

## Measuring Protein Concentrations by NMR Spectroscopy

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**Abstract:** In applications of NMR to biological macromolecules in solution, the concentration of the NMR sample is an important parameter describing the sample and providing information for the selection and planning of experiments. Although concentrations can be measured directly by NMR spectroscopy, other methods are usually preferred to measure the concentration of macromolecules in NMR samples. The reasons are the difficulties in the correlation of the sample of interest with the signal intensity representing a known concentration. This correlation is usually obtained by adding to the sample a reference compound with known concentration and comparing the integral over resolved resonance lines of the molecules with known and unknown concentrations. For solutions of biological macromolecules it is very difficult to find a compound that does not interact with the macromolecules and has a resonance outside their spectral range. We introduce PULCON which is a method that correlates the absolute intensities of two spectra measured in different solution conditions. PULCON is easy to implement and apply on all NMR spectrometers; it does not need any special hardware or software. PULCON is very robust and at the same time delivers accurate concentrations of samples in the NMR tube. We demonstrate that PULCON has the potential to replace UV spectroscopy for concentration measurements of NMR samples.

### Introduction

The concentration of macromolecules in biological NMR applications is of central importance for the characterization of the sample and the selection and planning of experiments.<sup>1,2</sup> Usually the concentration is determined from optical absorption measurements of the denatured protein at a wavelength of 280 nm or from a Bradford assay by binding of a dye to the protein.<sup>3</sup> Both methods cannot deal with the concentration of an average NMR sample which therefore has to be diluted substantially. Further, both methods are not generally applicable with any arbitrary protein, may interfere with additives in the NMR buffer, and are dependent on calibrations which not always can be determined exactly. It would definitely be an advantage to measure concentrations directly in the NMR tube. In principle, NMR spectroscopy would be very well suited to measure concentrations since the NMR signal is proportional to the number of molecules in the sample. However, the determination of the absolute concentration requires a reference compound of known concentration to be added to the sample which then permits determination of concentrations by direct comparison of integrals in one spectrum.<sup>4</sup> This method is used in chemical applications of NMR, but there is great reluctance in using it for biological applications of NMR. Adding substances to a solution of biological macromolecules bears the risk that the two types of molecules interact which is unacceptable. Further, the spectra are crowded with signals, and finding a reference

compound that resonates in an empty region of the spectrum will be rather difficult.

On principal grounds it would be very much preferable to determine concentrations of NMR samples directly in the final NMR tube using NMR spectroscopy. Such a procedure would be much less error prone and more efficient than currently used methods since it eliminates the need for a special preparation of samples to make them suitable, e.g., for optical absorption measurements. Since no internal reference compound can be used in NMR samples of biological macromolecules, an external means has to be found that correlates the NMR signal strengths of the sample of interest with a separate reference sample. The correlation must include properties of the receiving coil that change with the sample conditions, e.g., salt content, and influence the signal strength. The external reference should mimic as closely as possible the NMR signal received from the spins in different sample conditions which will make the method easy to use and less error prone. A technique applied in chemical and in medical magnetic resonance applications uses an external radio frequency (rf) signal of constant amplitude that is irradiated during acquisition and thus permits correlation of the intensities measured in two different samples.<sup>5,6</sup> In practical applications this method can be rather cumbersome and needs changes in the spectrometer hardware, special experiments, and special processing. For all of these reasons the method is not suitable for routine applications of concentration measurements of biological macromolecules and has so far

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not been applied in this field. Here we investigate the application of the principle of reciprocity<sup>7</sup> to concentration measurements. In short, this principle states that the NMR signal strength is inversely proportional to the 90° pulse length. The application of such a simple external correlation promises an easy to use and robust method which can readily be applied in routine concentration measurements.

## Materials and Methods

The determination of concentrations by NMR requires that the integral over a resonance in the spectrum of a reference sample is correlated with signal strength in the sample of interest with unknown concentration. The signal strength in two NMR spectra can be correlated based on the principle of reciprocity.<sup>7–9</sup> This principle can be expressed in the following way: if a given NMR coil creates a large radio frequency (rf) magnetic field at point A within a sample inserted in this coil, then the coil has also a large sensitivity to the nuclear magnetization at A. Or in other words, in an appropriate setup the length of the 90° pulse for a sample in a given coil is inversely proportional to the sensitivity that can be obtained.

Using the principle of reciprocity, the signal strength  $S$  measured by a given NMR coil can be expressed by<sup>7–9</sup>

$$S \propto B_{90} \int \frac{\partial}{\partial t} \left( \frac{\vec{B}_{1u} \cdot \vec{M}}{|B_{1u}|} \right) dV \quad (1)$$

where  $B_{90}$  is the rf field amplitude of a 90° pulse measured with the sample in the tuned and matched coil,  $B_{1u}$  is the field created by unit current in the volume element  $dV$ ,  $M$  is the magnetization perpendicular to the main magnetic field in  $dV$ , and the integration runs over the sample volume. For measurements in aqueous solutions the high-temperature approximation is fulfilled and the magnetization  $M$  becomes

$$M \propto c/T \quad (2)$$

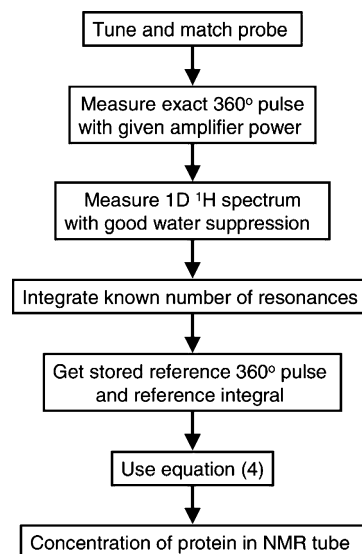
where  $c$  is the concentration, and  $T$ , the temperature in Kelvin. Combining eqs 1 and 2, the following proportionality of the signal strength  $S$  is obtained

$$S \propto \frac{cB_{90}}{T} \int \frac{\partial}{\partial t} \left( \frac{\vec{B}_{1u} \cdot \vec{M}}{|B_{1u}| \cdot |M|} \right) dV \quad (3)$$

For a given coil the integral in eq 3 is a constant if the sample in the NMR tube fills the active volume of the coil in the  $z$  direction.<sup>7–9</sup> The rf magnetic field strength  $B_{90}$  is inversely proportional to the duration of the 90° pulse and thus of the 360° pulse,  $\theta_{360}$ ; the latter is easier to measure in practice. Using eq 3 the unknown concentration  $c_U$  of a sample U can be calculated from the known concentration  $c_R$  of a reference sample R with

$$c_U = f_T c_R \frac{S_U T_U \theta_{360}^U n_R}{S_R T_R \theta_{360}^R n_U} \quad (4)$$

where  $S$  stands for the signal strength (i.e., the integrals over resonance lines),  $T$ , for the sample temperature in Kelvin,  $\theta_{360}$ , for the 360° rf pulse, and  $n$ , for the number of transients used for the measurements of the two samples U and R, respectively. The factor  $f_T$  accounts for variations in signal intensities in cases where different experimental schemes are used for the measurement of the samples U and R. The factor  $f_T$  is equal to 1 when both measurements are one-pulse experiments with the signal acquisition immediately following the rf pulse. With more than one scan per spectrum the relaxation period



**Figure 1.** Flowchart of a concentration measurement in the NMR sample tube using PULCON.

between scans has to be chosen long enough to exclude interference from differential longitudinal relaxation times of the resonances contributing to  $S_U$  and  $S_R$ , respectively. Equation 4 is valid when the measurements are done with the same NMR probe which is tuned and matched to the same amplifier delivering the same power. A properly tuned and matched coil is not a requirement of the principle of reciprocity, but is a consequence of the fact that this is the only state that can be reproducibly obtained on every spectrometer. The acquisition and transformation parameters can be adapted to the individual samples. It is most convenient to use the same receiver gain in the two measurements, otherwise the scaling introduced by different receiver gains has to be determined separately and included in  $f_T$  (eq 4). Further, the first value in the window functions applied during Fourier transformation must be one; otherwise the integral will be falsified. Please note that different scaling factors may be applied by the software during processing and hence care has to be taken to trace the proper scaling of the peak integrals.<sup>10</sup>

Based on eq 4 we propose the procedure PULCON (*pulse length based concentration determination*) summarized in Figure 1 for the measurement of protein concentrations in NMR tubes by NMR spectroscopy. After tuning and matching the probe with the sample of interest,  $S_U$ , the 360° rf pulse is determined. A single proton resonance or a group of resonances with a known number of contributing protons is integrated in the spectrum of  $S_U$ . The previously determined 360° pulse and the reference integral of the reference sample  $S_U$  are used to calculate the concentration of the sample of interest using eq 4.

The NMR measurements were done on a BRUKER DRX 600 and DRX 750 spectrometer using [<sup>1</sup>H,<sup>13</sup>C,<sup>15</sup>N]-triple resonance probes equipped with a shielded  $z$ -gradient coil. All samples contained 95%/5% H<sub>2</sub>O/D<sub>2</sub>O, and for the reference and test samples the solvent line was eliminated by presaturation. For protein samples presaturation may affect the intensities of resonance lines due to saturation of exchangeable protons and NOE effects. The problems with presaturation in protein samples were circumvented by measuring 1D <sup>1</sup>H spectra with a WATERGATE sequence<sup>11</sup> between the excitation pulse and the acquisition. However, relaxation and exchange during the WATERGATE element reduce the signal intensities, and for a determination of the original signal intensities the transfer efficiency of a WATERGATE element has to be measured.<sup>10</sup> The transfer efficiency was obtained from two experiments where one or two WATERGATE elements were applied before acquisition of the signal. From the ratio

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of the integrals in the two spectra the factor  $f_T$  (eq 4) can be calculated which quantizes the signal loss during one WATERGATE element, so that eq 4 can be applied.

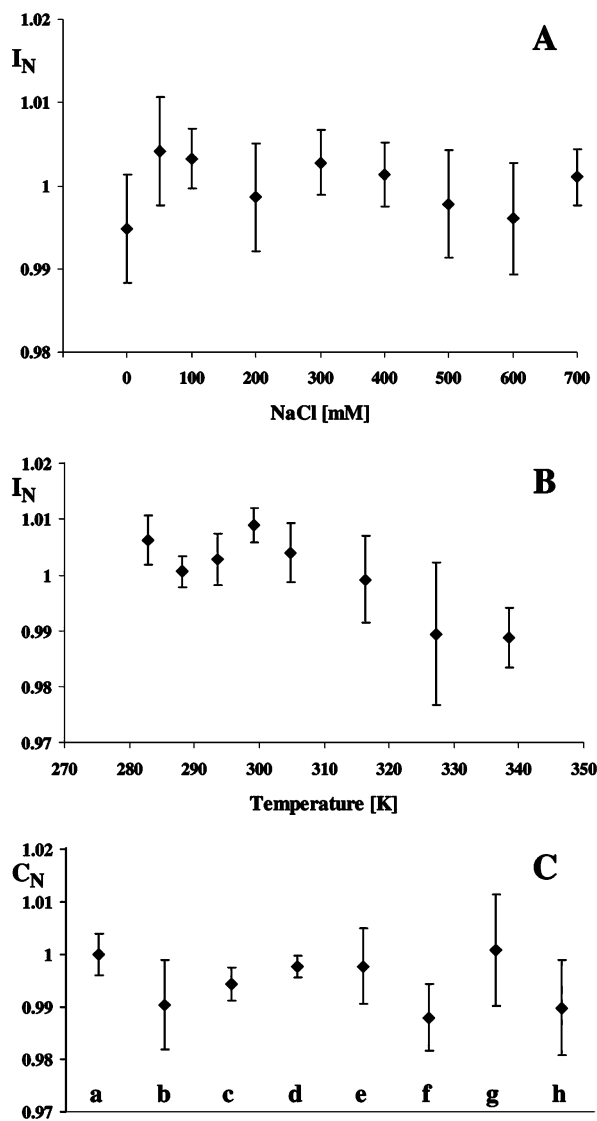
The concentration measurements by NMR were repeated 5 times for each sample, and the precision of the measurements was determined from the standard deviation. The accuracy of the measurement can suffer from interscan delays which are too short. To prevent such an interference we used interscan delays of 30 s which guarantee more than 99% recovery of signals with longitudinal relaxation times  $T_1$  shorter than 6 s which is longer than the  $T_1$  values of all nonprotein samples measured; for protein samples the interscan delay was 20 s. The acquisition (except for the individually determined  $90^\circ$  pulses) and processing parameters for the reference, test, and protein samples were the same. Prior to Fourier transformation the data were multiplied with a decaying exponential function (line broadening factor of 2 Hz). The spectra were carefully phased, and the offset of the flat baseline was corrected by subtracting a constant value. The integrals were determined without any further corrections for slope or bias. Integrals of different spectra were measured and calibrated using standard NMR software (e.g., command `lastcal` in XWINNMR 3.5, BRUKER, Germany), and eq 4 was applied with the values thus obtained.

The concentrations of the reference solutions containing sucrose (>99%; Fluka AG, Switzerland) and tyrosine (>99%, Fluka AG, Switzerland) were obtained gravimetrically. Stock solutions were prepared with a concentration of 100 mM, and the final reference samples were obtained from these solutions by dilution. Hen egg white lysozyme was obtained from Fluka (Switzerland; ~97%), and 20 mL of a 5 mM solution were prepared to minimize errors in the gravimetrically determined concentration. From this stock solution seven samples were produced with concentrations of 5, 2, 1, 0.5, 0.25, and 0.1 mM; two 1 mM NMR samples were prepared, one unaltered and one with the addition of the free amino acid Trp in a 1 mM concentration. Aliquots of the different solutions were diluted, and the concentration was measured by UV spectroscopy indicating the following concentrations: 5.05, 2.07, 1.02, 0.51, 0.26, and 0.10 mM. The UV extinction coefficient  $\epsilon = 37970$  at 280 nm (<http://www.expsy.org/tools/protparam.html>) was used for the denatured protein. A linear regression analysis against the gravimetrically determined concentrations on the  $x$ -axis resulted in a slope of 1.009, an offset of 0.012 mM, and a coefficient of determination  $R^2 = 1$ .

For the NMR measurements 5 mm precision NMR tubes (ARMAR Chemicals, Switzerland) with  $4.2 \pm 0.025$  mm inner diameter were used and filled with 600  $\mu$ L of solution. Based on this tolerance the volume may vary by  $\pm 1\%$  which translates to a possible error of  $\pm 1\%$  in addition to the measurement errors when the concentration is measured in this NMR tube using NMR spectroscopy.

## Results

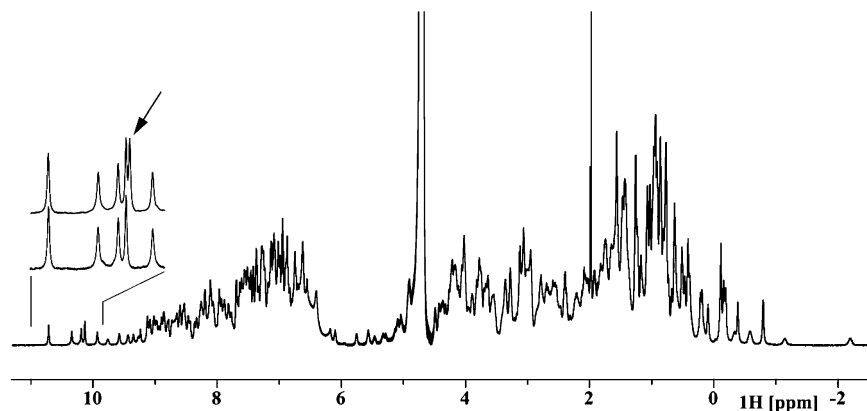
For an efficient determination of protein concentrations by NMR it is important that the method is independent of the sample conditions and measurement temperature, so that only one reference spectrum per probe is required for different samples. Accurate and efficient measurements which examine these features are best carried out with small, robust molecules. The performance of PULCON in practice was thus investigated with 10 mM samples of sucrose dissolved in 95%/5%  $H_2O/D_2O$  with different salt contents, measured at different temperatures, with different concentrations and using different additives. The influence of the sample conductivity (salt content) was tested using PULCON with 1D  $^1H$  NMR spectra of sucrose samples containing 0, 50, 100, 200, 300, 400, 500, 600, and 700 mM NaCl. The probe was tuned and matched to the individual samples, and the  $360^\circ$  rf pulse was determined; the other acquisition and processing parameters were the same. The



**Figure 2.** Plots of normalized intensities  $I_N$  and normalized concentrations  $C_N$  for different sample conditions and different samples. (A) Sum of the integrals of the resonances at 3.95 and 4.1 ppm in 10 mM sucrose samples in 95%/5%  $H_2O/D_2O$  measured at different concentrations of NaCl ranging from 0 to 700 mM. The normalized integrals  $I_N$  were obtained by dividing by the arithmetic average value of all 45 measurements; the error bars represent the standard deviation. (B) Measurements at different temperatures with 10 mM sucrose in 95%/5%  $H_2O/D_2O$ . The same data evaluation as that in part A was used. (C) Concentration measurements using eq 4 and 10 mM sucrose in 95%/5%  $H_2O/D_2O$  as a reference sample. The normalized concentrations  $C_N$  were obtained by using eq 4 and dividing the result by the concentration that was determined gravimetrically for the individual samples; the error bars represent the standard deviation. The following samples were measured: (a) 10 mM sucrose in 95%/5%  $H_2O/D_2O$  (reference sample,  $C_N = 1.0$ ); (b) 0.1 mM sucrose in 95%/5%  $H_2O/D_2O$ ; (c) sample a plus 50 mM phosphate buffer; (d) sample a plus 50 mM phosphate buffer and 50 mM NaCl; (e) sample a plus 3 mM CHAPS; (f) Sample a plus 3 M urea; (g) 1 mM tyrosine in 95%/5%  $H_2O/D_2O$ ; (h) 2 mM tyrosine in 95%/5%  $H_2O/D_2O$ .

results are shown in Figure 2A, where normalized integral values are shown. Each measurement was repeated 5 times with new tuning/matching and consecutive determination of the  $360^\circ$  pulse length. The data in Figure 2A convincingly show that the concentration measurements using PULCON do not depend on the salt content.

Equation 4 relates only the strengths of the signals and is not directly dependent on the noise created in the receiving coil;



**Figure 3.** 1D  $^1\text{H}$  spectrum of lysozyme in 95%/5%  $\text{H}_2\text{O}/\text{D}_2\text{O}$  in 20 mM phosphate buffer at pH 4. The magnified spectral regions on the left-hand side show the lowest field indole resonances of the lysozyme spectrum with (upper trace) and without (lower trace) the addition of 1 mM Trp; the indole resonance of the free amino acid Trp is indicated by an arrow. For the concentration determination, the four lowest field resonances, the two highest field resonances, and the aromatic/amide region between 6.1 and 10.9 ppm were integrated (see text).

thus the reference sample and the sample of interest can be measured at different temperatures. This prediction was verified with measurements in the temperature range from 10 to 60 °C using a 10 mM sucrose sample. The results shown in Figure 2B confirm that PULCON can be applied over the whole temperature range usually used with protein samples using only one reference sample. In addition to the results with different salt concentrations and different temperatures, Figure 2C shows measurements with sucrose samples containing phosphate buffer, CHAPS, and urea which are all common additives to NMR samples. Irrespective of the additives, the determination of concentrations using PULCON gives the correct result with an error of less than  $\pm 2\%$ . As expected, large concentration differences do not influence the precision and accuracy of the measurements (Figure 2C: measurements a and b); with lower concentrations only more transients have to be accumulated to obtain sufficient signal-to-noise in the resulting spectrum.

The accuracy and reproducibility of the concentration determination using PULCON was investigated with 10 mM sucrose as a reference sample which was used to determine the concentration of a 1 mM and a 2 mM sample of tyrosine (Figure 2C). All three samples were measured 5 times each. Between the measurements the samples were removed from the magnet and reinserted. The probe was newly tuned, shimming was optimized, and the  $360^\circ$  rf pulse was determined. All other acquisition and processing parameters were the same for the 15 measurements except for the number of transients which was 8 for the sucrose and 32 for the tyrosine samples, respectively. The standard deviation obtained for 10 mM sucrose was  $\pm 0.04$  mM. The average integral value obtained from the 5 measurements with 10 mM sucrose was defined to correspond to a concentration of 10 mM and used to determine the tyrosine concentration in the 10 individual measurements with Tyr; values of  $1.00 \pm 0.01$  mM and  $1.98 \pm 0.02$  mM were obtained for the 1 mM and 2 mM tyrosine samples (Figure 2C), respectively, confirming that PULCON can provide accurate concentration measurements for different samples.

For the application of PULCON to aqueous solutions of a protein, we prepared six samples with concentrations of 5, 2, 1, 0.5, 0.25, and 0.1 mM hen egg white lysozyme. Lysozyme is very well suited for the test measurements since it can be obtained pure and in large quantities; we used 1.5 g of protein to obtain very accurate gravimetrically determined concentra-

tions. PULCON can readily be applied when the 1D  $^1\text{H}$  spectrum contains separated resonance lines representing a known number of protons. In protein spectra this would usually be low field shifted amide resonances or high field shifted resonances. For proteins in aqueous solution water suppression techniques have to be used which do not saturate protein resonances. When we measured a 1D  $^1\text{H}$  spectrum of 1 mM lysozyme with presaturation, a concentration of only 0.8 mM was obtained with the four lowest field indole amide protons and a concentration of about 0.95 mM with the two resonances at  $-2.2$  and  $-1.2$  ppm (Figure 3); the reference sample was 10 mM sucrose.

The problems with presaturation were eliminated by using WATERGATE<sup>11</sup> which permits very good water suppression, a prerequisite for a reliable integration of 1D  $^1\text{H}$  protein spectra. The signal loss during the water suppression element was determined as described in Material and Methods. With the 1 mM lysozyme sample we obtained a concentration of  $0.95 \pm 0.04$  mM using the four lowest field indole resonances and a concentration of  $1.01 \pm 0.05$  mM using the two highest field shifted resonances (Figure 3). We investigated the robustness, reliability, and ease of use of PULCON with protein solutions with single measurements of six different concentrations of lysozyme in the range 0.1 to 5 mM. For the individual measurements, the  $360^\circ$  rf pulse was determined; the other acquisition and processing parameters were the same for the 6 measurements except for the number of transients which was stepwise increased from 8 for the 5 mM solution to 2048 for the 0.1 mM solution. The following concentrations were obtained with PULCON: 5.01, 2.02, 0.99, 0.501, 0.249, 0.096 mM. A linear regression analysis against the gravimetrically determined concentrations on the x-axis resulted in a slope of 1.003 (UV: 1.009), an offset of 0.001 mM (0.012 mM), and a coefficient of determination of  $R^2 = 1$  (1); results which compare favorably with the corresponding analysis of the UV measurements given in brackets (see Materials and Methods).

The situation of proteins without clearly resolved single resonance lines was evaluated by integrating the whole amide/aromatic region between 6.1 and 10.9 ppm (Figure 3). The number of protons was determined from the protein sequence counting all aromatic protons and all N-bound exchangeable protons, except the  $\epsilon\text{-NH}_3$  of Lys which are usually not observed and half of the guanidino  $\text{NH}_2$  groups which are only partially observed.<sup>12,13</sup> In this way a total number of 258 contributing

proton resonances was estimated. If the spectral properties of the protein resonances are not known, it is not possible to account for resonances which have unusual chemical shifts or which are missing due to conformational or chemical exchange processes. However, compared to the uncertainty in exchangeable protons this will usually be a small number. The concentration obtained from the integration of the amide/aromatic region was 0.94 mM; whereas the scaling and integration were very reproducible resulting in an error of  $\pm 0.05$  mM, the uncertainty in the number of (exchangeable) protons remains. The number of Arg residues in lysozyme is far above average for this size of protein; counting half of the 22  $\text{NH}_2$  groups of the 11 Arg could introduce a maximal error of  $\pm 8\%$  in the unlikely case that all or none of these resonances were observed. The maximal error in the concentration of lysozyme using the whole amide/aromatic spectral region may thus be up to  $\pm 13\%$  including the measurement errors, a value which is clearly not reached by the actual experimental result.

## Discussion

Concentration measurements of proteins in NMR samples are usually determined by UV absorbance measurements at 280 nm of a diluted and denatured aliquot of the NMR sample. The amount of the aliquot is typically small to minimize loss of protein, and for the same reason the concentration measurements are not repeated. In routine measurements rather large errors in the determination of the concentration may result. Further, proteins in denaturing conditions may contain residual structured parts<sup>14,15</sup> which are often associated with Trp residues. Since Trp has by far the largest absorption in UV spectra of proteins, large systematic errors may be introduced in UV measurements which cannot easily be accounted for. A real challenge is presented by mixed samples where the cosolute is also UV active, e.g., a mixture of proteins and nucleotides where the large absorption of the nucleotides will make a reliable determination of protein concentrations impossible.

In contrast to the problems one may encounter with UV measurements we have shown that direct measurements of concentrations in the NMR tube by PULCON can be very precise and accurate. A very wide range of different solvent conditions in the NMR sample are covered by eq 4. Different conductivities of the samples result in varying signal strength which are perfectly compensated by the changing  $360^\circ$  pulse length (Figure 2A and C) as predicted by the principle of reciprocity.<sup>7–9</sup> Temperature dependent properties of the coil are taken into account by proper tuning/matching and the determination of the  $360^\circ$  pulse; the explicit dependence on the temperature in eq 4 reflects the Curie law. Thus, the measurements of the reference sample and the sample of interest can be made at different temperatures (Figure 2B). Further, one reference sample can also cover a very wide concentration range (Figure 2C: measurements a and b) which is only limited by the dynamic range of the spectrometer. For solutes in  $\text{H}_2\text{O}$  one spectrum of a 1 to 10 mM reference sample is sufficient for concentration determinations between 10  $\mu\text{M}$  and 100 mM if

one works with the same receiver gain which is not a precondition but convenience (see Materials and Methods). This range will be even larger for nonprotonated solvents which do not limit the dynamic range by residual solvent resonances.

The reliability of PULCON depends on the rf power delivered to the coil in the NMR probe and perfect tuning/matching of the coil. The power at the coil depends not only upon the use of a stable amplifier but also upon identical rf cabling, rf filter, and other components in the path from the amplifier to the probe. If these conditions are met, one reference spectrum per probe and spectrometer can be used for many weeks<sup>16</sup> before the reference sample has to be remeasured to guarantee reliable concentration measurements. If proper matching cannot be obtained (e.g., due to high salt content), a reliable concentration measurement is not possible and only lower limits are obtained representing the NMR-active concentration which is still valuable information when planning NMR experiments.

The ability to use only one reference measurement for all concentration determinations relies on identical filling of the receiving coil. Thus, the NMR tube has to contain a minimal amount of solution which fills the active volume of the coil; any additional sample volume then does not influence the concentration measurement. On our probes this condition is fulfilled for properly centered NMR tubes containing about 400–450  $\mu\text{L}$  of solution. Conventional NMR tubes are usually filled with  $>450$   $\mu\text{L}$  of solution, and PULCON can be applied without restrictions. The situation is different for sample tubes that minimize the sample volume using a movable glass plug and a tube bottom which are both susceptibility matched to the solvent (“Shigemi tubes”). For this type of tube a calibration curve can be measured which correlates volumes (or sample heights) in the range of 100  $\mu\text{L}$  to 450  $\mu\text{L}$  against signal strength. With this correlation, the reference integral can be scaled appropriately to be used with Shigemi tubes. For our probes the plunger in these tubes is long enough to keep the solution above the plunger outside the active volume of the coil for sample volumes as small as 100  $\mu\text{L}$ .

The overall error of concentration measurement by PULCON critically depends on the quality and reproducibility of the integration. For separated resonance lines very reproducible integrals can be obtained. As soon as the lines are no longer separated close to the baseline, the error increases since the integration range is not clearly defined. But even in this situation the error stays within the limits of corresponding UV measurements unless there is severe overlap of resonances. In slightly overlapping spectral regions, the error could be reduced by line fitting which would provide more reliable integrals. To keep the method simple and applicable on all types of spectrometers with standard software, we propose to integrate the whole amide/aromatic region in the spectrum in cases where there are no resolved resonance lines. The integration region is then well defined, but since many resonances in this region are potentially exchanging with water, the number of contributing proton resonances is usually not exactly known (see Results) resulting in a larger error which seems still acceptable taking into account the simplicity of PULCON and the accuracy of alternative methods.

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## Conclusion

In this work we have shown that protein concentrations can be reliably measured in the NMR sample tube by NMR spectroscopy without adding any reference compound. The method PULCON is a direct consequence of the principle of reciprocity,<sup>7</sup> which is represented in an adequate form for concentration measurements by eq 4. From the 360° pulse measured with the sample of interest, the concentration can be determined based on an external reference sample. Only one reference spectrum is required per probe, and it can be used for all solution and measurement conditions as long as the probe can be tuned and matched. With the long-term stability of modern NMR spectrometers, the reference sample need not be measured with every application of PULCON; the integral value from the reference spectrum and the corresponding 360° pulse length can be used for several months.<sup>16</sup> PULCON works directly on the NMR sample of interest, can be applied on the most basically equipped NMR spectrometer, does not require

any special software or sophisticated experiments, and is at the same time very robust and easy to use. PULCON permits concentration measurements which are accurate and precise enough for all practical purposes. We expect that PULCON will find widespread applications for the determination of concentrations of biological macromolecules. PULCON is not limited to protein solution NMR, and the concentration of any solute producing a NMR signal can be determined. Thus, PULCON can be applied in solution NMR at large and may find applications in solid-state NMR as well as in magnetic resonance imaging and in vivo magnetic resonance spectroscopy for measurements of many kinds of compounds.

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