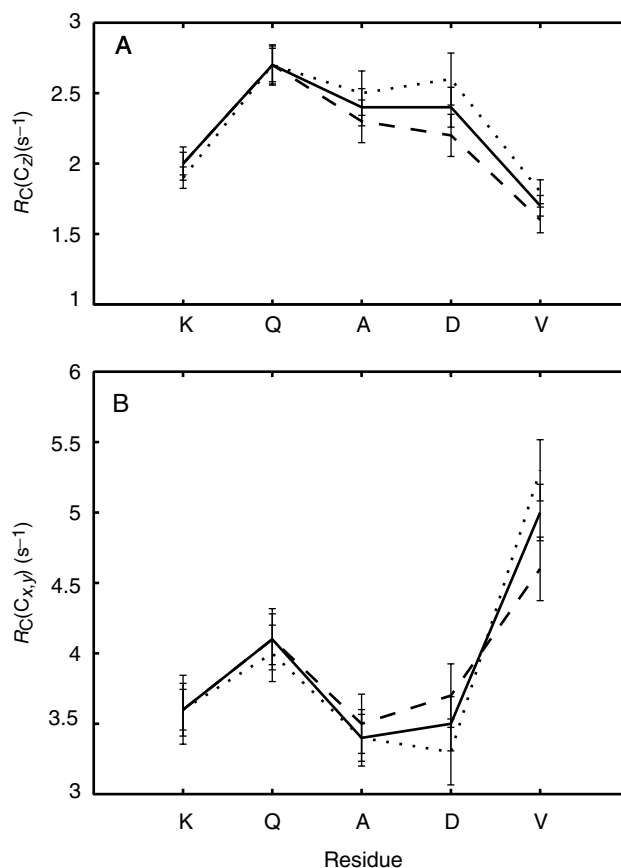


Figure 7. Comparison between relaxation rate constants obtained for the peptide KQAGDV from combination of the two accordion experiments, 'forward' and 'reverse' (solid line), and combination of one 'forward' (dashed line) or 'reverse' (dotted line) accordion experiment with the 'reference' experiment. The relative errors shown by error bars are estimated from the statistical studies described in the text. (A) Longitudinal relaxation rate constants $R_C(C_z)$; (B) transverse relaxation rate constants $R_C(C_{xy})$.

CONCLUSIONS

Accordion NMR spectroscopy has been shown to be a fast and reliable method for the determination of heteronuclear relaxation rate constants.¹⁷ A 2D accordion experiment is designed such that the relaxation rate $R_X(C_z)$ and $R_X(C_{xy})$ is encoded in the linewidth of the cross peak observed for the nucleus X in a 2D experiment. Two types of experiments have been designed: a 'forward' experiment, in which the intrinsic linewidth is increased, and a 'reverse' experiment, in which the linewidth is decreased. Mandel and Palmer proposed to combine the results of four of these experiments (two of each type) for measuring both the spin–spin and spin–lattice relaxation rate constants. In this paper, we introduce a 'reference' experiment which, when combined with two 'forward' or two 'reverse' experiments, provides the same information, resulting in a 25% gain in experimental time. This method also offers the possibility to choose between the 'forward' or 'reverse' experiment, based on the intrinsic relaxation properties of the molecule of interest.

We also propose a new hybrid non-linear least-squares method for linewidth determination of peaks in a 1D NMR

experiment, in which the model function is defined in the time domain, and the residual (or χ^2) that is minimized is computed from a selected region of the complex spectrum. This restriction to a region of the spectrum provides a useful gain in computing time, in addition to improved convergence since the number of parameters to be refined is kept small. This method, encoded into the program REFCOL, should be useful for other quantitative measurements of NMR spectra, such as the determination of coupling constants.

Numerical simulations have been performed and show that the relaxation parameters $R_x(C_z)$ and $R_x(C_{xy})$ could be extracted from the set of 2D experiments with good accuracy. These simulations provide useful guidelines for setting up accordion experiment parameters in order to achieve the best accuracy. Since the signal-to-noise ratio in accordion experiments is usually lower than that obtained from conventional pseudo-3D experiments, a direct comparison of the number of experiments that are needed to obtain the two relaxation rates is misleading. However, by fitting simulated exponential decays with fixed standard deviations (6–7%), it could be shown that the required number of transients in accordion experiment should be at least twice that used in conventional experiments. This rough calculation leads to an estimated gain in experimental time of 2–3 times when choosing the accordion strategy over the conventional method. This gain becomes critical if several datasets have to be recorded on the same system under various experimental conditions, or if the relaxation is to be measured at natural abundance of the ^{13}C isotope.

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