Rapid Multidimensional NMR: High Resolution by Spectral Aliasing

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1 INTRODUCTION

If sensitivity is the most important problem of NMR, resolution is probably the most interesting. In many instances, important developments in sensitivity have presented important challenges to resolution. For example, increasingly powerful magnets have increased sensitivity, but obtaining the full benefit with respect to signal resolution has required the development of techniques to produce exquisite field homogeneity. The introduction of Fourier transform NMR (see ***Fourier Transform Spectroscopy***), another decisive innovation with respect to sensitivity, called for the development of window functions and other processing methods to allow the balance between sensitivity and resolution to be manipulated.

The introduction of multidimensional NMR dramatically improved the accessibility of NMR data (see ***Multidimensional Spectroscopy: Concepts***). From a metaphorical side view, in which clusters of signals overlaid each other in a one-dimensional spectrum and therefore could not be analyzed, spectroscopists started to gain altitude, revealing that signals spread out into a two-dimensional map. The problem with multidimensional NMR is that each point in an indirect dimension requires the recording of an individual FID: the number of individual increments in evolution time determines the number of significant data points in the indirect dimensions. During the 1980s, progress was limited by computer memory and power, because of the difficulty of storing and processing large numbers of FIDs. However, when it became technically possible to record reasonably well-resolved COSY and NOESY spectra, a more fundamental limitation appeared: the experimental time. A chemical shift window with good resolution in an indirect dimension requires hundreds of increments, each in turn requiring multiple FIDs because of the need to cycle the phases of pulses (see ***Phase Cycling***). This problem grew even more serious when it became clear that 3-D experiments would be necessary to reduce signal overlap in biomolecular NMR (see ***Three- and Four-Dimensional Heteronuclear Magnetic Resonance***).

1.1 The Nyquist Condition

When using the classical sampling of data to be processed with the Fourier transform, the Nyquist condition imposes, for a given spectral width SW, a dwell time:

$$\Delta t_{\mathrm{sim}} = 1/\mathrm{SW} \text{ or}$$
$$\Delta t_{\mathrm{seq}} = 1/(2\ \mathrm{SW}) \quad (1)$$

depending on whether quadrature detection is implemented by simultaneous or sequential data sampling. In both cases, the acquisition time is

$$\mathrm{AQ} = N/\mathrm{SW} \quad (2)$$

where N is the total number of complex data points (real plus imaginary) acquired. In order to enjoy the maximum possible resolution, the acquisition should last a time approaching T_2^* which, in the case of small molecules, is typically of the order of a second or so. Table 1 shows what would be the typical conditions to record experiments with good resolution with quadrature detection.

1.2 Resolution in Indirect Dimensions

In ^{1}H 2-D spectroscopy, one can typically record about 600 FIDs per hour, assuming a phase cycle of two transients per FID and a 3 s recycle time. This means that a COSY 2-D spectrum recorded with equal F_1 and F_2 digitization on a 500 MHz ^{1}H Larmor frequency spectrometer would take over 16 h of acquisition time. In order to make full use of the digital resolution available, it is necessary to use a phase-sensitive, as opposed to absolute value or magnitude, display of 2-D data. This requires in turn that twice as many measurements be made in the indirect dimension as equation (2) would suggest, because of the need to cancel the double dispersion component of the 2-D "phasetwist" lineshape. Therefore, the hypercomplex or States–Haberkorn–Ruben method would acquire N cosine- and N sine-modulated increments, whereas the time-proportional phase incrementation (TPPI) method would use $2N$ increments but with half the dwell time in the indirect dimension (see ***COSY Spectra: Quantitative Analysis***).

With respect to resolution, one adverse consequence of high fields is that maintaining high resolution requires that the number of points acquired increase linearly with the field strength. This has little consequence in the detected dimension, but in the indirect dimension(s), it increases the total experimental time needed to maintain a given spectral digitization. At high field, especially when cryogenic probes are available, spectrometer users are tempted to take advantage of the high sensitivity and reduce the number of evolution time increments to increase throughput. This conflict of interest between high resolution and fast acquisition highlights the interest in escaping the limits imposed on the indirectly detected dimension(s) by the Nyquist condition.

In the case of heteronuclear experiments, the change from heteronuclear chemical shift correlation (HETCOR) to heteronuclear single quantum correlation (HSQC) as the experiment of choice is enlightening (see ***Heteronuclear Shift Correlation Spectroscopy***). In the HETCOR experiment, where carbon chemical shifts are detected directly, a spectrum

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Table 1 Number of data points necessary for 2-D spectra with good resolution[a]

Experiment	Nuclei F_1/F_2	Spectrometer 200 MHz[b]	500 MHz[b]
COSY	^{1}H/^{1}H	4k/4k	10k/10k
HETCOR	^{1}H/^{13}C	4k/20k	10k/50k
HSQC	^{13}C/^{1}H	20k/4k	50k/10k

[a]Calculated for 1 s acquisition time and spectral widths of 15 ppm in ^{1}H and 200 ppm in ^{13}C.
[b]Proton Larmor frequency.

with good resolution can, in principle, be recorded with the same number of t_1 increments as a COSY (Table 1). In the early days of heteronuclear 2-D spectroscopy, the HSQC experiment, which is in principle more sensitive because it is proton-detected, was not applicable to natural-abundance samples because of the poor suppression of the signals produced by protons bound to ^{12}C. The introduction of pulsed field gradient methods (see ***Field Gradients and Their Application***) allowed HSQC to realize its full potential with respect to sensitivity, but again introduced a problem with respect to resolution because now the wide ^{13}C chemical shift window is indirectly detected. When expressed in hertz, the width of the ^{13}C spectrum is about five times larger than that of ^{1}H, which would require the number of evolution time increments to be increased by the same factor to obtain spectra with good resolution (Table 1). The need for methods that avoid sacrificing too much spectral resolution when recording quick experiments is therefore even more acute for proton-detected heteronuclear experiments such as HSQC and heteronuclear multiple bond correlation (HMBC).

There are three possible approaches to obtaining well-resolved 2-D spectra. The first, and most common in small-molecule NMR, consists in fulfilling the Nyquist condition and recording, e.g., 256 increments. After processing, signals are over 100 times broader than they could be. Linear prediction can be applied to extend the sampled dataset, but the efficiency of the latter method depends on signal-to-noise ratio and the quality of the spectral data, and typically improves the resolution only by a factor of 2–4 (see ***Fourier Transform and Linear Prediction Methods***). This is certainly useful, but it is not a fully satisfactory solution to the problem. Covariance processing can result in spectacular resolution improvements, but should be interpreted with care in spectral regions where peaks overlap (see ***Multidimensional Correlation Spectroscopy by Covariance NMR***).

The second possibility is to avoid the Fourier transform. This allows increments to be sampled in a nonlinear manner, and uses alternative processing methods to obtain highly resolved spectra with fewer evolution time increments (see ***Rapid Multidimensional NMR: Decomposition Methods and their Applications***, ***Filter Diagonalization Methods for Time-Domain Signals***, and ***Maximum Entropy Reconstruction***). The problem is that many of the advantages of FT with respect to linearity of signal amplitude, ease of use, and reliability are lost.

The solution discussed in this article is intermediate. It retains the FT and its advantages, but escapes the Nyquist condition. First mentioned by Schmieder *et al.*[1] at the time of the development of quadrature detection in indirect dimensions, it consists in setting the dwell time above the value calculated with equation (1), undersampling with respect to the Nyquist condition, to increase the maximum evolution time and hence improve the digital resolution.

1.3 Violation of the Nyquist Condition

The reason for the low resolution of 2-D spectra recorded with only a few hundred time increments and wide spectral windows is that resonances with slightly different frequencies are not given enough time to distinguish themselves. A rational reaction is to increase the dwell time, to reach evolution times where the resonances are clearly different with fewer increments (Figure 1a). The drawback of such undersampling is that the Nyquist condition of equation (1) is not fulfilled. This arises because of the problem of spectral aliasing: if complex data points are sampled every 1/SW s (simultaneous sampling), signals with frequencies $f \pm n \times \mathrm{SW}$, where n is an integer, will have exactly the same amplitudes at the sampled data points as a signal of frequency f. This ambiguity is illustrated in Figure 1(b). When deliberately allowing aliasing to improve spectral resolution, a good choice of dwell time is very important because it determines the type of frequency ambiguity that is introduced, and in particular determines whether signals with different frequencies will overlap in the aliased spectrum.

Spectral aliasing can be considered as a "rapid method" for multidimensional NMR because any given resolution can be obtained much faster than with conventional techniques. More effective approaches have been developed for rapid acquisition of 2-D spectra. Hadamard techniques replace the systematic

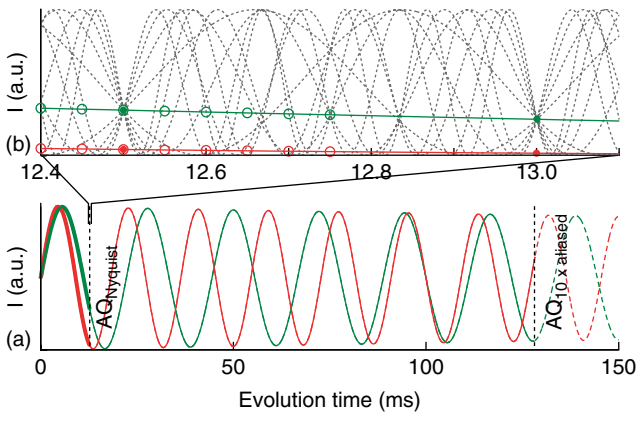

Figure 1 Pros and cons of normal sampling and undersampling. (a) When fulfilling the Nyquist condition (normal sampling), the signals induced by two carbons resonating at 45 (green) and 55 Hz (red) in a 20 kHz window (200 ppm at 100 MHz) have no time to distinguish themselves within the 12.8 ms acquisition time reached with 512 acquisition points (broad lines). Their signals will therefore overlap after Fourier transform. When the window is undersampled to 2 kHz (10-fold aliasing), the acquisition time is 10× longer, allowing the trajectories of the carbons to be sufficiently different (continuous lines) to be resolved in the spectrum. (b) The violation of the Nyquist condition causes the frequencies to be ambiguous because nine other sine functions with frequencies $\nu = \nu_a + N \times 2000$, where $N = \{-4, -3, -2, -1, 1, 2, 3, 4, 5\}$ (dotted gray lines) pass through the same points (filled circles) as the aliased frequency ν_a. In the spectrum, these nine resonances will be aliased in the reduced spectral window and all appear at ν_a

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sampling of the indirect dimensions with a tailored set of selective pulse experiments (see ***Fast Multidimensional NMR by Hadamard Spectroscopy***); the problem is that difficulties arise when trying to resolve close pairs of resonances.[2] Frydman's approach (see ***Ultrafast Multidimensional NMR: Principles and Practice of Single-Scan Methods***) is even more radical, completely replacing time incrementation of the indirect dimension by using a spatial distribution of evolution time within the acquisition of a single FID. This makes it possible to record 2-D spectra in a couple of seconds, but at the expense of poor spectral resolution in both dimensions. The unique advantage of spectral aliasing is to access the maximum resolution of multidimensional spectra within the total acquisition time of normal experiments.

2 SPECTRAL ALIASING

High resolution in the indirect dimensions of multidimensional spectra can be accessed with a small number of time increments by setting the duration of the dwell time above the value given by equation (1). In the frequency domain, this violation of the Nyquist condition corresponds to a reduction of the spectral window. Signals outside the window boundaries are aliased into the window and appear at deceptive frequencies. This phenomenon, the details of which depend on the quadrature detection method used, has not been named very consistently in the literature. Usually "aliasing" refers to the case where a signal falling just outside one end of the spectral window appears just inside the other end, whereas "folding" refers to such a signal being reflected about the edge of the spectrum and appearing just inside the same end of the spectrum (Figure 2). In 1-D spectra and directly detected dimension of multidimensional spectra, the oversampling and digital signal processing[3] of modern spectrometers effectively suppress any signals outside the spectral boundaries, so neither aliasing nor folding is seen.

In biomolecular NMR, it is quite common to set the spectral width in an ^{15}N dimension to about half the total width of the ^{15}N spectrum, to take advantage of empty spectral regions and effectively double the resolution. The section "Application of Spectral Aliasing to Proteins" shows that computer-optimized spectral aliasing can further reduce the spectral width needed.

2.1 Signal Positions in Aliased Spectra

The violation of the Nyquist condition can cause signals to appear at deceptive frequencies. In the case of spectral aliasing, the observed frequency is given by the modulo function:

$$\nu_a = \mathrm{mod}(\nu_0 + \mathrm{SW}_a/2 - \mathrm{CF},\ \mathrm{SW}_a) - \mathrm{SW}_a/2 + \mathrm{CF} \quad (3)$$

where ν_0 is the true frequency, SW_a the width of the spectrum, and CF the position of the carrier frequency. The opposite relation illustrates the ambiguities with respect to the true frequency:

$$\nu_0 = \nu_a \pm n \times \mathrm{SW}_a \quad (4)$$

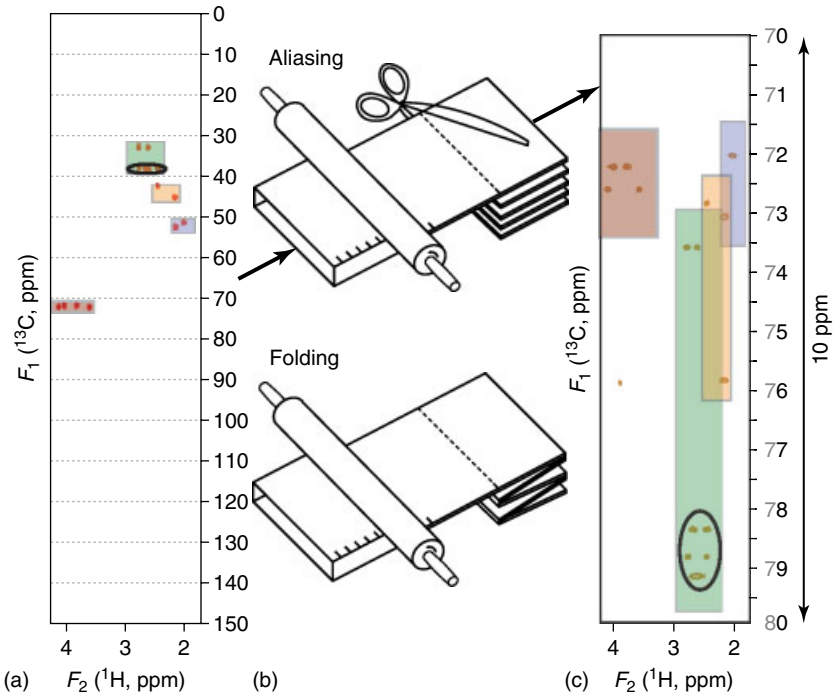

Figure 2 Illustration of the effect of reducing the spectral width of the carbon dimension of the HSQC spectrum of the epimers of cubebin from 150 (a) to 10 ppm (c). The 10 ppm spectrum looks (b) as if the full spectrum (a) has been converted into a laminate, by being stretched, thinned, cut into 15 equal pieces, and superimposed (c) in a 15-layer sandwich, in a process reminiscent of the preparation of puff pastry (pâte feuilleté). This results in a 15-fold increase in spectral resolution in F_1. With echo/anti-echo, states, or states-TPPI quadrature detection in F_1, the spectrum are "aliased", whereas TPPI quadrature causes "folding". The ellipses highlight a crowded region of the spectrum, which is only resolved clearly in the 10 ppm spectrum. The "7" digits of the 10 ppm chemical shift scale are grayed out to indicate that the high-order digits (tens, hundreds) of the chemical shift scale are undetermined because of the ambiguity introduced by aliasing

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where n is the unknown aliasing order. Such ambiguities in frequency may seem unmanageable, but in fact many solutions exist, some of which are trivial, to recover the missing information. In fact, aliased spectra are no more ambiguous than the classical low-resolution spectra; it is simply that the missing information is of a different order. Instead of an ordinary lack of precision making the last significant figure of the frequency undetermined, the unknown is the aliasing order n.

In most NMR analysis, a set of NMR spectra providing different types of information (^{1}H–^{1}H correlation, ^{1}H–^{13}C, or ^{1}H–^{15}N correlation, NOEs, etc.) is recorded. One can argue that measuring the true chemical shift in each experiment is irrational if it imposes acquisition times longer than necessary. A full and precise measurement of the relevant frequencies need only be done once, and then the other spectra can be recorded with spectral windows tailored to record the minimum number of time increments needed to distinguish between the known frequencies. Spectral aliasing allows this saving in time to be realized.

2.2 Improvement Factors

The resolution enhancement achievable in aliased 2-D spectra depends on relaxation properties, but is typically a factor of 10 to 200 for small molecules, because acquisitions under standard conditions only explore the first few tens of milliseconds of the signal evolution in the indirect domain (Figure 1b). In protein NMR, relaxation and the reduced signal dispersion limit the factor to 2 to 8 for ^{1}H–^{15}N HSQC of proteins with up to 150 residues or so. For larger proteins, 3-D and 4-D experiments are necessary to spread signals in additional dimensions, and spectral aliasing can be applied in each indirect dimension individually. Even if the improvement factors are not large in any given dimension, the product of the improvements in the different dimensions may nevertheless be considerable.

2.3 The Limits of Spectral Aliasing

How far the evolution time can be extended depends very much on the sample in question. In small molecules, field inhomogeneity often limits useful data acquisition to about 1 s, while T_2 relaxation is generally the limiting factor in protein NMR. In both cases, a maximum evolution time AQ $< T_2^*/4$ causes no significant loss of sensitivity. When sensitivity allows, one can record more points and adapt the weighting function used to allow for the fact that signal amplitude decays by about 75% for AQ $= 4T_2^*$. In very favorable cases, one can sample beyond $4T_2^*$ and apply window functions emphasizing the middle part of the FID to enhance resolution (see ***Data Processing***).

2.4 Resolution of Chemical Shift Ambiguities

Given aliased spectra, a natural wish is to reconstruct a normal spectrum with a reassuringly unambiguous frequency scale. A number of difficulties warn against this temptation. First, the resulting spectra may be quite large and very sparse because of the fine resolution. Their use may be less practical because of the need to constantly zoom in and out over huge chemical shift maps. More problematic is the misleading impression of safety. In the process of reconstruction, each signal has to be moved to the correct location in the artificial 2-D map. But what happens to weak signals below the threshold of the peak picking used, or to signals with more than one candidate location? Should they be eliminated, or plotted with a different color? Whatever the option selected, such a reconstruction would function as a black box, risking misleading the user. This would sacrifice a great advantage of the Fourier transform, which is that it functions in an entirely linear and predictable manner, an important reason for preferring spectral aliasing to other rapid methods. In fact, simple software tools or printed annotations near the signals are all that should be needed to exploit aliased spectra effectively. Their nature will depend on the situations.

3 APPLICATIONS OF SPECTRAL ALIASING TO SMALL MOLECULES

The choice of spectral width in an indirect dimension depends on the frequency range expected, typically determined by the range of chemical shifts for the nucleus involved. We will discuss ^{13}C in some detail because of its importance in the structure elucidation of organic compounds and the two orders of magnitude potential improvement in resolution obtainable and, say, only a few words about aliasing in homonuclear COSY and NOESY.

When considering aliasing in the proton dimension of 2-D spectra, a simple visual identification of the spectral regions where signals can be aliased without overlap is often the best way to determine where a spectrum should be divided. Arranging a set of printed copies of a spectrum with horizontal displacements equivalent to a trial spectral width makes it possible to see which, if any, signals would overlap were that spectral width used and the spectrum aliased. With sparse spectra, this method can result in resolution enhancement factors of 2–5. This may be quite useful when studying the fine structures of COSY multiplets[4] or NOESY spectra of mixtures of similar compounds.

Carbon spectra being much more sparse, it is difficult to determine an appropriate window to use for 2-D ^{1}H–^{13}C heteronuclear experiments by hand because of the larger scaling factor. Different approaches discussed in sections "Combinations of 2-D Spectra" and "Optimization of Aliasing Conditions" are illustrated in Figure 3.

3.1 Combinations of 2-D Spectra

Fundamentally, spectral aliasing sacrifices part of the information on frequencies in the indirect dimension—^{13}C chemical shifts in the case of HSQC/HMQC and HMBC—to increase resolution. Combining spectra with different spectral widths is a good solution to compensate for this loss. We will see that some choices of spectral window are particularly useful.

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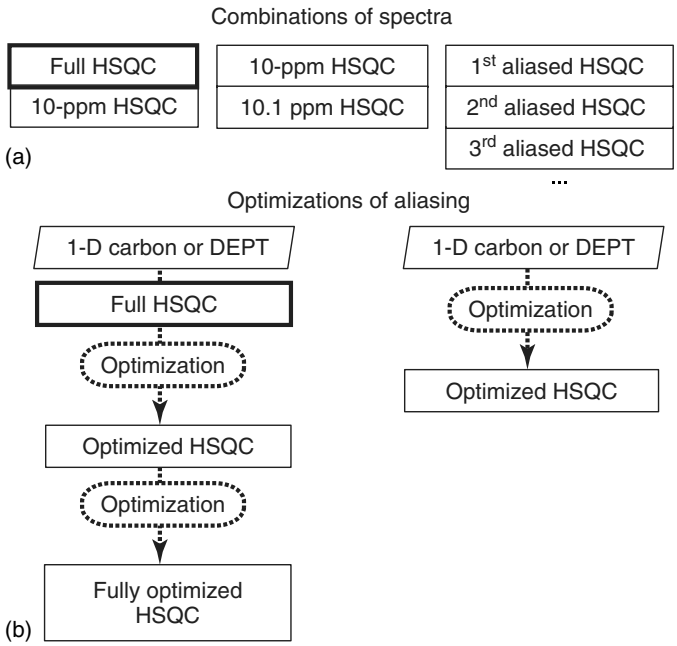

Figure 3 Strategies to include aliased spectra in the study of small molecules. Some use pairs or series of spectra (a); others are based on computer optimization (b)

3.1.1 *The Advantage of 10 ppm Spectra*

Setting the spectral width of the carbon dimension in HSQC and HMBC spectra to 10 ppm increases the resolution by a factor of 15 to 25 when compared with full-width spectra with the same number of increments. With 512 increments, the acquisition time becomes about 200 ms at 125 MHz ^{13}C frequency, which is reasonable with respect to the T_2 relaxation of small molecules. This spectral window has a great advantage with respect to the expression of the ambiguity of the chemical shifts. Equation (4) becomes

$$\delta_0 = \delta_a + n \times 10 \qquad (5)$$

where δ_0 is the true chemical shift and δ_a is the apparent chemical shift in our 10-ppm wide spectrum. The apparent chemical shift δ_a is then just the last three significant figures X.XX of the true chemical shift YYX.XX ppm. The missing significant figures YY can be determined from a complementary spectrum that digitizes the full-spectral window at low resolution.[5]

3.1.2 *Combination of 10 ppm and Normal Spectra*

In most situations, chemists first record normal full-width HSQC or HMBC spectra (as discussed above, at relatively low resolution). When analyzing these spectra, they often wish to zoom into problematic regions. One can therefore assume that normal spectra are available when recording aliased experiments.

The correspondence of signals between full width and 10 ppm spectra can be determined by looking for equal proton chemical shifts and compatible carbon chemical shifts. By compatible, we mean that signals have equal values for the middle figures of the chemical shifts (−X.X-). The full and precise carbon chemical shifts can be determined by simply combining the first two digits of the full spectrum (YYX.X) with the last three of the 10 ppm spectrum (X.XX).[5] A 1-D carbon spectrum could also be used to confirm the correspondence, but turns out to be not necessary. This is useful because directly detected ^{13}C experiments are less sensitive than inverse methods, making it possible to replace a DEPT experiment with a couple of aliased HSQC experiments.

3.1.3 *Combination of 10 and 10.1 ppm Spectra*

A disadvantage of combining full and 10 ppm spectra is the need to handle two very different spectra. When only a few signals cause difficulties, this complication is acceptable, but otherwise the following method should be used. Consider the difference between a spectrum with a 10 ppm spectral width and one with a 10.1 ppm width, recorded with a carrier frequency corresponding to a chemical shift of 75 ppm. Signals located in the middle strip, that is, between 70 and 80 ppm in F_1, will overlap perfectly in the two spectra when their F_1 scales are aligned. Any signal located between 80 and 90 ppm will appear at 0.1 ppm higher apparent shift in the 10.1-ppm wide spectrum than the corresponding signal in the 10-ppm spectrum, and so on. In general, the difference in position of corresponding signals is $-0.1 \times n$, where n is the aliasing order n in equation (5) (Figure 4).[6]

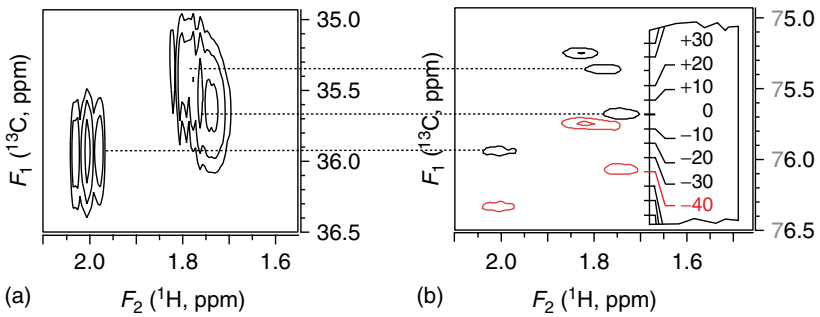

Figure 4 (a) Expansion of the normal full-width HSQC spectrum of cyclosporin A. (b) Overlap of the relevant parts of 10 ppm (black contours) and 10.1 ppm (red contours) wide aliased spectra. All spectra were recorded with the same number of time increments. The aliased spectra display a 15× resolution improvement, but each cross-peak requires a correction to its apparent ^{13}C chemical shift indicated by the ruler shown in the inset that corresponds to the vertical displacement between the black and the red signals. Thus the cross-peak at a ^{1}H shift of 1.75 ppm requires a correction of −40 ppm to its apparent ^{13}C chemical shift of 75.9 ppm

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3.1.4 Other Combinations of 2-D Spectra

The combinations of spectra discussed so far have practical advantages, but may not be ideal, especially for full automatic applications. The first publication about the systematic application of aliasing proposed the combination of a small, a medium, and a large width spectrum.[7] Berger and coworkers considered other combinations of spectral windows for a set of HMBC spectra recorded with the objective of determining the list of carbon chemical shifts, as an alternative to proton-decoupled ^{13}C spectra.[8] Pairs of aliased spectra have also been used as NMR fingerprints.[9]

3.2 Optimization of Aliasing Conditions

Thus far, we have considered combinations of predefined spectral windows, which are advantageous for automatic sample-changer operation. However, using fixed spectral widths does nothing to prevent accidental signal overlap. In nonroutine applications, it is worth spending some time to determine, for example using computer programs, the optimal acquisition conditions for a particular sample. The goals of computer-optimized spectral aliasing are twofold[10]: the avoidance of overlap due to aliasing, and the determination of the minimum number of time increments required to achieve a user-defined objective with respect to signal resolution.

3.2.1 Optimization Based on a 1-D Spectrum

When a 1-D carbon or DEPT spectrum is available, any given pair of carbon signals will show the same aliased frequency, and hence overlap in aliased heteronuclear correlation spectra, if the chemical shifts of coupled protons are the same and the spectral width

$$\mathrm{SW}_a = \Delta\nu / i \qquad (6)$$

where $\Delta\nu$ is the frequency separation between the resonances and i is an integer. An effective optimization algorithm consists in taking the list of carbon chemical shifts, calculating all the possible frequency differences and listing the spectral widths to avoid. The choice among the remaining possibilities is then made according to the number of time increments and the acquisition time needed to achieve the desired resolution.

This procedure only requires the experimenter to copy and paste a list of chemical shifts from a DEPT-135 or proton-decoupled ^{13}C spectrum into a web-based application to optimize HSQC or HMBC experiments respectively (Figure 5). Note that when considering the acquisition of series of experiments (say HSQC, HMBC, HSQC–TOCSY, HSQC–NOESY, etc.), it is preferable to record all spectra with the same spectral widths to facilitate analysis of the spectra.

3.2.2 Optimization Based on 1-D and 2-D Spectra

When considering recording series of HSQC-based spectra to map out variations in signal intensity as a function of relaxation, diffusion, NOE build-up, etc., it is possible to improve the efficiency of optimization by complementing the list of carbon chemical shifts from a 1-D spectrum with data from an HSQC experiment. This establishes, for each pair of carbon resonances, whether the potential for overlap in the proton dimension is actually fulfilled. Allowing pairs of CH cross-peaks with different proton chemical shifts to have the same position in the carbon dimension of the aliased spectrum makes the optimized spectrum more compact, and reduces the number of time increments needed.[10] For this purpose, a normal low-resolution HSQC spectrum is not as useful as a fully resolved one as the source of ^{1}H and ^{13}C chemical shift data for the optimization. Usually, two iterations are necessary to optimize the spectra fully.[10]

3.3 Compatibility with Standard Pulse Sequences

In most cases, recording aliased spectra only requires the spectral window to be changed and, sometimes, the number of time increments to be adjusted. However, in some

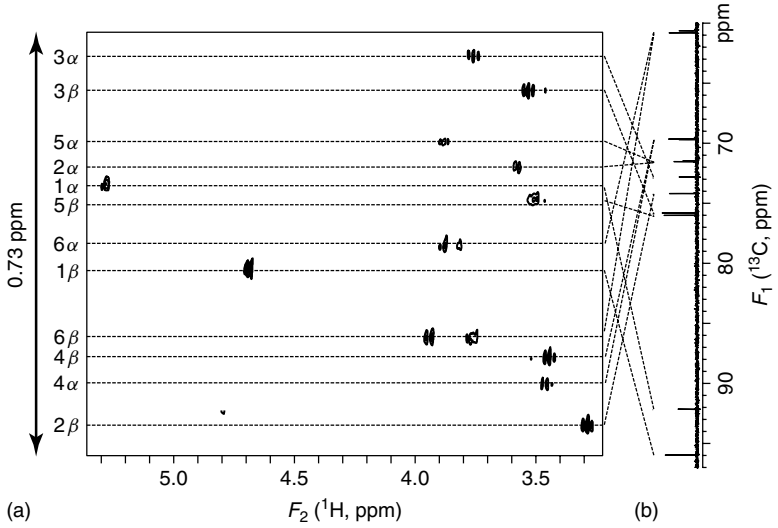

Figure 5 Results of optimization of the carbon dimension of the HSQC spectrum of the two anomers of glucose in D_2O. The ^{13}C spectral width was set to 0.73 ppm and sampled with 100 time increments. The factor of reduction in the spectral width is 60. C-4α and C-4β are only 5.5 Hz apart at 125 MHz but nevertheless clearly resolved in F_1

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HMQC and HMBC experiments where the magnetization is gradient-encoded during the evolution time, signals can decay quickly as a function of t_1 because of molecular diffusion[11] (see ***Diffusion-Ordered Spectroscopy***). Such effects are accentuated by low-viscosity solvents, small molecular size, and high temperature. In most cases, these adverse effects can be eliminated by changing the positions and amplitudes of the gradient pulses.

4 APPLICATION OF SPECTRAL ALIASING TO PROTEINS

In protein NMR, studies typically combine many different experiments requiring long acquisition times. It is therefore especially interesting to optimize the acquisition of multidimensional experiments using the knowledge obtained from previous ones. Two important differences would seem to decrease the potential utility of aliasing in biomolecules. First, transverse relaxation is much faster than for small molecules, which limits the potential extension of the evolution time. Second, the spectral windows are often much narrower. On the other hand, because the total gain in acquisition time is the product of the gains obtained in each indirect dimension, for experiments with three or more dimensions aliasing can still offer useful time savings when the experimental time is determined by the resolution needed. A couple of examples of applications of spectral aliasing to protein NMR are presented in Figure 6 and the following paragraphs. Note that many effective methods have been developed for reducing the time demands of multidimensional NMR (see ***Filter Diagonalization Methods for Time-Domain Signals***, ***Rapid Multidimensional NMR: Decomposition Methods and their Applications***, ***Multidimensional NMR by Projection-Reconstruction***, ***Fourier Transform and Linear Prediction Methods***, and ***Rapid Multidimensional NMR: Fast-Pulsing Techniques and their Applications to Proteins***), some of which could be combined with aliasing.[12,13]

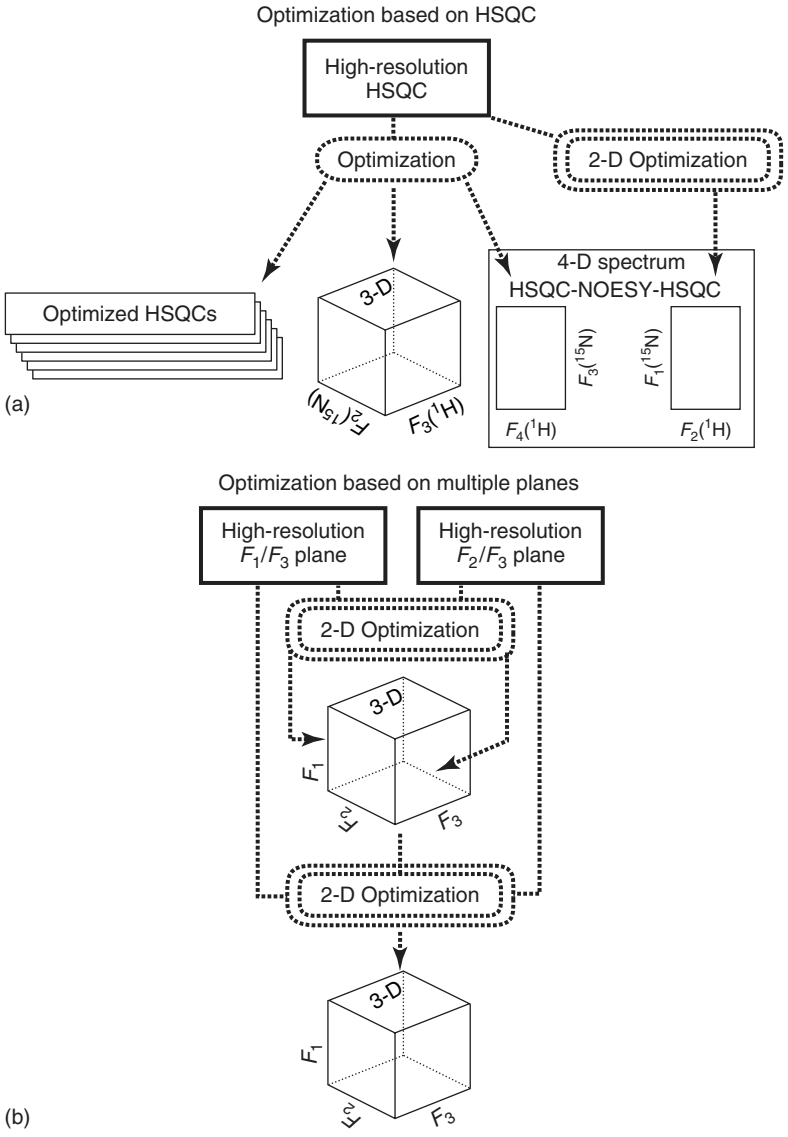

Figure 6 Examples of strategies to optimize spectral aliasing in multidimensional NMR of proteins

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4.1 Single-Dimension Optimization

The basic data required for the optimization of aliasing in multidimensional NMR protein are 2-D $^{1}H-^{15}N$ and $^{1}H-^{13}C$ HSQC or 2-D planes of 3-D or 4-D experiments recorded at the maximum resolution relaxation permits. These experiments typically involve a few hundred time increments. The optimization consists in a study of the overlap caused by aliasing as a function of the spectral width and number of time increments. Typical results are displayed in a form where the two most important parameters are clearly displayed: the number of increments and the acquisition time of the optimized dimension (Figure 7).

The choice of acquisition parameters depends on the type of experiment envisaged. When considering the acquisition of a series of 2-D spectra for relaxation measurements, one may wish to resolve all the signals and use aliasing conditions that give no overlap. When relaxation during the evolution time is the main concern, the acquisition conditions will be selected from among those located below the maximal acceptable evolution time. Finally, when preparing the acquisition parameters of 3-D and 4-D experiments, the priority will be to keep the number of time increments to a minimum and to warn the experimenter how much overlap should be expected for a given choice.

Statistics show that for typical small proteins of 100–150 residues, the reduction in the number of time increments for $^{1}H-^{15}N$ HSQC is typically three- to eightfold. For larger proteins, the optimization is not effective and one should consider additional dimensions and apply multidimensional optimization.

4.2 Multidimensional Optimization

When setting up the parameters of 3-D experiments such as HNCO, one can optimize the two indirect dimensions separately by analyzing the F_1/F_3 and F_2/F_3 planes recorded at maximum resolution. However, in cases where 3-D spectra are necessary, these 2-D spectra will be so crowded that unidimensional optimization will not be effective. A combined optimization of the two independent dimensions has a better potential. One solution is to consider, for any position in the F_3 dimension, the list of chemical shifts in F_1 and F_2 that need to be resolved in the 3-D spectrum envisaged. Consider the case of an HNCO experiment with three resonances at a given F_3 position. A maximum of 3×3 positions in the F_1/F_2 planes are candidate locations for the three resonances. These regions should not overlap in the aliased 3-D spectrum that is contemplated. When a normal 3-D spectrum (or a

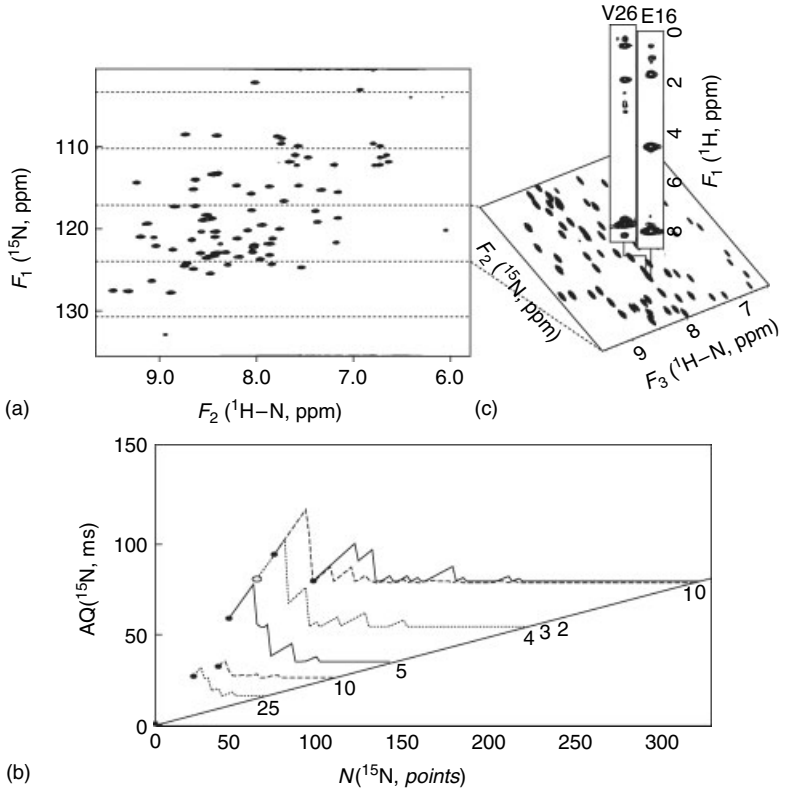

Figure 7 (a) High-resolution $^{1}H-^{15}N$ HSQC spectrum of human ubiquitin recorded with 400 time increments. (b) Analysis of overlap as a function of the acquisition parameters (SW_a, N) in the ^{15}N dimension. The ordinates of the points correspond to their acquisition times. The bullets indicate the conditions with minimum number of time increments for 0–5, 10, and 25 overlaps. The oblique line indicates the full spectral width and the remaining lines the acquisition time (ordinate) needed to give the indicated number of overlaps for a given number of increments (abscissa). (c) Projection of the 3-D NOESY–HSQC spectrum recorded with the conditions indicated by the small empty ellipse ($N = 60$, AQ = 87.3 ms). $SW_2 = 343.7$ Hz is about 1/6th of the full ^{15}N window. The numbers of evolution time increments were 160 and 60 in the $F_1(^{1}H)$ and $F_2(^{15}N)$ dimensions respectively. The strips illustrate the satisfactory resolution of the NOE observed along the F_1 dimension for the pair of partially overlapping NH signals

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3-D spectrum obtained after a first optimization) is available, one can test for the presence of signals at the 3×3 relevant positions and reduce the number of locations where overlap should be avoided to 3 in favorable situations (Figure 6b). This reduction in the requirements will improve the efficiency of the optimization. After a complete list of positions where overlap should not occur has been made by applying the same principle throughout the F_3 dimension, a simulation of the overlap as a function of SW_1, SW_2, N_1, and N_2 can be computed. The user can then select acquisition parameters according to the total number of overlaps, taking into account any specific needs for selected signals not to overlap. Possible criteria for optimization include the total number of time increments $N_{tot} = N_1 \times N_2$, and a measure of the cumulative effect of signal decay $T_w = AQ_1/T_{2*1} + AQ_2/T_{2*2}$, where T_{2*1} and T_{2*2} are the estimated values of the effective transverse relaxation time during t_1 and t_2 respectively.

5 RELATED ARTICLES

Heteronuclear Shift Correlation Spectroscopy; Three-Dimensional HMQC-NOESY, NOESY-HMQC, and NOESY-HSQC; Structural Analysis Enhancements for Natural Products; Fourier Transform and Linear Prediction Methods; Filter Diagonalization Methods for Time-Domain Signals; Heteronuclear Shift Correlation Spectroscopy; Fast Multidimensional NMR by Hadamard Spectroscopy; Rapid Multidimensional NMR: Decomposition Methods and their Applications; Ultrafast Multidimensional NMR: Principles and Practice of Single-Scan Methods; Rapid Multidimensional NMR: Fast-Pulsing Techniques and their Applications to Proteins; Multidimensional Correlation Spectroscopy by Covariance NMR; Multidimensional NMR by Projection-Reconstruction

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