# Cross-Fitting of Residual Dipolar Couplings

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**Abstract:** Residual dipolar couplings (RDCs) are important NMR-parameters for the structure determination of organic molecules. In this article we describe how RDCs can be used to effectively transfer structural information by cross-fitting the anisotropic parameters of molecules with similar overall structure. Using the example of 5-α-cholestan-3-one and cholesterol, it is possible to distinguish diastereomers of the compounds by cross-fitting with transferred alignment tensors, even when strongly reduced subsets of RDCs are used. It is also demonstrated that RDCs can be used for direct cross-fitting even in flexible parts of the molecules that are sufficiently similar in structure and dynamic behavior. The cross-fitting approach as a general tool is discussed in details.

**Keywords:** NMR spectroscopy, residual dipolar couplings, alignment tensor, crossfitting, steroids, cholesterol, dynamics.

## INTRODUCTION

Structure determination of organic molecules is one of the most challenging tasks for chemists and especially the determination of the stereo-configuration of natural or synthetic products is of high interest.

Among them steroids build a large class of molecules which are of special interest because of their variety of structures and biological activities [1]. Natural products with the steroid framework play an important role in medicinal chemistry and many strategies have been developed for their total synthesis or semisynthesis based on the modification of natural products [2]. In addition to the pharmaceutical and medicinal interests, steroid compounds are also quite suitable homochiral model compounds for chemo-, regio- and stereoselective investigations [3]. All this requires the reliable determination of constitution and configuration of the corresponding molecules.

While for X-ray-crystallography appropriate crystals are necessary, high resolution nuclear magnetic resonance spectroscopy (NMR) investigates molecules in solution. Based on classical NMR parameters like chemical shifts, *J*-couplings, and nuclear Overhauser enhancement (NOE) the structures of uncountable molecules have been solved. However, the structure determination by standard NMR parameters often fails because of a lack of information or because distant parts of the molecule can not be correlated.

In many cases a solution to the problem can be found by the use of residual dipolar couplings (RDCs) which contain information about internuclear distances and angles relative to an external reference. To measure anisotropic parameters like RDCs it is required to partially align the solute with the help of a so-called alignment medium. In recent years a variety of media for weak alignment of small to medium-sized organic molecules has been developed [4-6]. Especially mechanically stretched polymer gels are applicable to a wide range of solutes and solvents [7-18] and scaling of the alignment strength becomes very easy when combining polymer-based alignment media with an apparatus for arbitrary stretching [19-22].

Beside conformational studies of biologically active molecules [23-27] and e.g. the enantiomeric differentiation of small molecules in chiral alignment media, [15, 28-36] the central application for small organic molecules like natural or synthetic products is the determination of relative configurations of distant chiral and prochiral center [12, 17, 37-47]. Here, we describe the relative configurational analysis of molecules by cross-fitting of RDCs to RDCs from structurally similar molecules with known properties. After a brief theoretical introduction and a description of the RDC measurements performed on cholesterol and 5-α-cholestan-3-one as two test molecules with similar overall structure, cross-fitting based on transferring the alignment tensor or on direct comparison of RDCs will be demonstrated. In addition, the ability of distinguishing the diastereomeric 10-α-cholesterol from the measured cholesterol by cross-fitting with even strongly reduced RDC-datasets is studied in detail. As will be shown, the approach overcomes several limitations of classical RDC-analyses.

## THEORY

In partially aligned samples residual dipolar couplings, D, add up to the corresponding heteronuclear scalar couplings, J and therefore spectra on oriented, anisotropic samples contain the sum J+2D. In corresponding spectra of isotropic samples, e.g. in solution, only the scalar coupling, J, is present and therefore the difference between couplings measured under anisotropic and isotropic conditions gives the desired RDCs.

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As RDCs contain angular information relative to the external reference given by the magnetic field, their interpretation relies on the knowledge of the molecules orientation caused by the alignment medium. For rigid molecules this orientation is usually described by the so-called alignment tensor. Generally, the five independent components of the alignment tensor can be derived by mathematical methods like the singular value decomposition (SVD) as it is implemented in programs like PALES [48, 49] (-bestFit option). For this approach a set of 5 independent RDCs is required in which no two internuclear vectors for the RDCs are oriented parallel to each other and no more than three RDC vectors lie in a plane. Any further measured RDC directly contains valuable structural information given that the molecule can be considered as rigid.

As long as considerably more then 5 independent RDCs are given, this method can be used for the assignment of prochiral groups and/or for the configurational analysis of rigid molecules as various examples have shown (for reviews see [4, 6, 50]). Problems arise when the number of RDCs for fitting is not sufficient to unambiguously differentiate between different structural models. The situation gets significantly worse if flexible parts of a molecule must be taken into account [41-43, 51].

In steroids, for example, this lack of sufficient RDC-data might arise due to the small range in chemical shifts as most signals appear in the region between 15 ppm and 40 ppm in $^{13}C$ dimension and 0.5 ppm till 2.0 ppm in $^{1}H$ dimension respectively (see Fig. **1**). This does not only lead to signal overlap but might also cause immense strong coupling artifacts which prevent a reliable extraction of coupling constants [52]. In addition, a chair-like structure of six-membered rings in steroids reduces the number of independent RDCs as all axial CH-bonds are practically parallel. Therefore it might well happen that RDC-data measured on steroids are not sufficient for a desired configurational analysis.

Probably the most elegant and effective solution to the problem of a limited set of RDCs would be the prediction of alignment from first principles, which theoretically would allow to distinguish diastereomers by a single measured, decisive RDC. Many attempts have been made to predict the alignment of a molecule by considering steric [48] and electrostatic [53] interactions between the solute and the alignment medium, as it is for example done in PALES (-stPales option), [49] or by using inherent properties of the solute molecule like its tensor of gyration [54, 55]. The method works well for large biomacromolecules, but for small molecules, for which the effects of the fine-structure of the alignment medium and corresponding dynamics play a much more important role, it usually leads to wrong results with very few exceptions if the molecule of interest is void of functional groups [12].

A different approach, first used for the identification of absolute configuration *via* natural abundance deuterium NMR, [56] is the use of RDCs of a molecule of known, very similar structure for a cross-fit with the RDCs from the molecule of interest. As the alignment and therefore RDCs are strongly influenced by steric and charge interactions of the solute with the polymer of the alignment medium, the molecule used for cross-fitting should possess very similar overall shape and charge distribution and the dynamic behavior of the molecules should also match. If such a molecule is available, cross-fitting of RDCs should in principle allow the differentiation of diastereomers even in the case of otherwise insufficient RDC data.

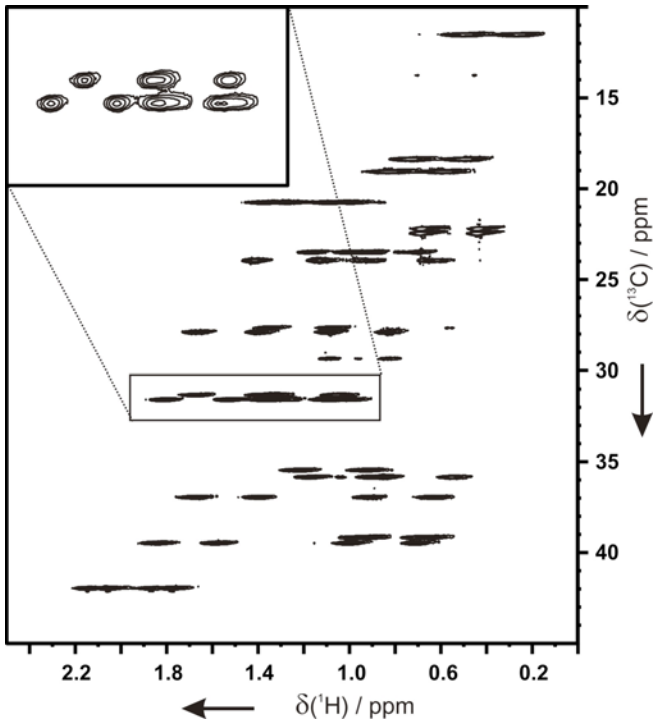


**Fig. (1).** Aliphatic region of the CLIP-HSQC spectrum [52] acquired on cholesterol in a stretched PDMS/$CDCl_3$ gel. Almost all steroid signals appear in a very narrow spectral range causing signal overlap and strong coupling artifacts. As an example of signal overlap the inset shows five doublets: C2-H2α, C2-H2β, C7-H7α, C7-H7β, and C8H8.

This approach can in principle be implemented in two ways: RDCs of the cross-fitting molecule can either be used to create an orientational model which is then applied to the solute of interest, or the couplings can be compared directly. If the molecule of interest contains large rigid parts and sufficient RDCs are available, simple singular value decomposition (SVD) [57] will allow to calculate an alignment tensor for the rigid parts which allows a detailed comparison of structural features in the solute. If the molecule is inherently flexible, more complex mean-field models should in principle be applicable like the AP-model, [58] the Chord-model, [59] the ME-model, [60] or combinations thereof [61-65].

If only the conservation of a specific part of the molecule shall be tested, a direct comparison of RDCs of this specific part should lead straightforwardly to the desired result without complicated fittings to a model.

In the following we test in how far the hypothesis of cross-fitting is applicable to real molecules. For this we apply the SVD-based alignment tensor model and the direct comparison of RDCs to two steroids with inherently high similarity. We also compare the result with steric RDC-prediction as implemented in PALES.

**Fig. (7).** Differentiation of diastereomers with reduced sets of RDCs. Compared are quality factors $n/\chi^2$ obtained for the direct SVD-fitting method (top) and the cross-fitting approach (bottom) using subsets of RDCs measured on cholesterol in a stretched PDMS/$CDCl_3$ gel against the structures of cholesterol (blue) and 10-α-cholesterol (orange). RDC-data was reduced by building various subsets of the 18 measured RDCs with 15, 12, 9, 8, 7 and 6 RDCs. The individual subsets are marked with letters (15A-C, 12A-E, 9A-H, 8A-J, 7A-L, 6A-O) for which the corresponding RDC combinations can be found in Tables **S6** and **S7** of the Supporting Information. For each fit the prochiral assignment of $CH_2$-groups was permutated and only the fit with the best result is shown for each subset of RDCs. For each asterisk (*) one methylene group was obtained with incorrect prochiral assignment for the best permutation. Fewer RDCs for the SVD-fitting lead to highly unreliable results and strongly increased $n/\chi^2$ values, while the cross-fitting approach maintains the correct differentiation of diastereomers and prochiral assignments even for sparse data.

(**6D**) by looking at the coupling deviations of individual RDCs, almost any RDC measured in the A-ring, for example, will be sufficient to differentiate the correct from the wrong diastereomer, while the whole set of RDCs of the C-ring will not lead to conclusive results.

### Comparison with PALES RDC-prediction

As mentioned in the theory section, RDC prediction would be the most elegant way of deriving the alignment tensor of a given molecule. However, established methods only work for large proteins and sometimes small, rigid molecules without functional groups. In addition, the prediction of alignment is highly susceptible to flexible parts, whose influence on the induced orientation is generally overestimated.

The steroids investigated in our study both contain only a single functional group and their orientational order is most certainly dominated by the relatively rigid ring systems. They therefore represent almost ideal candidates for predicting the alignment tensor by PALES (with the -stPales option), but actual RDCs predicted by PALES for the whole steroid structure fit only reasonably to the measured RDCs.

We tried to improve the RDC prediction by shortening the flexible side chain attached to C17 (see Supporting Information). Indeed, predicted results became much better this way: for cholesterol best prediction results were achieved with the cholesterol fragment C1-C24 (cholesterol shortened by C25, C26 and C27 and adjacent protons) with a very good $n/\chi^2$ value of 3.29 and for 5-α-cholestan-3-one prediction was best for the fragments C1-C23 (shortened by C24, C25, C26 and C27 and adjacent protons) and C1-C20 (shortened by the whole side chain) with $n/\chi^2$ values of 0.92 and 0.87, respectively. However, the optimal length of the flexible side chain for the PALES prediction differs even in this case of two very similar molecules, showing how fast and unpredictable the method can get unreliable.

More problems with the PALES prediction arise when the interaction between solute and alignment medium is not purely steric but charges need to be considered. The steric

PALES prediction performed for sodium cholate, for example, does not at all reflect RDCs measured for sodium cholate in PAA/$D_2O$ [39] (see Supporting Information). Considering how difficult and unreliable an accurate RDC-prediction with currently available methods is, cross-fitting appears to be the superior approach whenever applicable.

### Cross-fitting by Direct Comparison of RDCs

The cross-fitting *via* an alignment tensor or another model describing the average orientational behavior allows the indirect comparison of RDCs measured even in differing parts of two similar molecules. However, quite often it is only necessary to transfer the assignment of e.g. prochiral groups or to identify signals in a flexible chain for which the alignment tensor approach is not applicable.

As has been shown previously for residual quadrupolar couplings (RQCs) by Ziani *et al*., [56] the anisotropic couplings in this case can directly be transferred between two molecules with very similar orientational behavior without the detour over an orientational model. A comparison between RDCs measured on cholesterol and 5-α-cholestan-3-one (Table **1**) leads to a unique assignment for practically all prochiral protons with the exception of C7-H7 close to the structural difference in ring B. Especially in the flexible side chain attached to C17 (atoms C20-C27) the match of RDCs measured for the two molecules is striking.

For more or less identical parts of two molecules that fulfill the conditions for cross-fitting, the direct comparison of anisotropic NMR parameters like RDCs therefore must be considered as an highly effective tool to obtain an assign-

**Table 1. Comparison of $^1H,^{13}C$ one-bond RDCs Measured on Cholesterol and 5-α-Cholestan-3-one in Similarly Stretched PDMS/$CDCl_3$ gels. Individual Maximum Error Estimates are Propagated from the Individual Maximum Error Estimates of the Original Measurements of $^1J_{CH}$ and $^1J_{CH}+^1D_{CH}$ Couplings, Respectively (see also Tables S2 and S4 of the Supporting Information)**

| Group[a] | $^1D_{CH}$ (exp) cholesterol [Hz] | $^1D_{CH}$ (exp) 5-α-cholestan-3-one [Hz] |
|---|---|---|
| C19-H19 | -6.6 ± 0.6 | -7.2 ± 0.8 |
| C18-H18 | -7.0 ± 0.6 | -7.2 ± 0.5 |
| C16-H16a | 0.3 ± 5.8 | -1.9 ± 5.8 |
| C16-H16b | 5.1 ± 5.8 | 8.5 ± 5.4 |
| C15-H15a | –[b] | 11.6 ± 5.1 |
| C15-H15b | 14.0 ± 5.8 | 14.0 ± 5.1 |
| C7-H7β | 14.2 ± 5.8 | 4.1 ± 5.0 |
| C7-H7α | –[b] | 25.9 ± 1.5 |
| C8-H8 | 20.7 ± 8.5 | 27.2 ± 1.0 |
| C2-H2α | 11.3 ± 3.9 | 6.9 ± 3.2 |
| C2-H2β | 16.1 ± 3.9 | 21.1 ± 3.2 |
| C1-H1β | 9.0 ± 1.4 | 7.3 ± 1.2 |
| C1-H1α | 18.2 ± 1.4 | 20.7 ± 1.7 |
| C12-H12α | 22.4 ± 1.4 | 27.4 ± 1.6 |
| C12-H12β | 5.1 ± 1.4 | 4.3 ± 1.1 |
| C9-H9 | 23.6 ± 4.0 | 23.7 ± 1.1 |
| C21-H21 | -4.9 ± 0.6 | -5.0 ± 0.7 |
| C25-H25 | 10.8 ± 0.9 | 11.4 ± 0.8 |
| C20-H20 | 23.3 ± 1.1 | 23.3 ± 1.0 |
| C27-H27 | -0.2 ± 0.4 | -0.5 ± 0.4 |
| C26-H26 | 0.2 ± 0.4 | 0.2 ± 0.4 |
| C23-H23a | 18.1 ± 5.1 | 20.5 ± 8.2 |
| C23-H23b | 5.2 ± 2.7 | 8.5 ± 8.5 |
| C22-H22a | 25.6 ± 6.4 | 26.7 ± 5.8 |
| C22-H22b | 8.7 ± 10.4 | 8.7 ± 5.1 |

[a] Methylene protons marked with a, b indicate that prochiral assignment is not available. Protons marked with α or β are assigned following standard steroid nomenclature.
[b] Couplings not extracted due to signal overlap or strong coupling artifacts.

ment or to independently verify a given assignment. Especially for molecules like the presented steroids with many signals within narrow chemical shift ranges this can be very useful as the potentially slightly better resolution for closely related compounds can be directly used by transferring corresponding RDCs.

### Limitations and Potentials

In contrast to the prediction by PALES our approach does not need to consider structural details or charges of the alignment medium, as the orientational information is taken from experimental data. However, a number of conditions must be fulfilled: both the molecule of interest and the molecule used for cross-fitting RDCs must be rather similar in their overall shape and charge distribution, and RDCs must be measured for both molecules in the same alignment medium. Otherwise the cross-fitting of RDCs will fail.

The influence of the alignment medium and/or the influence of the molecules charge distribution might be seen when comparing experimental data measured on sodium cholate, another molecule with steroid scaffold, in PAA/$D_2O$ [39] with our approach (see Supporting Information). The alignment of cholesterol and 5-α-cholestan-3-one in PDMS/$CDCl_3$ is fairly different compared to that of sodium cholate in PAA/$D_2O$ and therefore the cross-fitting does not work. Notably the prediction of alignment with PALES in this case does also not work.

It should be mentioned that cross-fitting based on an alignment tensor requires rigid or at least partly rigid molecules, as only for those an alignment tensor is well-defined. The method, however, should be extendable to flexible molecules by the use of more general mean-field descriptions of orientational properties, which was not attempted here.

By directly comparing RDCs from a reference molecule with the molecule of interest, flexibility has no influence on the results as long as the molecules show similar dynamic behavior. The success of the method therefore is not limited by the cross-fitting approach in general, but by finding a reference molecule of known structure with sufficient similarity to the molecule of interest.

## CONCLUSION

We could show that the alignment of the two steroids cholesterol and 5-α-cholestan-3-one in stretched PDMS/$CDCl_3$ gels is rather similar and that it is therefore possible to use the alignment tensor derived for one of the molecules to fit the RDCs of the other one. As has been shown in detail, cross-fitted RDCs then can be used to unambiguously distinguish diastereomers to the two measured compounds even in the case of massively reduced RDC datasets.

The approach of cross-fitting RDCs can also be applied without the detour of fitting an alignment tensor but by directly comparing RDCs of a known reference molecule with RDCs from the solute of interest. In this case, as long as the molecules are sufficiently similar, no restriction in terms of rigidity of the molecules apply and molecules can be compared even when no overall alignment tensor can be defined.

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## SUPPORTING INFORMATION

Supporting information is available on the publishers Web site along with the published article.

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