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COMMUNICATION

Aliasing in reduced dimensionality NMR spectra: (3,2)D HNHA and (4,2)D HN(COCA)NH experiments as examples

David Pantoja-Uceda · Jorge Santoro

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Abstract Reduced dimensionality NMR spectra usually require very large spectral widths in the shared dimension. In this paper we show that aliasing can be introduced in reduced dimensionality NMR spectra either to decrease the acquisition time or increase the resolution of the experiments without losing information. The gains of introducing aliasing are illustrated with two examples, the (3,2)D HNHA and the (4,2)D HN(COCA)NH experiments. In both cases a reduction of the spectral width of more than 50% was possible.

Keywords Aliasing · Reduced dimensionality · HNHA · HN(COCA)NH · Proteins · Resonance assignment

Fast multidimensional NMR spectroscopy has received considerable attention in the last years with the presentation of several novel methods (Atreya and Szyperski 2005; Felli and Brutscher 2009; Freeman and Kupce 2003, 2004; Malmodin and Billeter 2005a; Szyperski and Atreya 2006). Among these methods, one of the most successful is the reduced dimensionality (RD) approach. RD was proposed many years ago (Szyperski et al. 1993a, b; Simorre et al. 1994), but has received special interest in the last years, specially after the introduction of the multiple quadrature detection (Brutscher et al. 1995; Kozminski and Zhukov 2003), that allows the editing of the RD spectra, and of the equivalent G-matrix Fourier transform (GFT) NMR spectroscopy (Kim and Szyperski 2003). In RD spectroscopy the evolution times of two or more indirect dimensions are jointly sampled, in that the corresponding delays are increased simultaneously. Consequently several chemical shifts are encoded on a single frequency axis, and the resulting spectral data contains peaks with positions defined by linear combinations of several chemical shifts rather than single chemical shifts. More precisely, if N chemical shifts are combined in a single dimension, as in a (N+1,2)D experiment, 2^{N-1} sub-spectra are obtained with signals at frequencies

$$v_0 \pm k_1 v_1 \pm k_2 v_2 \pm \cdots \pm k_{N-1} v_{N-1} \quad (1)$$

where k_i represents the scaling factor of the sampling increments used for the evolution of chemical shift i. Several methods of analysis of RD spectra have been proposed: reconstruction of the multidimensional spectrum (Kupce and Freeman 2003); use of peak coordinates to solve the linear Eq.(1) (Kim and Szyperski 2003); extraction of the orthogonal spectra corresponding to individual nuclei (Malmodin and Billeter 2005b, 2006; Mueller 2009).

The spectral range spanned by the peaks in the shared dimension of RD spectra is (Kim and Szyperski 2003)

$$\mathrm{SW} = \sum_{i=0,N-1} k_i \cdot \mathrm{SW}_i \quad (2)$$

where $\mathrm{SW_i}$ is the spectral width of nucleus i and $k_0 = 1$. Thus, spectral widths in RD spectra can be very large. Reduction of the spectral width is attractive, either to decrease the measurement time or to increase the resolution of the RD experiments. However, reduction of the spectral width beyond the range spanned by the signals produces signal aliasing, what seems to be incompatible with obtaining the values of the chemical shifts of the nuclei combined into one dimension. Nevertheless, it has been showed (Malmodin and Billeter 2005b, 2006) that it is

D. Pantoja-Uceda · J. Santoro (✉)
Instituto de Química Física Rocasolano, CSIC, Serrano 119, 28006 Madrid, Spain
e-mail: jsantoro@iqfr.csic.es

possible to correctly interpret aliased RD spectra using multi-way decomposition to obtain the orthogonal spectra. In this paper we will see that it is also possible to unambiguously obtain the values of the chemical shifts from the peak coordinates of aliased RD spectra, provided that the spectral width in the shared dimension satisfies some conditions. The utility of introducing aliasing in RD spectra is demonstrated on the (3,2)D HNHA and the (4,2)D HN(COCA)NH experiments.

Analysis of RD spectra using peak coordinates is straightforward. Obtaining the frequencies of the N nuclei of the shared dimension is relatively easy, once the corresponding peaks in the 2^{N-1} sub-spectra are identified. The value of ν_0, the frequency of the leading nucleus, corresponds to the mean frequency of the group. For the remaining $N-1$ nuclei there are 2^{N-2} pairs of sub-spectra with peaks appearing at frequencies

$$\nu_A = \nu_0 \pm k_1\nu_1 \pm \cdots + k_i\nu_i \pm \cdots \pm k_{N-1}\nu_{N-1} \quad (3a)$$

$$\nu_B = \nu_0 \pm k_1\nu_1 \pm \cdots - k_i\nu_i \pm \cdots \pm k_{N-1}\nu_{N-1} \quad (3b)$$

so that the frequency ν_i is obtained from the equation (one for each pair)

$$\nu_i = (\nu_A - \nu_B)/(2k_i) \quad (4)$$

In presence of aliasing, the equations (3) must be changed to

$$\nu_A = \nu_0 \pm k_1\nu_1 \pm \cdots + k_i\nu_i \pm \cdots \pm k_{N-1}\nu_{N-1} + m \cdot \mathrm{SW} \quad (5a)$$

$$\nu_B = \nu_0 \pm k_1\nu_1 \pm \cdots - k_i\nu_i \pm \cdots \pm k_{N-1}\nu_{N-1} + n \cdot \mathrm{SW} \quad (5b)$$

where m and n are unknown integers, corresponding to the number of times the peaks have been aliased. The frequency of nucleus i, with $i \neq 0$, is now

$$\nu_i = (\nu_A - \nu_B)/(2k_i) + (m-n) \cdot \mathrm{SW}/(2k_i) \quad (6)$$

Equation (6) corresponds to an infinite set of solutions that differ in a multiple of $\mathrm{SW}/(2k_i)$. However, as shown in Fig. 1, if $\mathrm{SW}/(2k_i)$ is larger than SW_i only one of the solutions appears in the frequency range expected for ν_i. Therefore, the condition to unambiguously determine ν_i is $\mathrm{SW} > 2k_i \cdot \mathrm{SW}_i$. Once all the ν_i ($i = 1, N-1$) have been determined, the value of ν_0 can be obtained from each of the 2^{N-1} equations of the form

$$\nu_0 = \nu_A \pm k_1\nu_1 \pm k_2\nu_2 \pm \cdots \pm k_{N-1}\nu_{N-1} - m \cdot \mathrm{SW} \quad (7)$$

We have again an endless set of solutions, now differing in a multiple of SW. As in the previous case, if SW is larger than SW_0, only one of the solutions appears in the chemical shift range expected for ν_0, and the value of ν_0 can be unambiguously established. Combining both constraints we obtain

$$\mathrm{SW} > \mathrm{SW}_0, 2k_i \cdot \mathrm{SW}_i \quad (8)$$

as the condition to allow signal aliasing in RD spectra.

In the previous derivation we have considered that the peaks $\nu_0 \pm k_1\nu_1 \pm \cdots \pm k_{N-1}\nu_{N-1}$ are correctly grouped into sets, each belonging to one multiplet. The unambiguous grouping of peaks usually requires the acquisition of "central peaks" (Kim and Szyperski 2003; Szyperski et al. 1995), i.e. peaks defining the centers of the chemical shift multiplets at the frequencies $\nu_0 \pm k_1\nu_1 \pm \cdots \pm k_{N-2}\nu_{N-2}$, $\nu_0 \pm k_1\nu_1 \pm \cdots \pm k_{N-3}\nu_{N-3}$,…,$\nu_0 \pm k_1\nu_1$, and ν_0. The use of central peaks allows a further reduction of the spectral width in RD spectra with aliasing. Assume we make a "bottom-up" analysis of the spectra, starting from the central peak, ν_0, and the doublet

$$\nu_A = \nu_0 + k_1\nu_1 + m \cdot \mathrm{SW} \quad (9a)$$

$$\nu_B = \nu_0 - k_1\nu_1 + n \cdot \mathrm{SW} \quad (9b)$$

From the doublet we obtain for ν_0

$$\nu_0 = [\nu_A + \nu_B - (m+n) \cdot \mathrm{SW}]/2 \quad (10)$$

and an equation identical to (6) for ν_1. Equation (10) permits the doublet to be correctly grouped and the determination of the value of $m+n$. Obviously, knowing the value of $m+n$ does not allow the calculation of the value of $m-n$ needed to unambiguously obtain the value of ν_1 from (6). However, we can take advantage of the fact that if $m+n$ is even, then $m-n$ is also even. Likewise if $m+n$ is odd, then $m-n$ is also odd. Therefore, every second solution to (6) can be discarded. The remaining solutions are separated by SW/k_i. Thus, the condition to unambiguously determine the value of ν_1 is now $\mathrm{SW} > k_1 \cdot \mathrm{SW}_1$. Once the value of ν_1 is determined, it can be used to analyze the sub-spectra $\nu_0 \pm k_1\nu_1 \pm k_2\nu_2$. Using the same analysis as before the condition $\mathrm{SW} > k_2 \cdot \mathrm{SW}_2$ is obtained. In general, the spectral width of the RD spectrum that incorporates the nucleus i in the shared dimension must satisfy the condition

$$\mathrm{SW} > k_i \cdot \mathrm{SW}_i \quad (11)$$

To illustrate the gains obtained by allowing aliasing in RD spectra we present two examples here. The first example corresponds to the (3,2)D HNHA experiment (Barnwal

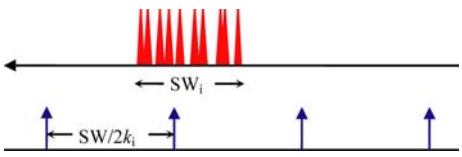

Fig. 1 Graphical representation of the solutions to (6). If the separation between solutions is larger than the range spanned by nucleus i, the chemical shift ν_i is unambiguously determined

et al. 2007) proposed as an alternative to the regular 3D HNHA experiment (Vuister and Bax 1993). The experiment provides two 2D sub-spectra with peaks along the indirect dimension at frequencies $\omega(^{15}N) \pm k_1 \cdot \omega(^1H_\alpha)$ and $\omega(^{15}N) \pm k_1 \cdot \omega(^1H_N)$. The scaling factor, k_1, allows to increase the dispersion of peaks or to restrict the ^1H chemical shift evolution time to reduce intensity losses due to transverse relaxation. A value $k_1 = 0.5$ is a reasonable compromise for small and medium sized proteins. To make an estimation of the spectral width of the indirect dimension we will consider that the ^1H carrier is placed at 4.7 ppm, the $^1H_\alpha$ signals appear in the interval 3–6 ppm and the ^1H_N ones in the range 6–10 ppm. Therefore, to completely cover the ^1H spectral range a SW_1 value of 10.6 ppm must be used. The typical interval covered by the ^{15}N resonances, SW_0, is 32 ppm. With these numbers the value of SW (expressed as ^{15}N ppm) for a non-aliased RD spectrum, obtained from (2), is 84.30 ppm. However, if aliasing is allowed and central peaks are employed it suffices, (11), to use a spectral width larger than k_1 times the spectral range spanned by the protons, 7 ppm. Expressing this as ^{15}N ppm we obtain $0.5 \cdot 7 \cdot \gamma_H/\gamma_N = 34.54$ ppm. Figure 2 shows (3,2)D HNHA spectra acquired for ubiquitin with $k_1 = 0.5$ and spectral widths of the shared dimension of 85 and 40 ppm, recorded at 298 K on Bruker AV 800 spectrometer using an inverse cryogenically-cooled triple resonance probe with Z-gradients. For both spectra 128 (t_1) × 1,024 (t_2) complex data points were accumulated with 8 transients per fid, 4 fids per t_1 value, and a relaxation time of 1 s. The total measuring time of each spectrum was 1 h and 22 min. Before Fourier transformation, the data matrices were pre-processed to separate the two sub-spectra, zero filled up to the duplicate number of data points, and apodized with shifted squared sine-bell functions in both dimensions. The spectra were analyzed using the central peaks provided by a ^{15}N-HSQC spectrum. In most cases both HNHA spectra provided the same information. However in some other cases the aliased spectrum, which has better resolution in the indirect dimension, proved to be superior. Examples are given in Fig. 3, that shows that the $\omega(^{15}N) + 0.5 \cdot \omega(^1H_\alpha)$ peaks of A28 and L56 overlap with the $\omega(^{15}N) + 0.5 \cdot \omega(^1H_\alpha)$ peaks of K48 and N60, respectively, in the 85 ppm spectrum, but are clearly resolved in the aliased spectrum. $^3J_{HNHa}$ couplings for 61 out of an expected 65 residues, excluding glycines, could be measured in the aliased (3,2)D HNHA spectrum. These couplings were compared with those measured in the non-aliased spectrum giving an r.m.s.d. of 0.26 Hz.

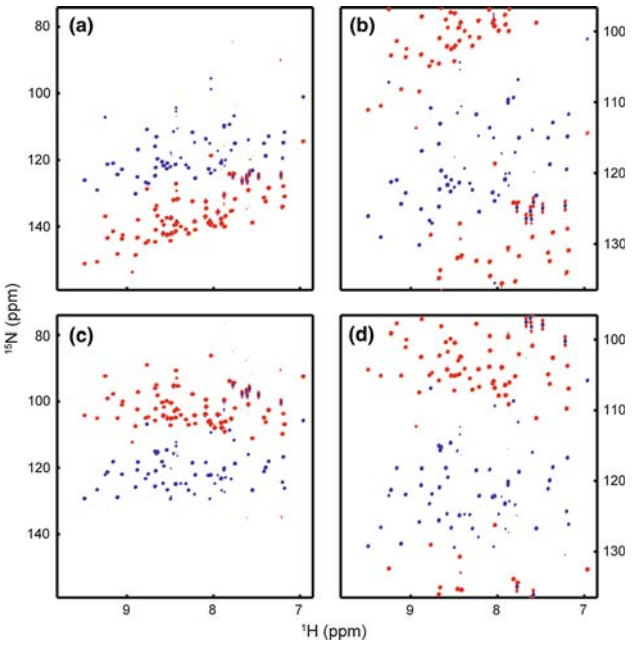

Fig. 2 (3,2)D HNHA spectra of ubiquitin recorded with 85 ppm (**a**, **c**) and 40 ppm (**b**, **d**) in the indirect dimension. The proton chemical shift in the indirect dimension were scaled by 0.5. The (3,2)D HNHA experiment provided two sub-spectra with peaks along the indirect dimension at frequencies $\omega(^{15}N) + 0.5 \cdot \omega(^1H_\alpha)$ and $\omega(^{15}N) + 0.5 \cdot \omega(^1H_N)$ (**a**, **b**), and $\omega(^{15}N) - 0.5 \cdot \omega(^1H_\alpha)$ and $\omega(^{15}N) - 0.5 \cdot \omega(^1H_N)$ (**c**, **d**). *Positive*, $\omega(^{15}N) \pm 0.5 \cdot \omega(^1H_N)$, and *negative*, $\omega(^{15}N) \pm 0.5\omega(^1H_\alpha)$, cross peaks are coloured in *red* and *blue*, respectively

The second example is the HN(COCA)NH experiment (Grzesiek et al. 1993) used to achieve amide connectivities without using ^{13}C chemical shift information. This 4D experiment requires high resolution to produce an error-free sequential connection of backbone amide protons, and therefore extremely long experimental times. Thus, 3D versions of the experiment have been proposed (Panchal et al. 2001; Sun et al. 2005). Even with 3D spectra, the time to obtain the required resolution can be very long. To

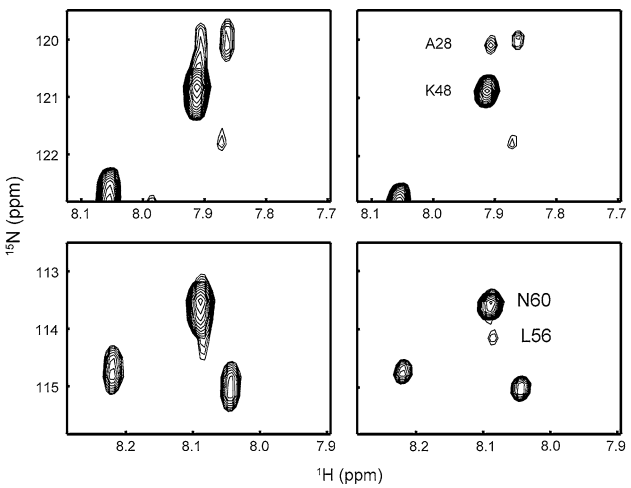

Fig. 3 Excerpts from the (3,2)D HNHA of spectra of ubiquitin recorded with 85 ppm (*left*) and 40 ppm (*right*)

reduce the experimental time, the group of Wagner has proposed (Sun et al. 2005) the use of non-uniform sampling followed by maximum entropy reconstruction. Obviously, the use of RD spectroscopy is a valid alternative, with the advantage of not requiring sophisticated processing methods. The pulse sequence of Wagner (Sun et al. 2005) was converted in the reduced dimensionality (4,2)D HN(CO-CA)NH pulse sequence shown in Fig. 4. This pulse sequence gives peaks at $\omega(^{15}N_i) \pm k_1 \cdot \omega(^{15}N_j) \pm k_2 \cdot \omega(^{1}H_{Nj})$, $\omega(^{1}H_{Ni})$ with $i = j$, $j - 1$. Sequential peaks, $i = j - 1$, are negative and stronger than the intraresidual peaks, $i = j$, that are positive, except when residue $j - 1$ is glycine. In these cases, the sequential peak $j - 1$, j is positive and the intraresidual peak j, j negative. Using $k_1 = k_2 = 0.5$ and the same spectral ranges for $^{1}H_N$ and ^{15}N as before, a spectral width of the shared dimension of 100 ppm, expressed as ^{15}N ppm, is calculated for the non-aliased spectrum, but only 40 ppm for an aliased spectrum to be analyzed without central peaks information. The pulse sequence was tested with a sample of ubiquitin $\sim$1.7 mM uniformly labeled with ^{15}N and ^{13}C. Spectra were recorded at 298 K on Bruker AV 800 spectrometer using an inverse cryogenically-cooled triple resonance probe with *Z*-gradients. A 2D spectrum was acquired with 128 (t_1) $\times$ 1,024 (t_2) time increments, 8 transients per fid, 8 fids per t_1 value, and a relaxation delay of 1 s. A scaling factor of 0.5 was used for proton evolution and for the first nitrogen evolution in the shared dimension. Spectral widths were 12 ppm in the direct dimension and 40 ppm in the indirect dimension, instead of the 100 ppm needed for a non-aliased spectrum. The total measuring time was 3 h. Before Fourier transformation, the data were pre-processed to obtain the sub-spectra, zero filled up to the duplicate number of data points and apodized with shifted squared sine-bell functions in both dimensions. Figure 5 shows the four sub-spectra obtained.

The typical analysis of RD experiments uses central peak information to properly group peaks from the different sub-spectra, and then calculates the chemical shifts of the nuclei from the frequencies of the grouped peaks. However, the analysis of the (4,2)D HN(COCA)NH experiment can be can be made in an alternative way. Since the ^{15}N and ^{1}H chemical shifts of all residues are known from the ^{15}N-HSQC spectrum of the protein, it is possible to calculate the expected frequency values in the indirect and the direct dimensions for all putative pathways $H_s \rightarrow N_s \rightarrow N_r \rightarrow H_r$. Therefore, the analysis is easily done by comparing the frequencies of the observed peaks with the

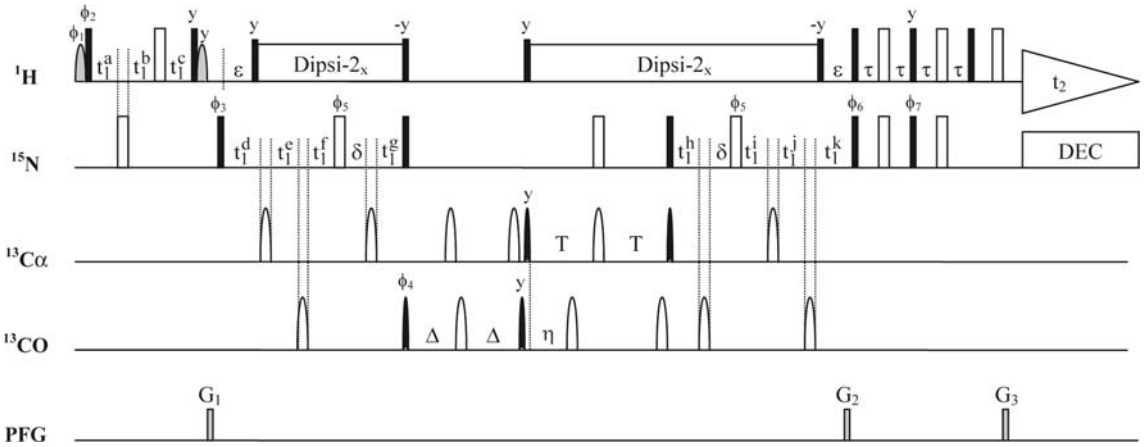

Fig. 4 Pulse sequence for the (4,2)D HN(COCA)NH experiment. All radiofrequency pulses are applied along the *x*-axis unless indicated. 90° and 180° rectangular pulses are represented by *filled* and *unfilled bars*, respectively. ^{13}C pulses have the shape of gaussian cascades Q5 (*black filled shapes*) and Q3 (*open shapes*) with durations of 307 μs and 192 μs at 800 MHz, respectively. $^{13}C\alpha$ pulses are centered at 54 ppm and CO pulses at 174 ppm. The ^{1}H carrier is centered on the water resonance, and the ^{15}N carrier is placed in the center of the ^{15}N dimension of the ^{15}N-HSQC spectrum. The fixed delays are adjusted to $\varepsilon = 5.5$ ms; $\Delta = 4.5$ ms; $\eta = 4.5$ ms; $T = 14.3$ ms; $\tau = 2.3$ ms. The sequence uses the semi-constant time evolution periods proposed in (Sun et al. 2005). The delays and increments for the ^{1}H evolution period are: $t_1^a = 2.3$ ms; $t_1^b = 3\mu s$; $t_1^c = t_1^a + t_1^b + p_N$; $\Delta t_1^a = 2k_2 \cdot \text{in} - \Delta t_1^b + \Delta t_1^c$; $\Delta t_1^b = \max(k_2 \cdot \text{in} - 2.75 \text{ ms}/n, 0)$; $\Delta t_1^c = -\min(k_2 \cdot \text{in}, t_1^c/n)$; p_N is the length of the 180° nitrogen pulse, n the number of time increments and $\text{in} = 1/(2 \cdot \text{SW})$. The delays and increments for the first ^{15}N evolution period are: $t_1^d = t_1^g = 12 \text{ ms} - p_C - \delta/2$; $t_1^e = t_1^f = 3\mu s$; $\Delta t_1^d = k_1 \cdot \text{in} + \Delta t_1^g$; $\Delta t_1^e = \min(k_1 \cdot \text{in}, 16.6 \text{ ms}/n)$; $\Delta t_1^f = k_1 \cdot \text{in} - \Delta t_1^e$; $\Delta t_1^g = -\min(k_1 \cdot \text{in}, t_1^g/n)$; p_C is the length of the 180° carbon pulse; the delay δ is equal to $p_C + t_1^e + t_1^f$ and compensates for the 180° ^{13}CO pulse. The delays and increments for the second ^{15}N evolution period are: $t_1^h = t_1^k = 13.5 \text{ ms} - p_C - \delta/2$; $t_1^i = t_1^j = 3\mu s$; $\Delta t_1^h = -\min(\text{in}, t_1^h/n)$; $\Delta t_1^i = \text{in} - \Delta t_1^j$; $\Delta t_1^j = \min(\text{in}, 15.7 \text{ ms}/n)$; $\Delta t_1^k = \text{in} + \Delta t_1^h$. A eight-step phase cycle is used with $\phi_1 = -x$; $\phi_2 = x$; $\phi_3 = x, -x$; $\phi_4 = 4(x), 4(-x)$; $\phi_5 = x$; $\phi_6 = x, x, -x, -x$; $\phi_7 = -y, -y, y, y$; $\phi_{rec} = x, -x, -x, x, -x, x, x, -x$. The phase cycle can be extended to 16 steps by inverting the sign of ϕ_5. Pulsed field gradients G_1 to G_3 of *sinusoidal shape* are applied along the *z*-axis with a 1 ms length and amplitudes of 15, 40 and 4.05 G/cm. Multiple quadrature detection requires 8 fid's for each t_1 value, and is achieved by incrementing ϕ_1 and ϕ_2 for ^{1}H, incrementing ϕ_3 for the first ^{15}N evolution, and inverting G_2 in concert with a 180° shift of ϕ_7 for the second ^{15}N evolution

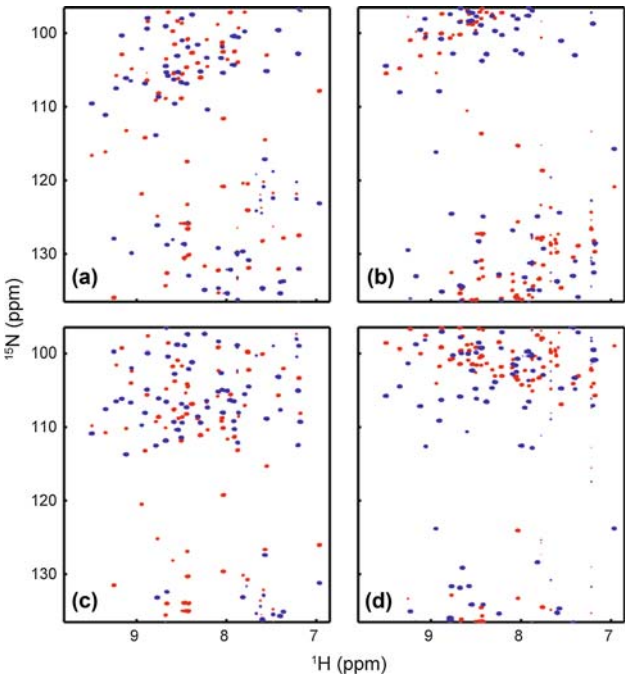

Fig. 5 (4,2)D HN(COCA)NH spectrum of ubiquitin. A scaling factor of 0.5 was used for proton evolution and for the first nitrogen evolution in the shared dimension. The experiment provides four sub-spectra with peaks at $\omega_i(^{15}N) + 0.5 \cdot \omega_j(^{15}N) + 0.5 \cdot \omega_j(^{1}H)$, $\omega_i(^{1}H)$ (**a**); $\omega_i(^{15}N) + 0.5 \cdot \omega_j(^{15}N)$-$0.5 \cdot \omega_j(^{1}H)$, $\omega_i(^{1}H)$ (**b**); $\omega_i(^{15}N)$-$0.5 \cdot \omega_j(^{15}N) + 0.5 \cdot \omega_j(^{1}H)$, $\omega_i(^{1}H)$ (**c**); and $\omega_i(^{15}N)$-$0.5 \cdot \omega_j(^{15}N)$-$0.5 \cdot \omega_j(^{1}H)$, $\omega_i(^{1}H)$ (**d**); with $i = j, j - 1$. *Positive* and *negative* cross peaks are coloured in *red* and *blue*, respectively

calculated values. First, peaks corresponding to the intra-residual correlation, s ← s, are identified and their sign determined. Then, peaks of opposite sign matching the expected frequency for every putative sequential correlation, r ← s, are searched in each sub-spectrum. The true sequential correlation will be that for which a matching is found in all four sub-spectra. If several NH's satisfy this criterion, the one best fitting the expected frequencies is taken as the correct solution.

The analysis of the spectrum of ubiquitin was greatly facilitated by the high resolution of the aliased spectrum and produced five stretches of consecutive NH's. These stretches have been mapped to the protein sequence, Fig. 6, using the

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 XXXXXXXXG X XXXXXXX XXXX XXXXXXXXXXG X
MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIP

 XXXXXXXXG X XXXX  X XXXXXXXXXXXXXXXXXXXXG X
PDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
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Fig. 6 Mapping of the stretches obtained in the analysis of the (4,2)D HN(COCA)NH spectrum to the sequence of ubiquitin. NH's identified as coming from residues following Gly appear in *bold*. *Underlined residues* in the sequence are not observed in the ^{15}N-HSQC spectrum

fact that intraresidual peaks of residues following Gly have opposite sign to the rest. The mapping gives the assignment of all NH's observed in the ^{15}N-HSQC spectrum. In more complicated cases the Gly anchor points might not be sufficient to achieve the assignment. In these circumstances more anchor points can be obtained by recording additional amino acid typing experiments, that can go from the simple and sensitive A-(i + 1)-HSQC experiment (Schubert et al. 1999) to more exhaustive amino acid typing methods (Lescop et al. 2008; Pantoja-Uceda and Santoro 2008).

The (4,2)D HN(COCA)NH experiment has similarities with the 6D-APSY-seq-HNCOCANH (Fiorito et al. 2006) and the 7D-APSY-HNCO(CA)CBCANH (Hiller et al. 2007) experiments. All these experiments use practically the same magnetization transfer steps and obtain amide connectivity's matching ^{15}N and ^{1}H chemical shifts. The (4,2)D experiment requires the recording of less projections, uses a pulse sequence shorter in time, and its analysis is very easy. On the contrary, the APSY experiments give higher resolution of the peaks, since they use 6 or 7 dimensions, and a very high precision of the NH chemical shifts. These properties are important in the analysis of large proteins. Therefore, what experiment is more adequate for the assignment of the amide NH resonances will highly depend on the characteristics of the protein under study.

In conclusion, we have shown that aliasing can be introduced in reduced dimensionality spectra either to decrease the acquisition time or increase the resolution of the experiments without losing information. Its incorporation in the (4,2)D HN(COCA)NH experiment provides an efficient method for the backbone amide assignment of small to medium sized proteins. The (4,2)D HN(COCA)NH pulse sequence in Bruker language, and a program to perform the linear combination to separate the sub-spectra can be obtained from the page http://rmn.iqfr.csic.es.

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