

R-factor, Free R, and Complete Cross-Validation for Dipolar Coupling Refinement of NMR Structures

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Abstract: NMR structure determination of macromolecules involves a minimization problem in which atomic models, subject to restraints relating to idealized covalent geometry and nonbonded contacts, are fitted to experimental observables. The latter comprise restraints between atoms separated by $\leq 6 \text{ \AA}$, such as NOE-derived interproton distances, torsion angles, coupling constants, and chemical shifts, as well as restraints that provide direct information on long-range order such as dipolar couplings. An expression for the dipolar coupling *R*-factor is derived which provides a quantitative and readily interpretable measure of the agreement between observed and calculated dipolar couplings. The dipolar *R*-factor expresses the ratio of the observed rms difference between observed and calculated values with that expected for a totally random distribution of vectors. The latter can be calculated exactly from the magnitude of the alignment tensor. The dipolar *R*-factor scales between 0 and 1, where a value of 0 indicates perfect agreement between observed and calculated dipolar couplings, and a completely random structure yields a value of 1. The dipolar coupling *R*-factor is readily amenable to complete cross-validation, with multiple pairs of working and test data sets, thereby permitting one to assess the quality of the fit to the experimental dipolar couplings and to avoid overfitting the experimental data. The application of the dipolar *R*-factor with complete cross-validation is demonstrated using experimental data for the protein cyanovirin-N.

Introduction

One of the main indicators of quality in crystal structures is the *R*-factor which quantitatively measures the agreement between observed and calculated reflections.¹ The principal source of geometric information used in NMR structure determination is the NOE which provides interproton distance restraints between protons separated by $\leq 6 \text{ \AA}$.² Although attempts have been made to define an analogous *R*-factor for NOE intensities in NMR structure determinations,^{3,4} the NOE *R*-factor is rarely used, particularly in the case of proteins, since direct refinement against NOE intensities is problematic both from a theoretical standpoint as well as a computational one.^{3–5} Moreover, the meaning of the NOE *R*-factor is difficult to ascertain and relate to. Residual dipolar couplings,⁶ generally measured on protein samples partially oriented in dilute liquid crystals of bicelles^{7a} or rod-shaped virus particles,^{7b,c} have

recently been introduced into protein structure determination.⁸ Dipolar couplings provide unique long-range structural restraints for NMR structure determination that are not accessible by most other NMR observables, such as the NOE, chemical shifts and couplings constants, which are dependent on close spatial proximity of atoms.^{6–9} Consequently, dipolar coupling restraints represent a particularly powerful set of restraints for defining the orientation of one structural element to another, one domain to another, or one component of a complex to the other(s) in cases where the information content provided by the NOE restraints (which require a proton–proton separation of $\leq 6 \text{ \AA}$) is insufficient, either due to lack of NOEs or to accumulation of errors over long distances. In addition to providing long-range information, the use of various different dipolar couplings (e.g., D_{N-H} , $D_{N-C'}$, $D_{HN-C'}$, $D_{C\alpha-H}$, $D_{C\alpha-C'}$) along the protein backbone provide restraints that narrowly restrict the range of accessible ϕ and ψ backbone torsion angles.^{8a,b} In this paper, we will show that an expression for a readily interpretable

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R -factor for dipolar couplings can be derived which provides a useful indicator of the quality of NMR structures. In addition, we will demonstrate that the R -factor for dipolar couplings is amenable to complete cross-validation in a straightforward manner.

Results

Theoretical Background. The observed dipolar coupling between two atoms A and B is given by^{9a}

$$D^{AB}(\theta, \phi) = D_a^{AB}(3 \cos^2 \theta - 1) + \frac{3}{2} D_r^{AB}(\sin^2 \theta \cos 2\phi) \quad (1)$$

where D_a^{AB} and D_r^{AB} in units of hertz are the axial and rhombic components of the traceless second rank diagonal tensor \mathbf{D} given by $\frac{1}{3}[D_{zz}^{AB} - (D_{xx}^{AB} + D_{yy}^{AB})/2]$ and $\frac{1}{3}[D_{xx}^{AB} - D_{yy}^{AB}]$, respectively, with $|D_{zz}^{AB}| > |D_{yy}^{AB}| \geq |D_{xx}^{AB}|$; θ the angle between the A–B interatomic vector and the z axis of the tensor; and ϕ the angle which describes the position of the projection of the A–B interatomic vector on the x – y plane, relative to the x axis. D_a^{AB} subsumes various constants, including the gyromagnetic ratios of the two nuclei γ_A and γ_B , the inverse cube of the distance between the two nuclei $\langle r_{AB}^{-3} \rangle$ where the $\langle \rangle$ brackets indicate vibrational averaging, and the generalized order parameter S for fast angular fluctuations of the internuclear vector which provides a first order correction for the effect of rapid internal motion on D^{AB} . The various types of dipolar couplings can be normalized relative to say the N–H dipolar couplings, ${}^1D^{NH}$, using the equation¹⁰

$$D^{AB}(\text{NH}) = D^{AB}(\gamma_N \gamma_H \langle r_{NH}^{-3} \rangle / \gamma_A \gamma_B \langle r_{AB}^{-3} \rangle) \quad (2)$$

where $D^{AB}(\text{NH})$ is the dipolar coupling between atoms A and B, normalized relative to ${}^1D^{NH}$. By this means, it is a simple matter to weight the force constants for the other dipolar couplings relative to that for the N–H dipolar couplings on the basis of their respective experimental measurement errors. In addition, with all the dipolar couplings normalized, global R and free (cross-validated) R -factors are readily calculated (see below).

The effect of internal motion on dipolar couplings has been discussed in detail previously.⁶ Since the observed dipolar couplings scale with S and not S^2 and since peptide backbone amide S^2 values in structured regions of a protein typically fall in the 0.85 ± 0.05 range, the assumption of a uniform S value introduces a negligible error of at most a few percent in the dipolar coupling.⁶ Even relatively mobile residues with S^2 values of 0.6 will have an S value of 0.77 and therefore only introduce minimal errors. For residues with S^2 values less than 0.6, the harmonic potential employed for the dipolar couplings^{6,9a} should be replaced by a half-harmonic potential which sets a lower limit for the calculated coupling but no upper limit.^{9b} The presence of a significant degree of internal mobility in the backbone can be readily ascertained by measurement of ${}^{15}\text{N}$ – $\{^1\text{H}\}$ NOE values where a value of less than 0.6 is diagnostic of the presence of internal motion. In the case of side chains, particularly those at the surface of a protein, one should generally adopt the half-harmonic potential.

Derivation of a Dipolar R -Factor. Traditionally, the R -factor employed in crystallography is defined as $\sum(|\text{obs} - \text{calc}|/|\text{obs}|)$ where obs and calc are the observed and calculated experimental values, respectively. Because the tensor \mathbf{D} is unitary, $\langle D \rangle$ is zero. Consequently, for an infinite number of randomly distributed

vectors $\langle (D_{\text{obs}} - D_{\text{calc}})^2 \rangle / \langle D_{\text{obs}}^2 \rangle = 2$ (where D_{obs} and D_{calc} are the observed and calculated values of the dipolar couplings, respectively). For an infinite number of measurements, a more useful definition of the R -factor for dipolar couplings, R_{dip} , is therefore given by

$$R_{\text{dip}} = \{ \langle (D_{\text{obs}} - D_{\text{calc}})^2 \rangle / (2 \langle D_{\text{obs}}^2 \rangle) \}^{1/2} \quad (3)$$

which expresses in effect the ratio of the observed rms difference between observed and calculated values with that expected for a totally random distribution of vectors, $(2 \langle D_{\text{random}}^2 \rangle)^{1/2}$, given by $(2 \langle D_{\text{obs}}^2 \rangle)^{1/2}$. R_{dip} scales between 0 and 1 and is a factor of $\sqrt{2}$ smaller than the Q -factor defined by Cornilescu et al.¹¹ Thus, $R_{\text{dip}} = 0$ for perfect agreement between observed and calculated dipolar couplings, and $R_{\text{dip}} = 1$ for a completely random structure. In practice, the distribution of vectors is not completely random and more importantly the number of observed dipolar couplings is far from infinite. Hence, the experimentally determined value of $(2 \langle D_{\text{obs}}^2 \rangle)^{1/2}$ will not in fact be equal to the expected rms difference between observed and calculated values for a random distribution of vectors, $(2 \langle D_{\text{random}}^2 \rangle)^{1/2}$. Fortunately, the value of $\langle D_{\text{random}}^2 \rangle$ for a random distribution of vectors can be computed exactly since it is given by the second moment of the powder pattern

$$\langle D^2 \rangle_{\text{random}} = (4\pi)^{-1} \int_0^\pi \sin \theta \, d\theta \int_0^{2\pi} d\phi D^2(\theta, \phi) = (D_a^{AB})^2 [4 + 3\eta^2] / 5 \quad (4)$$

where η is the rhombicity given by D_r^{AB}/D_a^{AB} and is always positive. Thus, R_{dip} can be expressed exactly as

$$R_{\text{dip}} = \{ 5 \langle (D_{\text{obs}} - D_{\text{calc}})^2 \rangle / [2 (D_a^{AB})^2 (4 + 3\eta^2)] \}^{1/2} \quad (5)$$

Complete Cross-Validation of Dipolar Couplings. Analogous to the crystallographic free R -factor,¹² it is also possible to define a cross-validated $R_{\text{dip}}(\text{free})$ for dipolar couplings in which the agreement between the observed and calculated dipolar couplings are computed for a subset of the dipolar couplings which are not included in the refinement. By this means one can ascertain how well the dipolar couplings in the test data set are predicted by those in the working set, thereby permitting one to estimate the quality of the fit to the experimental data. Unlike in crystallography, however, it is insufficient to simply define one working data set and one test data set. This is because a single dipolar coupling does not contain information regarding the whole molecule, in contrast to a single reflection which contains information relating to the whole crystal structure. Thus, just as in the case of the free R -factor for the NOE,¹³ it is necessary to carry out complete cross-validation, which involves using different pairs of working and test data sets for each calculation, where the contents of the data sets are chosen at random but partitioned according to some set size (e.g., a working data set comprising 10% of the whole data set).

To examine the behavior of $R_{\text{dip}}(\text{free})$ and $R_{\text{dip}}(\text{work})$, we carried out a series of simulated annealing calculations^{9a,14} with

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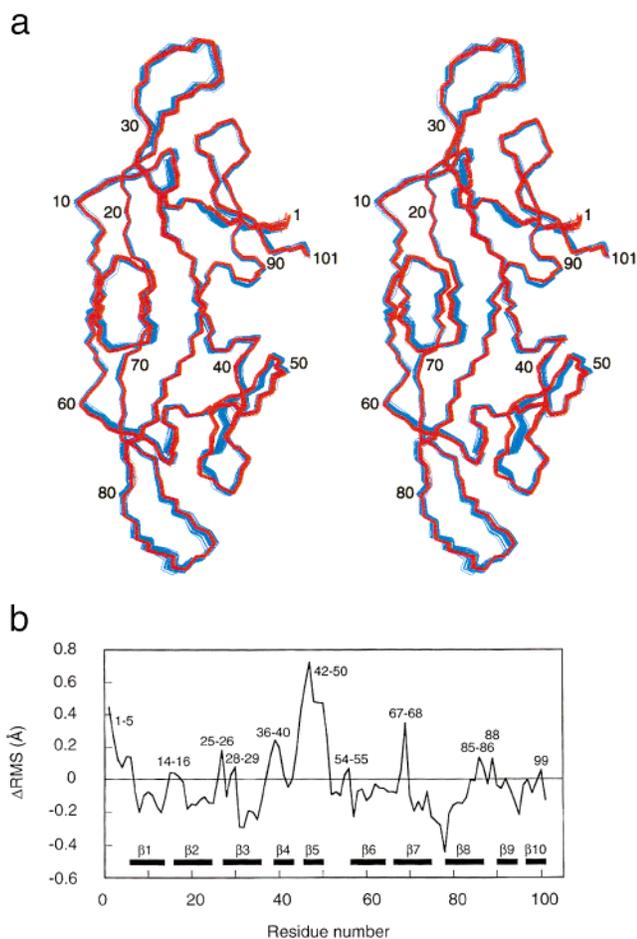


Figure 1. Comparison of the structure of CVN calculated with and without dipolar couplings. (a) Stereoview showing a best fit superposition of the backbone (N, C α , C') of the ensembles of simulated annealing structures (40 in each ensemble) calculated with (red) and without (blue) dipolar couplings. (b) Quantitative description of the differences between the two sets of structures plotted as Δ RMS versus residue number, where Δ RMS is given by the rms difference between the two mean coordinates minus the sum of precision of the coordinates for the two ensembles of simulated annealing structures. Positive values of Δ RMS indicate regions of real difference between the two sets of coordinates (i.e., regions where the two bundles of simulated annealing structures do not overlap). The overall precision of the backbone coordinates for the ensembles of structures calculated with and without dipolar couplings is 0.16 and 0.27 Å, respectively.

the program XPLOR/CNS¹⁵ using experimental data for the 101 residue protein cyanovirin-N.^{8a} The experimental data comprise 2147 restraints consisting of 1157 approximate interproton distance restraints, 84 distance restraints for 42 hydrogen bonds, 334 torsion angle restraints, 81 $^3J_{\text{HN}\alpha}$ coupling constant restraints, 157 $^{13}\text{C}\alpha/^{13}\text{C}\beta$ secondary shift restraints, and 334 dipolar coupling restraints.^{8a} The latter were measured in 4% 3:1 DMPC:DHPC bicelles and comprise 84 N–H, 77 C α –H, 44 C α –C', 66 N–C', and 63 HN–C' dipolar couplings.^{8a} The values of D_A^{NH} and η are -17.0 Hz and 0.17, respectively.^{8a}

Figure 1a shows a superposition of the two ensembles (consisting of 40 simulated annealing structures each) for CVN calculated with and without dipolar couplings, and the differences between the two sets of coordinates are expressed

quantitatively as a function of residue number in Figure 1b. Significant differences between the two coordinates sets occur when the backbone rms difference between the two mean coordinates (with and without dipolar couplings) exceeds the sum of the precision of the two sets of coordinates (measured by the average rms between the members of each ensemble of simulated annealing structures and their respective mean coordinate positions). These regions comprise residues 1–5 (N terminus plus helical turn), 25–26 (turn between strands β_2 and β_3), 36–40 (helical turn between strands β_3 and β_4), 42–50 (turn between strands β_4 and β_5 and strand β_5), 54–55 (helical turn preceding strand β_6), 67–68 (beginning of strand β_7), and 85–86 (end of strand β_8), 88 (helical turn between strands β_8 and β_9), and 99 (end of strand β_{10}).

Figure 2 shows the results from a series of calculations with 10 pairs of working and test data sets chosen at random and partitioned in a ratio of 90% to 10% in which the force constant for the N–H dipolar coupling restraint, $k_{\text{dip}}(\text{NH})$, is varied from 0.008 to 4 kcal·mol⁻¹·Hz⁻². (The force constants for the normalized C α –H, C α –C', N–C', and HN–C' dipolar couplings are fixed relative to that for the N–H dipolar couplings by factors of 1.0, 0.035, 0.050, and 0.108, respectively; this is based on measurement errors of ~ 0.5 , ~ 1 , ~ 1 , and ~ 1.0 Hz for the actual N–H, C α –H, C α –C', N–C', and HN–C' dipolar couplings, respectively, which correspond to errors of ~ 0.5 , 5.4, ~ 4.5 , and ~ 3 Hz for the normalized C α –H, C α –C', N–C', and HN–C' dipolar couplings, respectively). By this means, one can ascertain the optimal value for $k_{\text{dip}}(\text{NH})$, that is to say when the value of $R_{\text{dip}}(\text{free})$ reaches its minimum value such that the dipolar couplings in the test data set are best predicted by refinement against those in the working data set. Also plotted are the dependencies of the working set R -factor, $R_{\text{dip}}(\text{work})$, the R -factors for the individual dipolar coupling types within the working set, the energies for the NOE and covalent geometry restraints, and the backbone coordinate precision and accuracy. For operational purposes, accuracy is defined as the rms difference between the mean coordinates for each ensemble and a reference structure, which in this case is the mean structure obtained with the complete dipolar coupling data and a value of $k_{\text{dip}}(\text{NH}) = 1$ kcal·mol⁻¹·Hz⁻². For $k_{\text{dip}}(\text{NH}) = 0.008$ kcal·mol⁻¹·Hz⁻², $R_{\text{dip}}(\text{work})$ and $R_{\text{dip}}(\text{free})$ are approximately equal, indicating that for this value of the force constant, the dipolar couplings have little influence on the structure. As $k_{\text{dip}}(\text{NH})$ increases, $R_{\text{dip}}(\text{free})$ and $R_{\text{dip}}(\text{work})$ (both overall and individual) progressively decrease reaching their minimum value for $k_{\text{dip}}(\text{NH})$ in the range 2–2.25 kcal·mol⁻¹·Hz⁻². Note that $R_{\text{dip}}(\text{work})$ decreases more rapidly than $R_{\text{dip}}(\text{free})$, so that at the minimum, $R_{\text{dip}}(\text{work})$ is a factor of about two smaller than $R_{\text{dip}}(\text{free})$. Likewise, the coordinate accuracy increases substantially as $k_{\text{dip}}(\text{NH})$ increases, from 0.55 Å for $k_{\text{dip}}(\text{NH}) = 0.008$ kcal·mol⁻¹·Hz⁻² to a minimum of 0.08 Å for $k_{\text{dip}}(\text{NH}) = 1.5$ kcal·mol⁻¹·Hz⁻². Thus, there is a good correlation between backbone coordinate accuracy and $R_{\text{dip}}(\text{free})$. The coordinate precision and the energies for the NOE and covalent geometry restraints (as well as those for the torsion angle, coupling constant, and ^{13}C chemical shift restraints which have not been plotted), on the other hand, remain fairly constant for $k_{\text{dip}}(\text{NH}) = 0.008$ to 2.25 kcal·mol⁻¹·Hz⁻². Concomitantly, up to $k_{\text{dip}}(\text{NH}) = 2.25$ kcal·mol⁻¹·Hz⁻², the NOE, torsion angle, coupling constant, and ^{13}C chemical shift restraints are satisfied within their respective experimental errors. For $k_{\text{dip}}(\text{NH}) > 2.25$ kcal·mol⁻¹·Hz⁻², there is a rapid deterioration in all parameters which display steplike function behavior. One can therefore conclude that the force constant for the dipolar coupling

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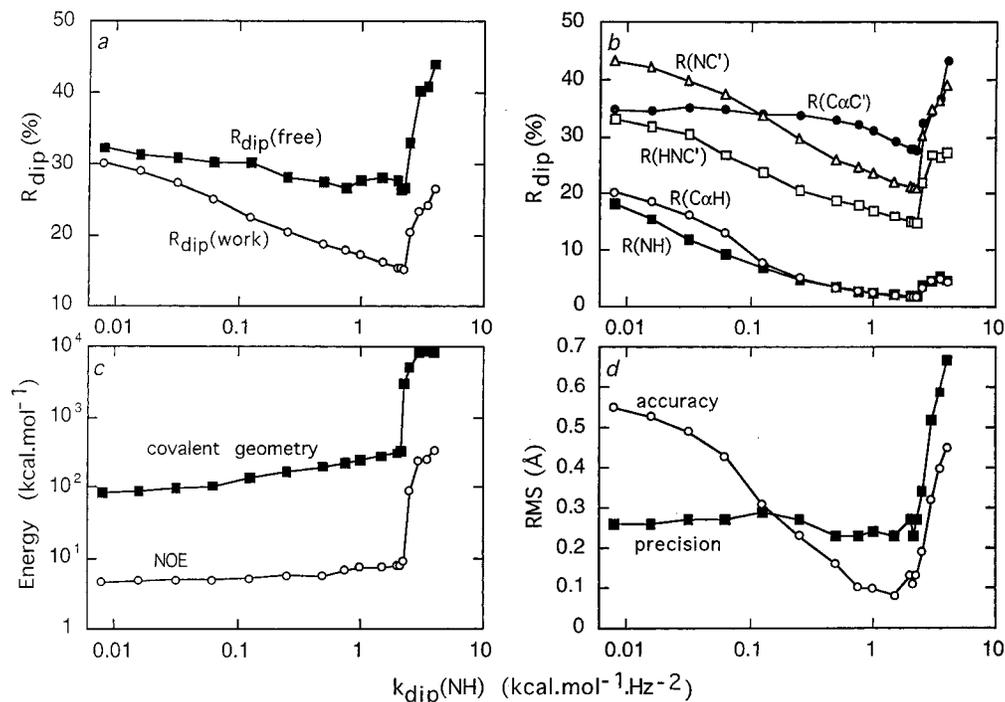


Figure 2. Influence of the force constant for the dipolar coupling restraints on the quality of the resulting structures of CVN. The dipolar couplings are partitioned into 10 pairs of test and working sets comprising 10 and 90% of the data, respectively. For each ensemble of simulated annealing structures, the precision of the backbone coordinates (N, C α , C', O) is defined as the average backbone rms difference between the individual simulated annealing structures and their mean coordinate positions. The backbone coordinate accuracy is estimated by the rms difference between the reference structure and the mean coordinates for each ensemble. In this case the reference structure is the mean structure obtained with the complete dipolar coupling data set and a value of $k_{\text{dip}}(\text{NH}) = 1 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{Hz}^{-2}$. This reference structure almost perfectly fits all the experimental and covalent restraints, and by this definition, the accuracy of the reference structure is 0 Å. The force constants for the C α -H, C α -C', N-C', and HN-C' dipolar couplings are fixed relative to that for the N-H dipolar couplings as described in the text.

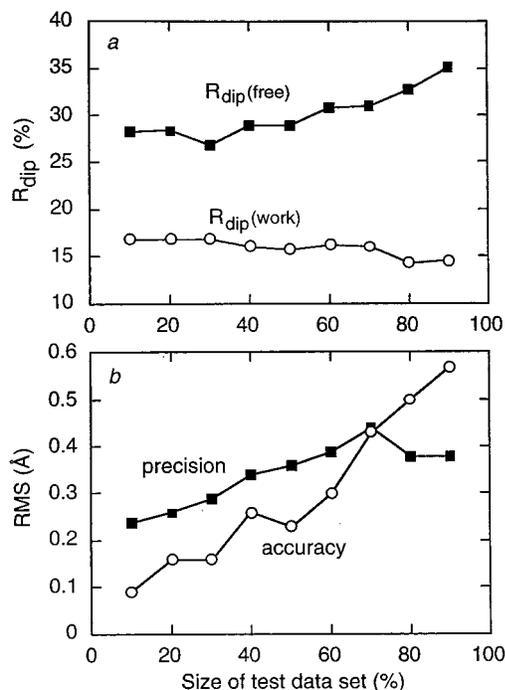


Figure 3. Influence of completeness of the dipolar coupling restraints (a) on $R_{\text{dip}}(\text{work})$ and $R_{\text{dip}}(\text{free})$ and (b) on the backbone precision and accuracy of the CVN structure.

restraints can be set to a value close to the largest value that is still compatible with the other experimental, covalent and van der Waals terms in the target function for simulated annealing.

In the particular set of calculations shown in Figure 2 in which the partitioning ratio between working and test dipolar coupling

data sets is constant, $R_{\text{dip}}(\text{free})$ is positively correlated to $R_{\text{dip}}(\text{work})$. This reflects the good quality and correctness of the other experimental restraints. In other instances where there are errors in some of the experimental restraints (e.g., due to incorrect NOE assignments), it is quite likely that an increase in the force constant for $R_{\text{dip}}(\text{work})$ may actually be accompanied by an increase rather than a decrease in $R_{\text{dip}}(\text{free})$, depending on the structural significance of these errors.

Figure 3 analyses the effect of dipolar coupling data set completeness by systematically increasing the size of the test data set from 10 to 90% of the measured dipolar couplings and reducing the size of the working data set from 90 to 10%. For test data sets in the range 10–30%, both $R_{\text{dip}}(\text{work})$ and $R_{\text{dip}}(\text{free})$ remain fairly constant. As the test data set increases above 30% (i.e., the working data set falls below 70%), $R_{\text{dip}}(\text{work})$ decreases (since there is less data to satisfy) but $R_{\text{dip}}(\text{free})$ increases, as the predictability of the test data set on the basis of the working data set decreases. Concomitantly, both the backbone precision and accuracy of the coordinates worsen as the size of the test data set increases. The accuracy decreases in an approximately linear manner while the precision reaches a plateau value for test data sets >60%. As a result, for test data sets below 70%, the coordinates are still more accurate than they are precise, but above this value they are more precise than they are accurate.

Concluding Remarks

In conclusion, the R -factor for dipolar couplings defined by eq 5 is easily calculated, and since it scales between 0 and 1, its meaning is simple to interpret. Complete cross-validation can also be carried out, typically with multiple pairs of working and test data sets partitioned in a 90%:10% ratio, thereby

permitting one to assess the quality of the fit to the experimental dipolar couplings and to avoid overfitting the experimental data. In the example shown in this paper, only dipolar couplings relating to the backbone were considered. Since side chain dipolar couplings can also be measured,^{9b} the same methodology can be applied equally well to side chains. In such cases, it is best to calculate *R*-factors for the backbone and side chains separately, since the various backbone dipolar couplings are

influenced to a large degree by long-range order, whereas those for the side chains are likely to be accommodated by small local changes in side chain torsion angles.

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