

Concentration measurements by PULCON using X-filtered or 2D NMR spectra

Lars Dreier and Gerhard Wider*

Institut für Molekularbiologie und Biophysik, ETH Zurich, CH-8093 Zurich, Switzerland

Received 10 January 2006; Revised 14 March 2006; Accepted 16 March 2006

Sample concentrations can be measured by nuclear magnetic resonance (NMR) spectroscopy without an internal reference compound using pulse length based concentration determination (PULCON) with 1D NMR spectra. PULCON delivers most accurate results if the spectrum of the sample of interest contains a resolved resonance; but can also be applied to spectral regions with overlapping resonances. If the exact number of lines contributing to the overlapping spectral region is not known, a corresponding error makes the result less precise. The uncertainty about the number of contributing resonances can be reduced with experiments that discriminate different classes of resonances by filtering techniques or by extending PULCON to two-dimensional NMR spectra. We demonstrate the application of PULCON with a 1D ^{15}N -filtered experiment where aromatic resonances of a protein can be observed without interference from ^{15}N -bound protons. Further, we extend PULCON to 2D NMR spectra that permits to determine the exact number of resonances. This extension can readily be applied with sample that contain different solutes. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: NMR; protein; concentration; reciprocity; pulse length

INTRODUCTION

Quantitative nuclear magnetic resonance (NMR) is an efficient method for the determination of concentrations due to the direct proportionality of the intensity of the NMR resonances to the number of molecules. For the calibration of the signal intensities a reference compound of known concentration is usually added.¹ The choice of such an internal reference is not straightforward since it must fulfill a number of conditions: the reference signal must be distinct from the signals of the sample, the reference compound shall not interact with sample molecules and it must be cosoluble with the sample. In addition, for efficient measurements, the NMR relaxation properties of the reference and the sample molecules should be similar. Concentration determination by NMR becomes easier and much more efficient if an internal reference is avoided and instead an external reference is correlated with the signal strength of the sample of interest. It was proposed that this correlation can be obtained with a calibrated external radio-frequency (r.f.) signal that is irradiated during acquisition.^{2,3} In practical applications this method seems to be rather cumbersome. Recently, an alternative technique, PULCON (pulse length based concentration determination), was introduced that provides an easy, straightforward and efficient determination of concentrations of NMR samples.⁴

The method PULCON correlates the absolute intensities in two one-dimensional (1D) NMR spectra measured in

different solution conditions based on intrinsic properties of the samples. The method is a direct consequence of the principle of reciprocity⁵ and signal intensities in spectra of different samples are correlated by the measurement of a precise 360° r.f. pulse.⁴ PULCON is easy to implement and apply on all NMR spectrometers; it does not need any special hardware or software. PULCON is most efficient and accurate when the spectrum of the sample contains separated resonances, but can be applied to whole spectral regions without resolved lines.⁴ For accurate concentration measurements, the number of resonances contributing to the overlapping spectral region must be known, a condition that cannot always be fulfilled.

A reduction of overlapping resonances can be obtained by various techniques which exploit, e.g. relaxation properties, chemical exchange or coupling constants. In our applications we used experiments discriminating resonances based on their heteronuclear coupling constants. We applied PULCON with 1D heteronuclear filter experiments and two-dimensional (2D) heteronuclear correlation experiments. As an example, we discuss the extension of PULCON to ^{15}N -isotope labeled proteins using ^{15}N -filtered 1D ^1H spectra and 2D [$^{15}\text{N}, ^1\text{H}$] correlation spectra.

EXPERIMENTAL

Concentration measurements of NMR samples with PULCON are based on the principle of reciprocity, which states that the length of the 90° pulse for a sample in a given r.f. coil is inversely proportional to the sensitivity that can be obtained.^{5–7} Based on this principle, the unknown

*Correspondence to: Gerhard Wider, Institut für Molekularbiologie und Biophysik, ETH Zurich, CH-8093 Zurich, Switzerland.
E-mail: gsw@mol.biol.ethz.ch

concentration c_U of a sample U can be calculated from the known concentration c_R of a reference sample R with

$$c_U = f_T c_R \frac{S_U T_U \theta_{360}^U n_R}{S_R T_R \theta_{360}^R n_U} \quad (1)$$

where S stands for the signal strength (i.e. integrals over resonance lines), T for the sample temperature in Kelvin, θ_{360} for the 360° r.f. pulse, and n for the number of transients used for the measurements of the two samples U and R, respectively.⁴ The factor f_T accounts for any signal loss that occurs between the excitation and the acquisition of the signal,⁸ for a possible use of different receiver gains and for incomplete relaxation in the case of short interscan delays (e.g. in 2D experiments). Equation (1) is valid when the measurements are done with the same NMR probe, which is tuned and matched to the individual samples.⁴

Without resolved resonances lines in the 1D ^1H spectrum, PULCON can be applied using whole spectral regions, e.g. the aromatic/amide region in protein spectra.⁴ However, a reduced precision may result since usually the exact number of amide protons contributing to the signal cannot be determined. A more precise number of contributing resonances can be obtained by the application of heteronuclear filter techniques.^{9,10} For example, the pulse sequence in Fig. 1 suppresses resonances of protons attached to ^{15}N nuclei and in ^{15}N -labeled proteins aromatic resonances can be observed separately. Since the spectrum of the reference compound is obtained from a one-pulse 1D ^1H spectrum, the signal loss during the heteronuclear filter has to be determined so that the factor f_T in Eqn (1) can be calculated.⁴ This factor can be obtained from the ratio of the signal intensities measured

from two experiments where one or two filter elements are applied.⁸ Thus, the experiment shown in Fig. 1 is performed twice: once with and once without the repetition of the filter element (shown in brackets in Fig. 1). For improved suppression of the ^{15}N -bound proton resonances a z-filter element with a gradient was used and a composite 180° pulse on ^{15}N . The inversion of the ^{15}N magnetization is crucial for best suppression of ^{15}N -bound proton resonances; we suggest using a 90–240–90 composite pulse, which inverts at least 99% of the magnetization over a bandwidth of $1/p_{360}$ where p_{360} is the 360° pulse on ^{15}N . The water resonance was suppressed by presaturation, which further reduced the residual intensity of the resonance of the exchangeable amide protons. The factor f_T would also account for the use of different receiver gains for the measurement of the reference sample and the sample of interest. However, the two spectra can often be measured with the same receiver gain without compromising spectral quality.

Application of PULCON with 2D spectra requires the correlation of integrals in 1D and 2D spectra since the reference signal is most efficiently measured in a 1D spectrum. Without relaxation the integral values are identical for a resonance in a 1D spectrum and the corresponding resonance in a 2D spectrum. However, this fact is not reflected in current NMR software packages, which provide seemingly uncorrelated integrals for 1D and 2D spectra. Intransparent automated scaling procedures make it very difficult in practice to obtain a general relation between the 1D and 2D integrals. For this reason, we implemented our own correlation by adding a synthetic signal to the time domain data. After Fourier transformation, the synthetic

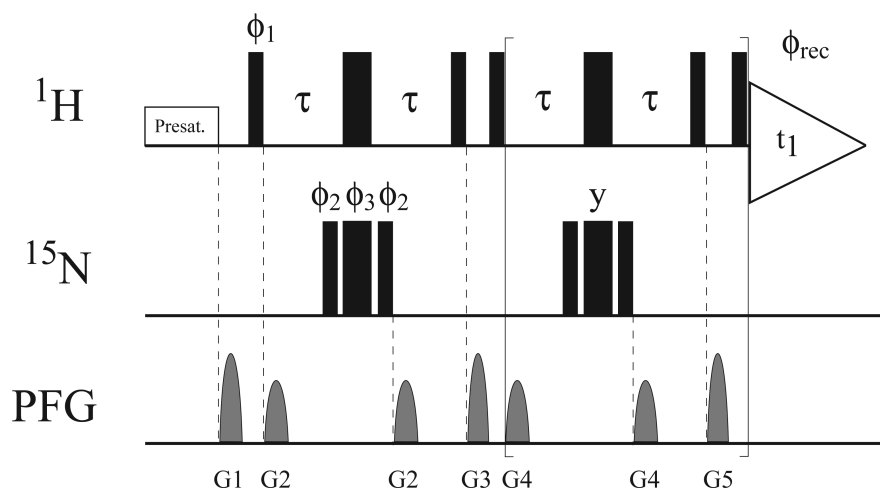


Figure 1. Pulse sequence of the ^{15}N -filtered 1D ^1H experiment used with PULCON. The pulse sequence element in brackets is used to determine the factor f_T in Eqn (1) (see text). Black bars represent rectangular r.f. pulses, which were applied at the proton frequency (on line ^1H) or at the nitrogen frequency (on line ^{15}N); narrow bars stand for flip angles of 90° , wide bars represent 180° pulses on ^1H and 240° pulses on ^{15}N , respectively. The pulse phases were set to x unless indicated otherwise above the pulse bar. The following phase cycles were applied: $\phi_1 = 2(x), 2(-x)$; $\phi_2 = (x, -x, y, -y, -x, x, -y, y)$; $\phi_3 = (y, -y, -x, x, -y, y, x, -x)$; receiver $\phi_{\text{rec}} = 2(x), 2(-x)$. The ^1H and ^{15}N carrier frequencies were positioned at the water frequency and at 118 ppm, respectively. The period τ was set to 2.7 ms corresponding to $1/(4 \ ^1J_{\text{NH}})$ where $^1J_{\text{NH}}$ is the one bond coupling constant in the polypeptide backbone amide moiety. On the line marked PFG, curved shapes indicate sine bell-shaped pulsed magnetic field gradients applied along the z-axis, with the following durations and strengths: G1, 1 ms, 25 G cm^{-1} ; G2, 0.8 ms, 10 G cm^{-1} ; G3, 1 ms, 30 G cm^{-1} ; G4, 0.8 ms, 15 G cm^{-1} ; G5, 1 ms, 20 G cm^{-1} . The interscan delay for protein samples is typically 20 s, whereas presaturation of the water resonance is applied only during the last second before the gradient G1 is applied.

signal represents identical signal strengths in 1D and 2D spectra irrespective of the actual integral value calculated by particular software. The k th data point of the complex 1D FID of the synthetic signal S^t was calculated as

$$S_k^t = A \exp(2\pi k \Delta (i(\nu_s + \phi_{pc}) - l/2)) \text{ with} \\ k = 0, 1, 2, \dots, N_2 - 1 \quad (2)$$

where A is the amplitude, N_2 is the number of real data points, Δ stands for the dwell time, ν_s is the offset of the synthetic signal from the carrier frequency, ϕ_{pc} is the phase correction (zeroth and first order) in the spectrum from which the concentration shall be determined and l is the line broadening factor in Hertz. Please note that positive values of ν_s shift the synthetic signal to a lower field. Truncation effects of the synthetic signal were avoided by multiplying the last 10% of the data points of S_k^t with $\cos^2(x)$ with $x \in [0, \pi/2]$, so that the last data point was multiplied by $\cos^2(\pi/2)$.

Real and imaginary points of S^t were written alternating in the FID file corresponding to the BRUKER data format for simultaneous acquisition. On BRUKER spectrometers using digital filtering, the filter delay is part of the FID. This delay has to be either added to the synthetic signal or removed from the real signal. For simplicity, we chose to remove the delay from the measured signal (command 'convdta' in XWINNMR software, BRUKER, Germany) before adding the synthetic signal to the measured FID; the integrals in spectra obtained from FIDs with and without the filter delay are identical.

The synthetic 2D time domain signal (S_{km}) was calculated as

$$S_{km}^t = S_k^t S_m^t = S_k^t \cdot \exp(2\pi m \Delta_1 (i(\nu_{s1} + \phi_{pc1} + \phi) - l_1/2)) \text{ with} \\ m = 0, 1, 2, \dots, N_1 - 1 \quad (3)$$

where all parameters containing 1 in their subscript refer to the indirect dimension; N_1 is the number of real data points, ν_1 is the offset of the synthetic peak from the carrier frequency and Δ_1 the increment, ϕ_{pc1} is the phase correction (zeroth and first order) in the indirect dimension of the spectrum from which the concentration shall be determined and l_1 is the line broadening factor in Hertz. The parameter ϕ is a phase factor that is used to obtain different types of complex data storage in the indirect dimension. The last 10% of the points in the 2D time domain data FID were multiplied with a \cos^2 window function as described for S_k above. As for S_k above we removed the filter delay from the measured time domain signal before adding the experimental and synthetic 2D time domain data.

For the NMR measurements a BRUKER DRX 600 spectrometer equipped with a [$^1\text{H}, ^{13}\text{C}, ^{15}\text{N}$]-triple resonance probe with a shielded z-gradient coil was used. The concentrations of the reference solution containing 10 mM sucrose (>99%; FLUKA AG, Switzerland) was obtained gravimetrically.⁴ ^{15}N -labeled ubiquitin was obtained from ASLA BIOTECH Ltd (Latvia). All samples contained 95%/5% $\text{H}_2\text{O}/\text{D}_2\text{O}$ and for the reference samples, the solvent line was eliminated by presaturation. Except for the evolution time, the signal loss during all time periods between the

first excitation pulse and the acquisition must be accounted for⁸ and included in the factor f_T (Eqn (1)). Since all experiments were done in H_2O solutions, we could use the same receiver gain; different receiver gains have to be calibrated separately and included in f_T (Eqn (1)).

For the NMR measurements, 5 mm precision NMR tubes (ARMAR Chemicals, Switzerland) with 4.2 ± 0.025 mm inner diameter were used and filled with 600 μl of solution. Based on this tolerance the volume may vary by $\pm 1\%$, which adds to the experimental errors in PULCON.

RESULTS

The experimental examples demonstrating the extension of PULCON to applications with isotope filtered 1D spectra and to 2D spectra were measured with ^{15}N -labeled ubiquitin. With a ^{15}N -filter experiment (Fig. 1), we obtained an average factor f_T of 0.90 ± 0.01 (Eqn (1)) for the ^{15}N -filter element for ubiquitin measured at a temperature of 25 °C. In the spectra obtained with the pulse sequence in Fig. 1, individual as well as groups of resonances of aromatic protons can be observed free from interference with ^{15}N -bound protons. PULCON was applied with the resulting ^{15}N -filtered spectrum in Fig. 2 and a sample of 10 mM sucrose as external reference.

The aromatic resonances between 6.75 and 7.65 ppm can reliably be integrated and the number of contributing protons can be calculated from the amino acid composition of the protein. A concentration of 1.28 mM was thus obtained. The concentration determination with a ^{15}N filter gives an upper limit since residual signals of ^{14}N -bound protons and of ^{15}N -amide moieties with a heteronuclear coupling constant $^1J_{\text{NH}}$ that does not match $\tau = 1/(4^1J_{\text{NH}})$ (Fig. 1) add to the integral. The latter can be neglected since $^1J_{\text{NH}}$ values of backbone amide groups are very homogenous in proteins.¹¹ In Fig. 2, residual signals can for example be observed close to the borders of the spectral regions labeled II. The contribution of residual amide proton resonances can be determined from the percentage of ^{15}N labeling (typically about 98%) and the number of amide resonances in the integration region that can be obtained from a 2D [$^1\text{H}, ^{15}\text{N}$] correlation spectrum. In the present example, there are 20 amide proton resonances in the integration region (Fig. 4). With a labeling degree of 98%, a residual signal intensity of 0.4 protons is obtained that corresponds to 2.5% of the 16 aromatic protons in the integration region; thus the actual concentration is reduced from 1.28 to 1.25 mM. Ubiquitin contains much less aromatic residues than an average protein of its size and thus the systematic error from residual ^{14}N -bound protons will in general be smaller and can be neglected for routine concentration determinations.

For error estimation we integrated separately over the five different spectral ranges indicated in the spectrum in Fig. 2 where the number of contributing protons can easily be determined. An average concentration of 1.26 ± 0.06 mM was obtained (Table 1). We excluded $\text{H}^{\text{C}^\ominus}$ at 7.6 ppm from this integration because of presaturation of the water resonance (Fig. 1). $\text{H}^{\text{C}^\ominus}$ is very close to the exchanging $\text{H}^{\text{N}^\delta}$ and saturation transfer from water protons reduces its intensity, whereas saturation transfer to other aromatic protons is negligible.

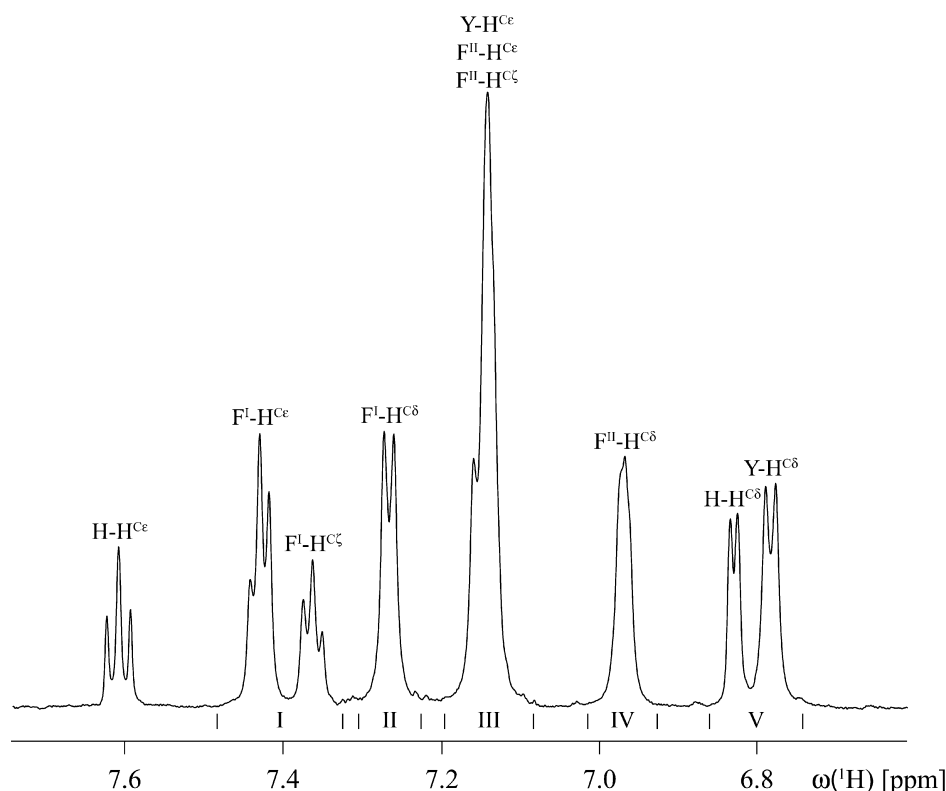


Figure 2. ^{15}N -filtered 1D ^1H spectrum obtained with the pulse sequence shown in Fig. 1 of ^{15}N -labeled ubiquitin in 95%/5% $\text{H}_2\text{O}/\text{D}_2\text{O}$ in 20 mM phosphate buffer at pH 6.3, interscan delay 20 s. Based on this spectrum, a protein concentration of 1.26 ± 0.06 was determined using PULCON⁴ with an external reference of 10 mM sucrose in 95%/5% $\text{H}_2\text{O}/\text{D}_2\text{O}$ (Fig. 3). The roman numbers I–V indicate the integration regions that were used alternatively to continuous integration over the whole aromatic region from 6.75 to 7.5 ppm. Ubiquitin contains 2 Phe, 1 Tyr, and 1 His; resonances are identified using the one-letter amino acid code supplemented by the proton name. The spectrum was recorded at 25 °C at 600 MHz and 128 transients were accumulated.

Table 1. Concentration determination by PULCON^a

Experiment	Concentration (mM)
1D ^1H spectrum ^b with presaturation ^c	1.25 ± 0.03 ($\pm 3\%$)
^{15}N -filtered 1D spectrum ^d with presaturation ^c	1.26 ± 0.06 ($\pm 5\%$)
2D [$^1\text{H}, ^{15}\text{N}$]-HSQC spectrum with water flip-back ^e	1.28 ± 0.10 ($\pm 8\%$)

^a ^{15}N -labeled ubiquitin in 95%/5% $\text{H}_2\text{O}/\text{D}_2\text{O}$; reference sample for PULCON: 10 mM sucrose in 95%/5% $\text{H}_2\text{O}/\text{D}_2\text{O}$ (gravimetric concentration).

^b PULCON with the resolved methine/methyl resonances at $-0.5/-0.27$ ppm.

^c Recycle delay $d_r = 20$ s, presaturation only during the last second of d_r .

^d PULCON with resonances in the aromatic region.

^e PULCON with 14 (30) resolved 2D peaks; the concentration is corrected by the ^{15}N -labeling degree of 98%.

For the application of PULCON with 2D NMR spectra we correlated the signal intensities of the 1D reference spectrum and the 2D spectrum using the time domain signals defined in Eqns (2) and (3). In the 1D reference spectrum the offset ν_s of the synthetic signal from the carrier frequency was set to 1000 Hz resulting in a resonance at about 6.4 ppm in the

1D ^1H spectrum of 10 mM sucrose shown in Fig. 3; a line broadening factor of $l = 10$ Hz was applied.

The synthetic 2D time domain data S_{km}^t was obtained by setting $\phi = (4D_1)^{-1}$ (Eqn (3)) that results in a complex FID with TPPI in the indirect dimension;¹² real and imaginary FIDs were stored sequentially and alternating into a file, which produces the BRUKER data format 'States-TPPI' (software XWINNMR, Bruker, Karlsruhe, Germany). The offsets $\nu_s = 3300$ Hz and $\nu_{s1} = -850$ Hz from the carrier frequency in the direct and indirect dimensions, respectively, were chosen to position the synthetic signal in a spectral region of a [$^1\text{H}, ^{15}\text{N}$]-HSQC spectrum that does not contain protein resonances (Fig. 4).

In the [$^1\text{H}, ^{15}\text{N}$]-HSQC experiment the water magnetization was kept along the z -axis at all times using selective pulses¹³; the residual water magnetization was dephased before acquisition using WATERGATE,¹⁴ which was integrated in the magnetization transfer step preceding acquisition. During the pulse sequence of the [$^1\text{H}, ^{15}\text{N}$]-HSQC experiment, the original signal intensity is reduced by relaxation, limited transfer efficiency (nonperfect 180° pulses and $\tau \neq 1/(4^1J_{\text{NH}})$) and exchange. In addition, measuring the 2D experiment with 20 s interscan delay as required for full recovery of the signal is very inefficient and we chose to use only 1 s interscan delay for the 2D spectrum. The complete signal loss compared to a one-pulse 1D ^1H spectrum

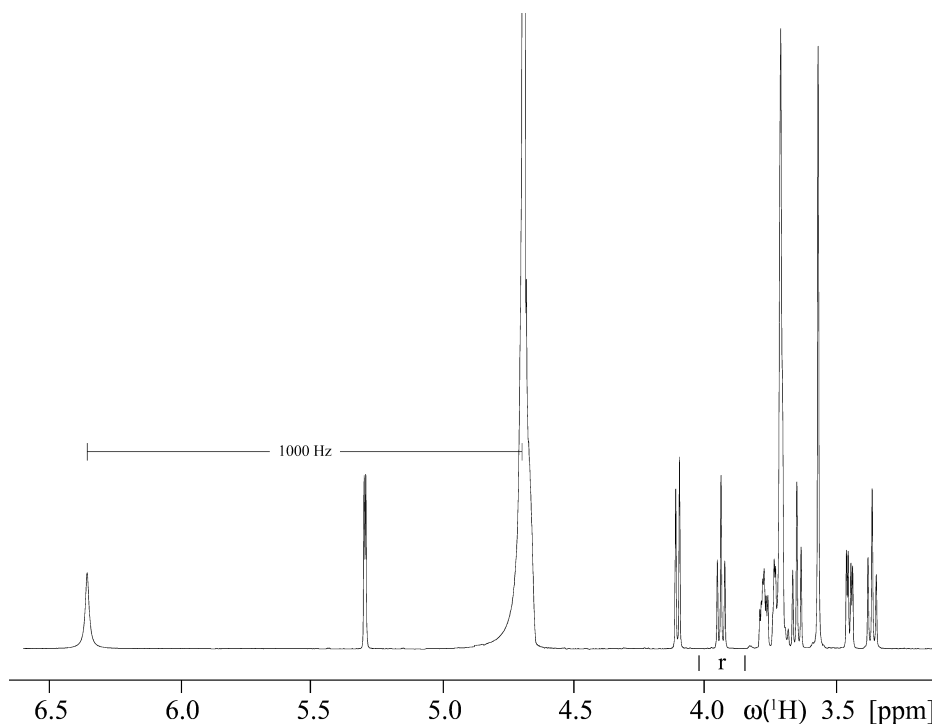


Figure 3. 1D ^1H spectrum of 10 mM sucrose in 95%/5% $\text{H}_2\text{O}/\text{D}_2\text{O}$ measured at 600 MHz including the synthetic peak at about 6.4 ppm with an offset $\nu_s = 1000$ Hz from the carrier frequency and a line broadening factor $l = 10$ Hz (Eqn (2)). The sucrose spectrum was recorded at 25 °C and eight scans were accumulated. The spectral region indicated by 'r' was integrated to obtain a reference integral for PULCON.

was assessed from two 1D ^1H spectra obtained by measuring only the first FID of two $[^1\text{H},^{15}\text{N}]$ -HSQC experiments with an evolution time of 2 μs . In the first experiment (I) the $[^1\text{H},^{15}\text{N}]$ -HSQC pulse sequence was run with an interscan delay of 20 s and 64 transients were accumulated. In the second experiment (II) the whole $[^1\text{H},^{15}\text{N}]$ -HSQC pulse sequence was doubled (except for the first excitation pulse) following the procedure outlined in Fig. 1 (Ref. 8) using an interscan delay of only 1 s, otherwise the same experimental parameters were used as in I. An average factor f_T (Eqn (1)) can be obtained by dividing the integral over the amide spectral region in spectrum II by the one in I, which also takes into account the reduced steady-state magnetization in the experiment with 1 s interscan delay. Alternatively, f_T can be obtained by minimizing the signal obtained from subtracting f_T -times spectrum I from spectrum II, which is the procedure we applied; $f_T = 0.38 \pm 0.02$ was obtained from visual minimization of the difference.

The concentration was calculated with Eqn (1) from integrals of individual peaks in the carefully phased and baseline-corrected 2D $[^1\text{H},^{15}\text{N}]$ -HSQC spectrum shown in Fig. 4. The spectrum of 10 mM sucrose shown in Fig. 3 was used as reference. From the average integral of 14 resolved peaks (indicated by an asterisk in Fig. 4), a concentration of 1.25 ± 0.08 mM was determined; only peaks were selected that had an integral within $\pm 30\%$ of the average integral. Peaks with integrals outside this range cannot be expected to be represented properly by the average factor f_T . Integration of all 30 resolved peaks provided the same concentration and error range; two resolved peaks were excluded since their integral was outside $\pm 30\%$ of the average value. The

peaks used for integration had an average signal-to-noise (S/N) ratio of about 200, where S is the signal height and N is the peak-to-peak noise. The error was estimated from independent integration of the 14 and 30 peaks, respectively, and the error of f_T . The concentration measurement based on a 2D $[^1\text{H},^{15}\text{N}]$ -HSQC spectrum reflects the NMR active, i.e. the ^{15}N -labeled material. For the actual concentration of the protein solution, the labeling degree has to be taken into account, which was 98% for the ^{15}N -labeled ubiquitin used in this study and a total concentration of ubiquitin of 1.28 ± 0.10 mM is thus obtained.

The error obtained for the 2D PULCON measurement is acceptable for a routine concentration determination when the integrals of about 15 peaks are averaged and comparable to a corresponding UV measurement. However, the average factor f_T used for the calculation may limit the precision that can be obtained. For a quantitative comparison we measured the experiments I and II described in the previous paragraph both with 20 s interscan and as full 2D spectra. In this way, the factor f_T can be determined for individual peaks in the 2D spectrum. Using the same 14 peaks described above (Fig. 4), the error based on the standard deviation of the integrals corrected by individual f_T factors was 6%, whereas it was 8% with the averaged factor f_T ; additionally, with individual correction factors f_T , the two peaks that were outside the integral range above could be used for concentration determinations, i.e. they had f_T values deviating substantially from the average.

The performance of PULCON with regard to S/N was investigated by adding noise to the 2D $[^1\text{H},^{15}\text{N}]$ -HSQC spectrum shown in Fig. 4. The S/N ratio (defined as peak

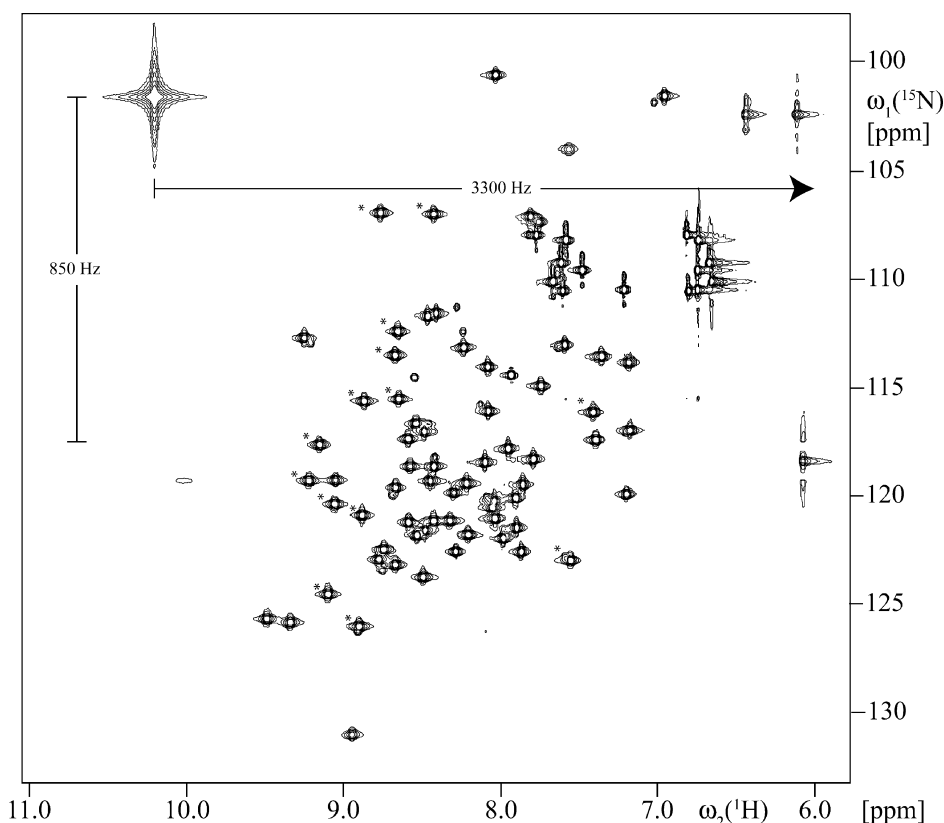


Figure 4. [^1H , ^{15}N]-HSQC spectrum of ^{15}N -labeled ubiquitin in 95%/5% $\text{H}_2\text{O}/\text{D}_2\text{O}$ measured at 600 MHz including the synthetic peak with an offset of $\nu_s = 3300$ Hz in the direct and $\nu_{s1} = -850$ Hz in the indirect dimension from the respective carrier frequencies (Eqn (3)); for the synthetic signal a line broadening factor of 15 Hz was applied in both dimensions. The ubiquitin spectrum was recorded at 25 °C, 16 scans were accumulated per FID and the maximal evolution time was 85 ms; before 2D Fourier transformation, the time domain data (sum of experimental and synthetic data) was multiplied with a cosine function in both dimensions.

height divided by peak-to-peak noise) was stepwise reduced from 200 to 1.7 (Table 2). In the individual spectra, the 14 peaks indicated by asterisks in Fig. 4 were integrated. The standard deviations of the 14 integral values in the individual spectra were used as measure for the error (Table 2). These results indicate that a S/N ratio of 20 still provides the same quality result as the original S/N of 200; for a S/N between 10 and 20 the error only slightly increases, whereas at a S/N of 5 the error has doubled and increases rapidly below 5 (Table 2). These considerations on S/N are valid only if the base plane of the 2D spectrum has been properly corrected. The same applies to 1D spectra where good results with PULCON can be obtained with a S/N of 10, but only if the baseline is flat and without offset in the integration region.

DISCUSSION

It was shown recently that PULCON can deliver as accurate protein concentrations for NMR samples as UV spectroscopy by being at the same time more efficient and less error prone.⁴ PULCON is applied most easily with 1D ^1H spectra and delivers very accurate concentrations if the spectrum contains one resonance or a small group of resonances that are separated from the bulk of the spectrum. If the spectrum lacks resolved resonances, PULCON can be used with the complete aromatic/amide region of a protein spectrum albeit with reduced accuracy since the exact

Table 2. Dependence of PULCON on S/N^a

S/N ^a	Concentration (mM)
200	1.28 ± 0.10 (±8%)
70	1.27 ± 0.10 (±8%)
19	1.25 ± 0.10 (±8%)
12	1.24 ± 0.11 (±9%)
4.7	1.17 ± 0.18 (±15%)
2.6	1.20 ± 0.32 (±27%)
1.7	0.97 ± 0.81 (±85%)

^a Signal height divided by peak-to-peak noise.

number of resonances cannot be determined due to exchange of nitrogen-bound protons with water.⁴ In this situation more accurate concentrations can be determined with isotope labeled compounds and X-filter experiments or with 2D spectra as shown in this work. The data obtained with ^{15}N -filtered 1D ^1H spectra and [^{15}N , ^1H] spectra demonstrates that PULCON can be applied with a much wider selection of experiments than 1D ^1H spectra.

Table 1 summarizes the results obtained with PULCON using a simple 1D, a ^{15}N -filtered 1D or a 2D spectrum. The 1D ^1H spectrum with ubiquitin calibrated against 10 mM sucrose thereby delivers the reference concentration as it was

shown that PULCON applied to spectra containing resolved resonance lines provides as accurate concentrations as UV spectroscopy.⁴ The concentrations obtained with ¹⁵N-filtered 1D ¹H and 2D [¹⁵N,¹H]-correlated spectra are somewhat less precise than those obtained with PULCON from simple 1D ¹H spectra (Table 1), however, they are still precise enough for routine concentration determinations. In special cases, where very precise measurements are required, exact volumes of NMR tubes, precise labeling degrees and factors f_T for individual 2D resonances can be determined (see Section on Results).

PULCON applied with X-filtered 1D spectra is almost as simple and efficient as the use with 1D ¹H spectra and may replace the latter method with labeled proteins in cases where the 1D ¹H spectra do not contain separated resonance lines. The application of PULCON with 2D spectra is more demanding and requires more setup and measurement time. Please note that for X-filtered or heteronuclear 2D experiments, the labeling degree has to be known for the determination of the total protein concentration, otherwise only upper or lower limits, respectively, are obtained. For planning of NMR experiments, lower limit concentrations from heteronuclear 2D experiments are of interest since they represent NMR-active concentrations (for heteronuclear experiments), which is important information, e.g. in cases with high basal expression that result in reduced labeling degrees.

The main fields of application for 2D PULCON experiments may become mixtures of compounds where individual concentrations have to be determined and separate lines cannot be resolved in 1D NMR spectra. Biological macromolecules and many of their ligands have strong UV absorption and individual concentration determinations in mixtures are not possible by UV spectroscopy. In contrast, 2D PULCON can be applied as long as it is known which peak belongs to which compound. More elaborate filtering/editing NMR techniques^{9,10} and isotope labeling schemes than presented here may become necessary to determine accurate factors f_T (Eqn (3)) for the individual compounds. The only alternative method to PULCON, also

based on NMR spectroscopy, requires external r.f. sources to correlate the intensities in two different samples.^{2,3,15} In practice, this method can become rather cumbersome and is difficult to implement for 2D applications; further, it requires special hardware that may not be available on all spectrometers. On the other hand, 2D PULCON will become even easier to use once the numerical correlation of integrals of 1D and 2D spectra becomes part of the NMR processing software.

PULCON and its extension to heteronuclear filtered and 2D NMR experiments are not limited to protein solution NMR and isotope labeled compounds, but can be used in different applications of solution NMR and other NMR applications. For example, for small molecules a COSY experiment can be used for concentration measurements,³ or for solute concentrations larger than 1 mM natural abundance ¹³C may be used, especially with methyl groups, which can easily be detected even at rather low concentrations.

Acknowledgement

Financial support by the 'Schweizerischer Nationalfonds' (project 3100A0-100399) is gratefully acknowledged.

REFERENCES

1. Pauli GF, Jaki BU, Lankin DC. *J. Nat. Prod.* 2005; **68**: 133.
2. Akoka S, Barantin L, Trierweiler M. *Anal. Chem.* 1999; **71**: 2554.
3. Michel N, Akoka S. *J. Magn. Reson.* 2004; **168**: 118.
4. Wider G, Dreier L. *J. Am. Chem. Soc.* 2006; **128**: 2571.
5. Hoult DI, Richards RE. *J. Magn. Reson.* 1976; **24**: 71.
6. Hoult DI. *Conc. Magn. Reson.* 2000; **124**: 173.
7. Van der Klink JJ. *J. Magn. Reson.* 2001; **148**: 147.
8. Braun D, Wüthrich K, Wider G. *J. Magn. Reson.* 2003; **165**: 89.
9. Otting G, Wüthrich K. *Q. Rev. Biophys.* 1990; **23**: 39.
10. Wider G. *Prog. Nucl. Magn. Reson. Spectrosc.* 1998; **32**: 193.
11. Tjandra N, Grzesiek S, Bax A. *J. Am. Chem. Soc.* 1996; **118**: 6264.
12. Marion D, Ikura M, Tschudin R, Bax A. *J. Magn. Reson.* 1989; **85**: 393.
13. Grzesiek S, Bax A. *J. Am. Chem. Soc.* 1993; **115**: 12593.
14. Piotto M, Saudek V, Sklenar V. *J. Biomol. NMR* 1992; **2**: 661.
15. Barantin L, LePape A, Akoka S. *Magn. Reson. Med.* 1997; **38**: 179.