Practical ssNMR

Interpretation of two-dimensional MAS solid-state NMR spectra of a microcrystalline protein

***Goal of this tutorial*:**

a) To give an introduction to using CCPNMR Analysis software for the analysis of solid-state NMR (ssNMR) spectra recorded under MAS conditions, and more especially techniques in which ^{13}C and ^{15}N are observed.
b) To manipulate and interpret in a qualitative manner a set of 2D ^{13}C-^{13}C and ^{13}C-^{15}N ssNMR correlation spectra of a uniformly ^{13}C,^{15}N-labeled microcrystalline protein prior to start a structural study
c) To evaluate if an optimization of the ssNMR experimental setup e.g by using higher MAS frequencies or choosing other alternative isotope labelling strategies will be beneficial to the ssNMR study of the protein of interest.
d) To analyse ssNMR resonances and compare these data with available X-ray and solution NMR datasets. An emphasis on how interpreting the differences in terms of sample properties will be given.

Tools:

- A database of ssNMR spectra of a microcrystalline protein under different conditions
- Sequential CCPNMR projects to facilitate the analysis progress of ssNMR spectra
- A handbook for the analysis of solid-state NMR spectra: AA structures, Graph for the characteristic ^{13}C chemical shifts of the 20 AA, 2D ssNMR correlation patterns for different isotope labelling patterns (adapted from V. Higman : http://www.protein-nmr.org.uk/ccpnmr-analysis/ccpnmr-analysis-solid-state-tips), procedure to predict backbone CS from 3D structure, procedure to generate synthetic peak lists from predicted or experimental chemical shifts.

1) *Manipulation of ssNMR spectra using CCPNMR Analysis*

- **Open CCPNMR project**
  - Open a terminal window and go to the folder TP-ssNMR/cristaux > cd …
  - Open ccpnmr project > Analysis ccpnmr_cristaux_a

- **Switching Spectra on/off**
  - In the top left hand corner of each window there is a Spectra button. Toggle this on/off
  - to see all the spectra which can be displayed in each window. Each spectrum can be toggled on/off using its button, so you are able to display as many or as few spectra in each window as you wish.
  - Open further windows by going to the Windows menu and selecting any of the windows in the lower section of the pull-down menu. Note the nomenclature of the spectrum names – they indicate the spectrum type, the mixing time and the MAS frequency.

    DARR50-12, 50 ms mixing time, MAS frequency 12 kHz

PDSD50-18, 50 ms mixing time, MAS frequency 18 kHz
PDSD150-10, 150 ms mixing time, MAS frequency 10 kHz
NCACX50-12, 50 ms mixing time, MAS frequency 12 kHz
Etc.

- **Zooming / Moving around in Spectra**
  - There are several ways to zoom (note that the mouse-based ones may not work with Windows!)
    - Rotate the mouse wheel
    - Middle-click the mouse while holding down Shift and drag the mouse to zoom in/out
    - Use the Page Up / Page Down keys
  - Move to a different part of the spectrum using the scroll bars or simply click on the middle mouse button and drag the spectrum.
  - To move through the z-planes of a 3D spectrum, either use the z-plane scroll bar or rotate the mouse wheel while holding down Ctrl.
  - If you want to go to a specific position on the z-axis of a 3D window, simply type the ppm value into the box in the bottom left hand corner.

- **Identifying Side-band Peaks**
  - It is possible to add the spinning speed at which an experiment was recorded by going to the Experiments popup in the Experiment menu and then going to the Experiment Details tab. Analysis will then automatically add dashed side-band diagonals to your spectrum where relevant. You can also use V, H and M to draw vertical rulers, horizontal rulers or marks which are repeated at side-band intervals. This is a convenient way to identify whether for instance a peak in the aromatic region of the spectrum is in fact a side-band peak from the carbonyls or not.
  - If you have identified a peak as being a side-band peak you may want to mark it in some so that you don't forget that it is a side-band peak. There are two possibilities of how you can do this. Either, right-click, go to Peak and then Set merit and 0.0. Alternatively, right-click, go to Peak and then Set details – you can then type something like side-band into the comment box. Now go to the Peak menu and select Draw Parameters. Make sure that Merit Symbol or Details are selected, depending on which one you opted to go with. If you are using the Merit Symbol, then go to the Merit Sympbols tab and enter a symbol such as * or ! into the Poor merit box and click on Set Symbols. Now your side-band peak should be marked either with a symbol or a comment to remind you that it is not a real peak.
  - It is likely that a rather more sophisticated system of properly identifying side-band peaks will be introduced some time in the future. But in the mean time this is a reasonably good fix.

2) ***Spin System Identification***

The protein of interest contains 8 threonines. This section will show you how to identify them based on their chemical shifts, pick their peaks, generate resonances and spin systems for them, and assign their atom and amino acid types.

For this section, you will need to refer to the following figures provided in the ssNMR-handout:
Figure 1: Structure of the amino acids (AA)
Figure 2: Graph for the characteristic ^{13}C chemical shift for the 20 AA
Figure 3: Diagrams illustrating the spectrum types used in this practical
Figure 4: Typical intra-residue CC correlation patterns for the 20 AA

- **Shortcut: Open CCPNMR project**
  - Open a terminal window and go to the folder TP-ssNMR/cristaux > cd …
  - Open ccpnmr project > Analysis ccpnmr_cristaux_b

- **Enter Sample information**
  - It is possible to enter the protein sequence by going to Chains popup into the Molecules menu and then click on "add sequence". You need to copy the amino-acid sequence of the protein totoH that is provided in the totoH.seq file.

- **Set the Double Cross-hair Mouse**
  - By default, your mouse will form a single crosshair with one vertical and one horizontal line. However, when you have two axes belonging to the same atom type (e.g. in a carbon-carbon correlation spectrum) it is really useful to have a double crosshair (with two vertical and two horizontal lines) which will trace equivalent points on either side of the diagonal.
  - In order to set a double crosshair mouse, go to Windows in the main menu and select Window. When you click on a window in the upper part of this pop-up, you will see the axes displayed in the lower part. One of the columns is headed Panel Type. By default, these are all set to be different, so for a ^{13}C-^{13}C window, they will be called C1 and C2. If you set the panel types to be the same, then you will obtain a double crosshair mouse. I find it useful to go through all windows and make sure that all panel types are C1, N1 and H1 and then you will always see a double crosshair mouse in diagonal spectra and you will always see equivalent mouse lines in all other windows, too.

- **Make use of Marks and Rulers**
  - Often it is useful to draw lines through your spectrum to check whether two peaks occur at the same chemical shift or not.
  - A Mark is drawn through all dimensions at the position where it is placed. To draw a Mark, place the mouse where you want it to be and press m. If you are close to a peak, then the mark will automatically be drawn through the peak.
  - Rulers only go through one dimension. To draw a Ruler, place the mouse where you want it to be and press v for a vertical ruler or h for a horizontal ruler.
  - You can remove all marks and rulers by pressing n. Alternatively you can right-click the mouse and go to Markers– here you can place marks and rulers or you can selectively remove only Marks or only Rulers.

- **Identifying and Peak Picking the threonine residues**
  - Based on their characteristic chemical shifts (see ssNMR-handout, figures 2 and 4), try to identify the Cα-Cβ, Cα-$C\gamma_2$, Cβ-$C\gamma_2$, Cα-CO and Cβ-CO cross peaks of the 8 threonines in the PDSD50-12 spectrum and peak pick them. Drawing marks through your peaks will help you connect them.
  - The best way to pick peaks is to let the program find the correct peak maximum for you. To do this, simply left-click and drag the mouse over a peak (or several peaks) while holding down Ctrl and Shift. You can do this both in 2D and 3D spectra.
  - Sometimes several peaks are overlapped and it is better to place the peaks manually. Simply place the mouse where you want to have your peak and then right-click the mouse and select Peaks and then Add New Peak.
  - A peak is selected when a box is drawn around it. To select a peak either click on it, or drag the mouse over it. To deselect your peaks just left-click the mouse somewhere in a spectrum window. To select several peaks at the same time, drag the mouse over several peaks in one go, or keep the Shift button pressed down while you select each peak individually
  - If you are having trouble, you can use the chemical shift table:

^{13}C (ppm)	ThrA	ThrB	ThrC	ThrD	ThrE	ThrF	ThrG	ThrH
CO	173.8	-	-	-	-	-	172.7	-
Cα	66.4	64.0	62.1	60.8	59.9	59.4	58.1	-
Cβ	68.6	66.6	68.8	68.4	67.8	68.9	69.4	-
Cγ2	20.1	20.2	19.0	21.5	20.4	19.0	18.4	-

- **Assigning Resonances, Spin systems and Amino-acid Type**
  - Go to one Cα-Cβ cross-peak, place the mouse on it press **a**. This will bring you to the **Assignment Panel**.
  - In the **Assignment Panel**, select a new resonance for each dimension by clicking on **New** in both the top and the bottom sections of the panel. Then create a new spin system which both of these resonances are part. Click on **Set Same Spin System**. Note that the [resonance numbers] are given in square brackets and the {spin system number} is given in curly brackets. Finally, you need to set the atom type for each of the resonance (left hand side) first. Then click on **Set Atom Type** which will bring up the **Atom Browser panel**. Here, you will first have to toogle on the carbon atoms by clicking on **C**. Then select any **Cα** button (doesn't matter which) and do the same for the lower resonance, but selecting the **Cβ** as the atom type.
  - Repeat the procedure for other Cα-Cβ crosspeaks
  - Once you have identified all threonine spin systems – each one containing a Cα and Cβ resonances - you need to complete them with the $C\gamma_2$ and the CO resonances
  - Find and assign Cα-$C\gamma_2$, Cβ-$C\gamma_2$, Cα-CO and Cβ-CO cross-peaks of each identified spin system by using Marks and Rulers and the procedure described for Cα and Cβ.

- **Identifying the threonine nitrogen chemical shifts**
  - **Shortcut:** Open ccpnmr project > Analysis ccpnmr_cristaux_c
  - Now identify the threonine nitrogen chemical shifts using the 2D NCA-12 and 2D NCACX50-12 spectra
  - Select one Cα-Cβ cross-peak on the PDSD50-12 spectrum and press **m**. This will create Marks at Cα and Cβ frequencies on the 2D NCA-12 and 2D NCACX50-12 spectra. Select the Cβ- $C\gamma_2$ cross-peak of the same spin system and press **v**. This will create a third Mark at the $C\gamma_2$ frequency on the 2D NCA-12 and 2D NCACX50-12 spectra.
  - Now, you should be able to identify a set of N-Cα correlations in the NCA-12 spectrum that match the Cα frequency of the considered spin-system. Go to these N-Cα cross-peaks and type ***h*** to mark the ^{15}N frequencies. Next, find the ^{15}N frequency for which other expected N-C correlations are detected. If you look at the ssNMR handout, you will see that intraresidue N-Cα, N-Cβ and N-$C\gamma_2$ should be observable on the NCACX using this mixing time i.e. 50 ms.
  - Peak pick your N-C cross peaks and assign the nitrogen chemical shifts using the procedure you used above for the carbon resonances. Bring up the **Assignment Panel** with **a**. Select the Cα, Cβ and $C\gamma_2$ resonances suggested as assignment options. Click on **New** in the nitrogen dimension. Click **Set Same Spin System**. Select the nitrogen dimension resonance and click **Set Atom Type** and then select an **N** atom in the **Atom Browser**.
  - **Shortcut:** Open ccpnmr project > Analysis ccpnmr_cristaux_d

3) Evaluation of the quality of the ssNMR dataset and optimization

Structural studies by solid-state NMR of proteins is rapidly advancing as a result of recent developments of samples and experimental methods. Multidimensional correlation spectroscopy of low-gamma nuclei such as ^{15}N and ^{13}C is an important step for making resonance assignments and measurements of distance and angular restraints in proteins. However, the spectral resolution and the efficiency of coherence transfer predominantly depend upon the strength of the dipole–dipole interaction, and this can vary from site to site and between sample, for example, during the mixing of ^{13}C and ^{15}N magnetization in MAS samples.

There are a number of different solid-state NMR approaches that utilize refined sample preparations procedures and advanced experimental methods to emphasize the measurement of distances or angles - ideally both, as sources of structural constraints - without compromising magnetization transfer efficiency and the underlying spectroscopic sensitivity. Here, you will explore if the use of higher MAS frequencies or refined isotope labelling schemes can be beneficial for the structural analysis of the microcrystalline protein of interest.

For this section, you will need to refer to the following figures provided in the ssNMR-handout:
Figure 5: Alternative isotope labeling strategy used in ssNMR studies of proteins

- **Evaluation of the spectral resolution in ssNMR correlation spectra**
  The protein of interest contains 140 residues. So far, we have focused our attention on Thr residues that give well-dispersed and well-isolated intraresidue CC correlations. However, this observation is no longer valid in 2D PDSD spectra with longer mixing times e.g. 150 ms and in 2D NC-type experiments.
  - Overlay the PDSD50-12 and PDSD150-12 spectra
    - ➔ ***What do you observe?***
  - Overlay the 2D NC-12 and the 2D NCACX50-12 spectra
    - ➔ ***What do you observe?***

- **Effect of the MAS frequency on the quality of CC and NC correlation spectra**
  The spectral resolution might be improved by several ways, notably by increasing the MAS frequency from 12 kHz to 18 kHz.
  - Overlay the PDSD50-12 and PDSD50-18 spectra
    - ➔ ***What do you observe?***
  - Overlay the PDSD50-18 and DARR50-18 spectra
    - ➔ ***What do you observe?***
  - Overlay the NCA-12 and NCA-18 spectra
    - ➔ ***What do you observe?***
    - ➔ ***In this case, is a higher MAS frequency beneficial to the spectral resolution.***
    - ➔ ***What are the advantages and potential limitations of using higher MAS frequencies?***
    - ➔ ***Think about alternative ways to improve the spectral resolution without modifying the ssNMR sample***

- **Other isotope labeling strategies to improve the ssNMR data analysis**

  In most cases (except in cell-free labelling), the protein is expressed by bacteria. The isotopic labels are introduced by feeding the bacteria specific nutrients. In most cases the basis will be so-called minimal medium which contains all the salts and trace elements needed by the bacteria but contains no carbon or nitrogen sources. These elements can then be introduced using a variety of different isotopically labelled carbon and nitrogen sources.

In this preliminary ssNMR study, we have used $^{13}C_6$-Glucose and ^{15}N-NH_4Cl as sole carbon and nitrogen sources and produced a (U-^{13}C,^{15}N)-labeled microcrystalline protein sample in order to collect a maximum amount of information. However, ssNMR spectra from large proteins can exhibit significant resonance overlapping that hamper the detailed analysis of ssNMR spectra. In this situation, fractional ^{13}C labeling schemes can be used to alleviate NMR assignment ambiguities and collect a large number of structural restraints.

The most popular fractional labeling schemes result from the supplementation of the unlabeled M9 medium with either glycerol or glucose labeled at specific positions:

- [1,3-^{13}C]-Glycerol or [2-^{13}C]-Glycerol
- [1-^{13}C]-Glucose or [2-^{13}C]-Glucose

➔ ***Why fractional labelling schemes can alleviate NMR assignment ambiguities***
➔ ***Why the use of fractional labeling schemes results also in sharper signals in microcrystalline proteins?***

4) Analysis of the ssNMR resonances and comparison with other available structural data

You have successfully identified the spin-systems of threonine residues from the protein. This section will show you how to use ssNMR ^{13}C and ^{15}N chemical shifts to predict secondary structure elements in the protein, and how to compare them to other available structural data arising from solution NMR or crystallography.

For this section, you will need to refer to the following figures provided in the ssNMR-handout:
Figure 6: Averaged chemical shift (in ppm) and standard deviation values categorized according to secondary structure type
Figure 7: Procedure used to predict backbone chemical shifts from a 3D structure
Figure 8: Procedure used to plot NC and CC correlation patterns from predicted or experimental chemical shifts

- **Probe secondary structure elements in the microcrystalline protein by measuring ^{13}C secondary chemical shifts from assigned ^{13}C resonances**

  - **Shortcut:** Open ccpnmr project > Analysis ccpnmr_cristaux_e

  - The backbone and side-chain resonances of 7 threonines have been unambiguously assigned by the combined analysis of the 3D NCACX, NCOCX, CANCOCX and CONCACX spectra with different mixing times and the ^{13}C chemical shifts are reported in the table below.

^{13}C (ppm)	Thr104	Thr37	Thr101	Thr10	Thr136	Thr6	Thr55	Thr23	*Thr (RC)*
CO	173.8	-	-	-	-	-	172.7	-	*174.6*
Cα	66.4	64.0	62.1	60.8	59.9	59.4	58.1	-	*62.3*
Cβ	68.6	66.6	68.8	68.4	67.8	68.9	69.4	-	*69.7*
Cγ2	20.1	20.2	19.0	21.5	20.4	19.0	18.4	-	*21.6*

  - Note that resonances and chemical shift assignment table are accessible by selecting in the main Menu the **Resonance** popup and setting **Resonances** and **Chemical Shift Table,** respectively.
    ➔ ***By adjusting the contour levels of the spectra, try to identify the Cα/Cβ resonances of Thr 23 that remained unassigned so far. Complete the chemical shift table if necessary***

- The ^{13}C, $^{1}H\alpha$ and ^{15}N NMR chemical shifts have been known to have a strong correlation with secondary structure. Several techniques have been developed to characterize and quantify protein secondary structure using chemical shift data through comparing the observed chemical-shift with the random coil value

- The secondary structure analysis of protein in solid-state NMR studies often rely on the calculation of ^{13}C secondary chemical from CO, Cα and Cβ resonances, which is the difference between the experimental chemical shift ($\delta C_{measured}$, in ppm) and the random coil chemical shift ($\delta C_{randomcoil}$, in ppm) given in the **BMRB** database (http://www.bmrb.wisc.edu/ref_info/statsel.htm)

$$\Delta\delta C \text{ (ppm)} = \delta C_{measured} - \delta C_{randomcoil}$$

➔ ***Calculate the CO, Cα and Cβ secondary chemical shifts for each threonine residue of the protein . Report the values in the table. Indicate which Thr is more likely located in a α-helical (α), β-sheet (β) or unstructured (rc) protein segment.***

^{13}C (ppm)	Thr104	Thr37	Thr101	Thr10	Thr136	Thr6	Thr55	Thr23	*Thr (BMRB)*
CO	173.8	-	-	-	-	-	172.7	-	*174.6*
Cα	66.4	64.0	62.1	60.8	59.9	59.4	58.1	-	*62.3*
Cβ	68.6	66.6	68.8	68.4	67.8	68.9	69.4	-	*69.7*
SCS (ppm)	**Thr104**	**Thr37**	**Thr101**	**Thr10**	**Thr136**	**Thr6**	**Thr55**	**Thr23**	
ΔδCO									
ΔδCα									
ΔδCβ									
SS (α,β,rc)									

- **Analyze the ssNMR data in reference with available structural data**

➢ **Highlight the Thr residues on the protein 3D model**

- **CcpNmr Analysis** contains a simple **3D structure viewing module** that is used to display NMR derived information on macromolecular coordinates. The structural model may be moved and rotated by various means listed below. Also, specific atoms may be selected and de-selected in the display by left clicking.

- Load the 3D structure model i.e. TPssnmr/cristaux/pdb/totoH.pdb into the CCPN project via the main **Structures** popup window. A structure is chosen for display by selecting from the "**MolSystem**", "**Ensemble**" and "**Model**" pulldown menus, i.e. at present only one conformational model is displayed at a time.

- The "**Peak List**" selection is used in combination with the [**Show Peaks**] button at the bottom, which brings up a table listing all of the peaks, within the selected peak list, that relate to that atoms chosen (left mouse click) in the structural view.

- To move and rotate the three-dimensional coordinate display the following keyboard controls may be used:
  - Rotate: Arrow keys
  - Zoom: Page Up & Page Down keys
  - Translate: Arrow keys + Control key

- Or alternatively the following mouse controls:
- Rotate: Middle button click & drag
- Zoom: Mouse wheel or middle button click + Shift key & drag up/down
- Translate: Middle button click & drag + Control key
- Also an options menu appears when the right mouse button is clicked and the left mouse button is used to select and de-select atoms in the current model view.

- The "**Residues"** window is used to highlight certain types of residues on the 3D model. For instance, select Thr in the **CcpCode** pulldown menu in order to locate the threonines on the structure. Select one of the Thr residue in the table panel and click on **View Residue**

- **Shortcut:** Open ccpnmr project > Analysis ccpnmr_cristaux_f

➢ **Compare ssNMR data with chemical shifts predicted from the X-ray structure of the protein crystallized in similar conditions**

- Go to the **Other** popup window and open **Format Converter** to import synthetic peaklists (in sparky format) that have been generated from predicted chemical shifts (see ssNMR handout figures 6 and 7)
- Go to **Import > Single files > peaks > Sparky**
- Browse the peaklist files to import and type **Select**: TPssnmr/cristaux/pred/totoH-sparta-CCintra5.peaks
- In the Additional options, uncheck the box "Match existing Resonances" and select IMPORT.
- Load the solution NMR peaklist on the PDSD50-12 spectrum by selecting **Peaklist** menu in the **Peak** popup window. Make the synthetic peaklist "**active**" and change the color in skyblue.
- **Shortcut:** Open ccpnmr project > Analysis ccpnmr_cristaux_g

➔ ***Compare the ssNMR and predicted chemical shifts qualitatively (cross-peak positions)***

➔ ***Why some cross-peaks are not visible in the synthetic peaklist stemming from Sparta + CS prediction?***

➢ **Compare ssNMR data with chemical shifts predicted from the X-ray structure of the protein crystallized in similar conditions**

- Go to the **Other** popup window and open **Format Converter** to import synthetic peaklists (in sparky format) that have been generated from predicted or solution NMR chemical shifts (see ssNMR handout figures 6 and 7)
- Go to **Import > Single files > peaks > Sparky**
- Browse the peaklist files to import and type **Select**: TPssnmr/cristaux/pred/totoH-sparta-CCintra5.peaks
- In the Additional options, uncheck the box "Match existing Resonances" and select IMPORT.
- Load the solution NMR peaklist on the PDSD50-12 spectrum by selecting **Peaklist** menu in the **Peak** popup window. Make the synthetic peaklist "**active**" and change the color in red.
- **Shortcut:** Open ccpnmr project > Analysis ccpnmr_cristaux_h

➔ ***Compare the ssNMR and solutionNMR chemical shifts qualitatively (cross-peak positions) and quantitatively (cross-peak intensity).***

➔ ***Which threonine residues are experiencing larger changes? Are they located in a particular region of the protein?***

➔ ***What hypothesis can you make concerning the protein properties in the ssNMR sample?***

SUCCESS !!!

ssNMR Handout

Interpretation of two-dimensional MAS solid-state NMR spectra of a microcrystalline protein

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Summary :

Figure 1. Structure of the 20 amino-acids
Figure 2. Averaged aliphatic carbon chemical shifts
Figure 3. 2D and 3D ssNMR experiments
Figure 4. Characteristic intraresidue CC correlation patterns for the 20 amino acids
Figure 5. Averaged chemical shift and standard deviation values categorized according to secondary structure type
Figure 6. Procedure used for predicting backbone chemical shift form a crystal structure and generating synthetic shift lists
Figure 7. Procedure used to plot NC and CC correlation patterns from synthetic shift lists
Figure 8: Alternative isotope labeling strategy used in ssNMR studies of proteins
And leading to fractional ^{13}C labeling schemes

Figure 1. Structure of the 20 amino-acids

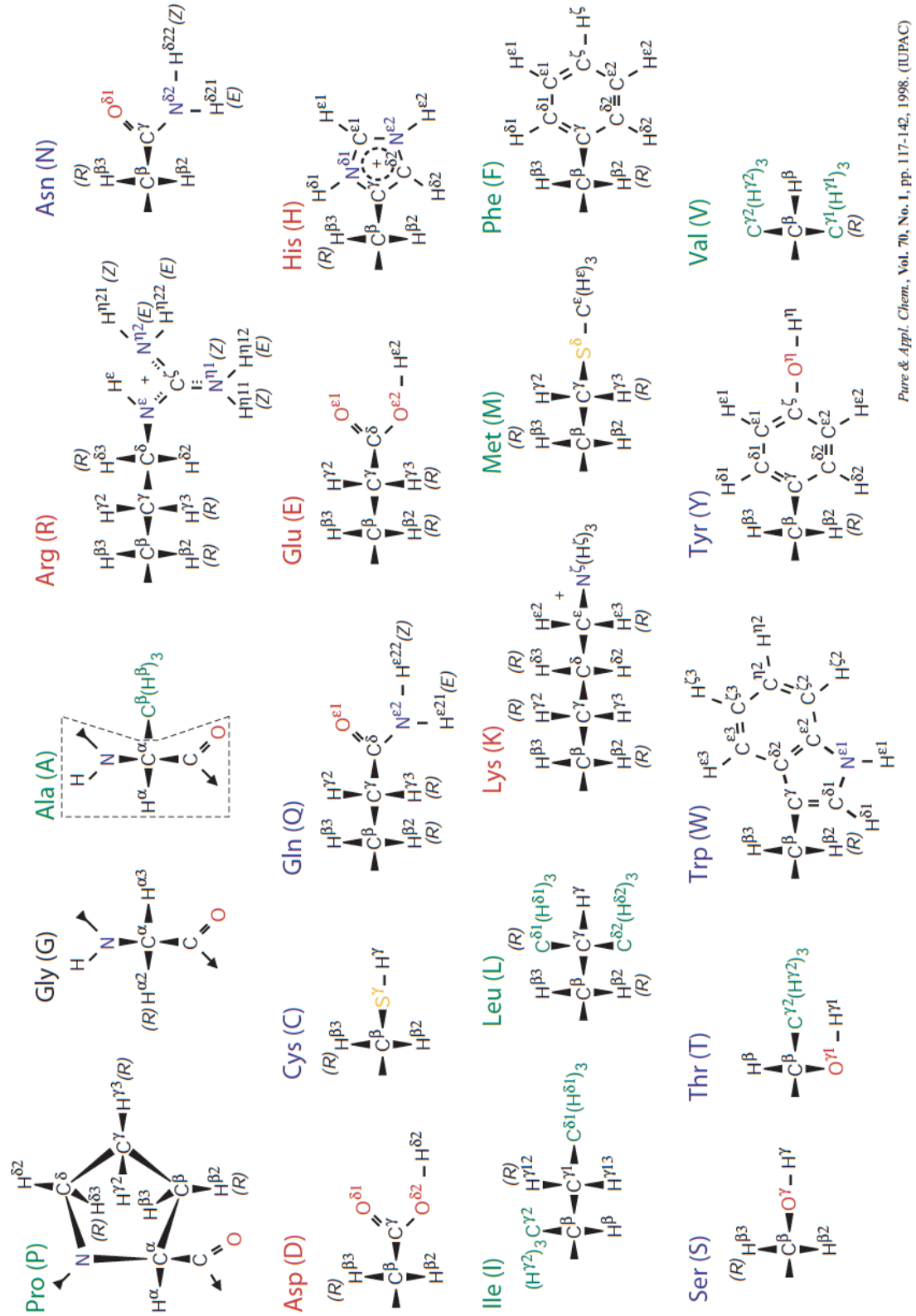


Figure 2. Averaged aliphatic carbon chemical shifts

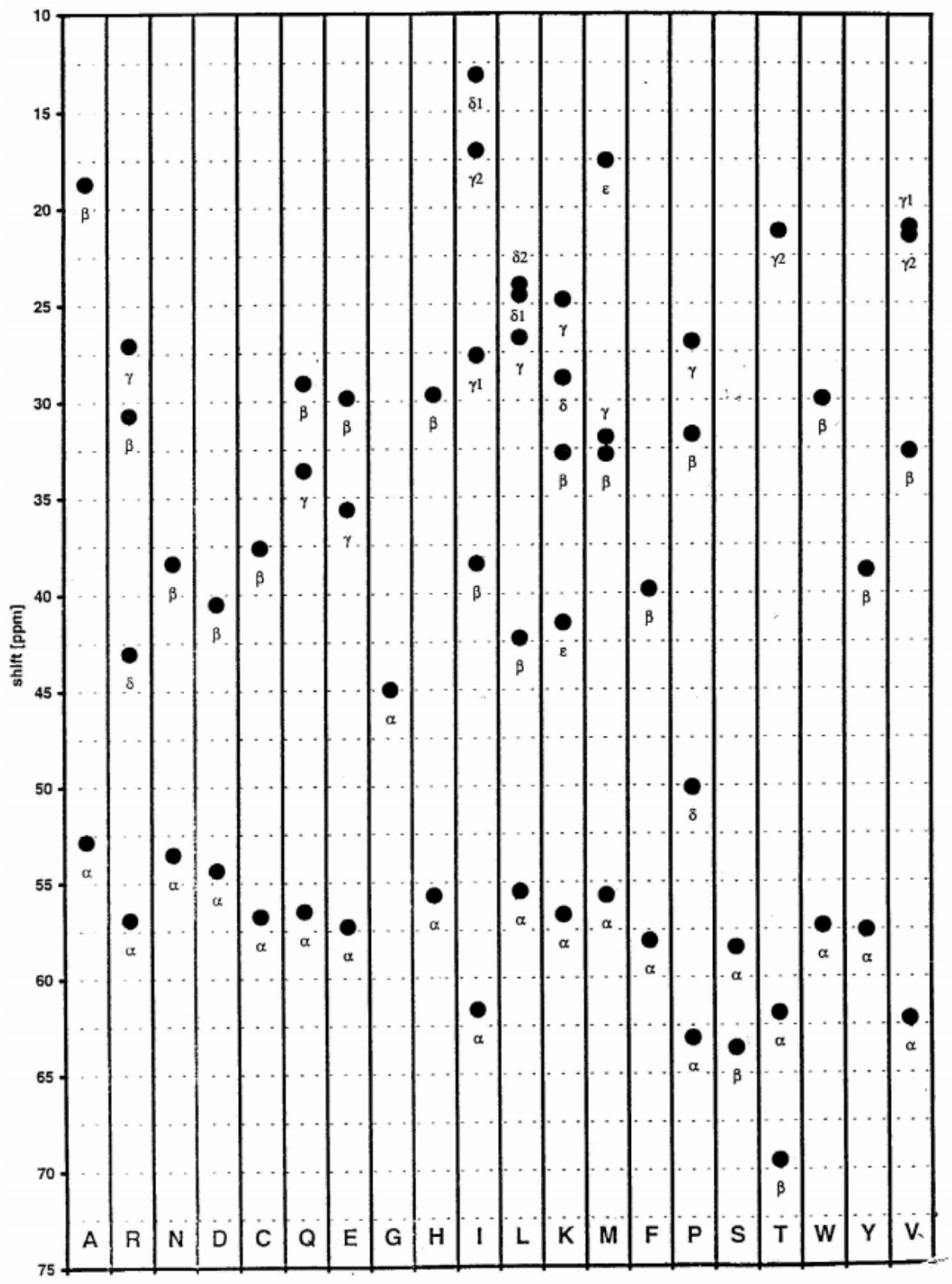
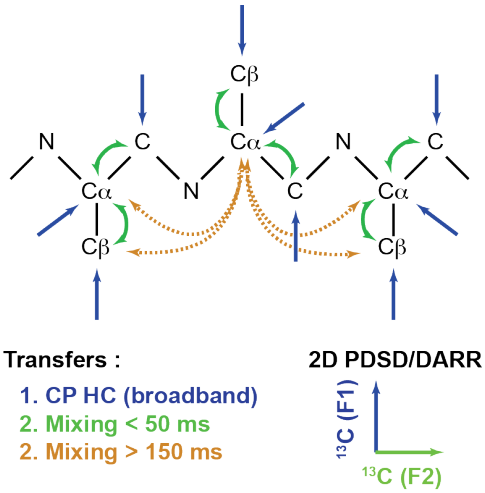


Figure was taken from http://www.protein-nmr.org.uk/wp-content/uploads/2012/10/ccpnmr_analysis_sh3_tutorial_figures.pdf

Figure 3. 2D and 3D ssNMR experiments

PDSD/DARR



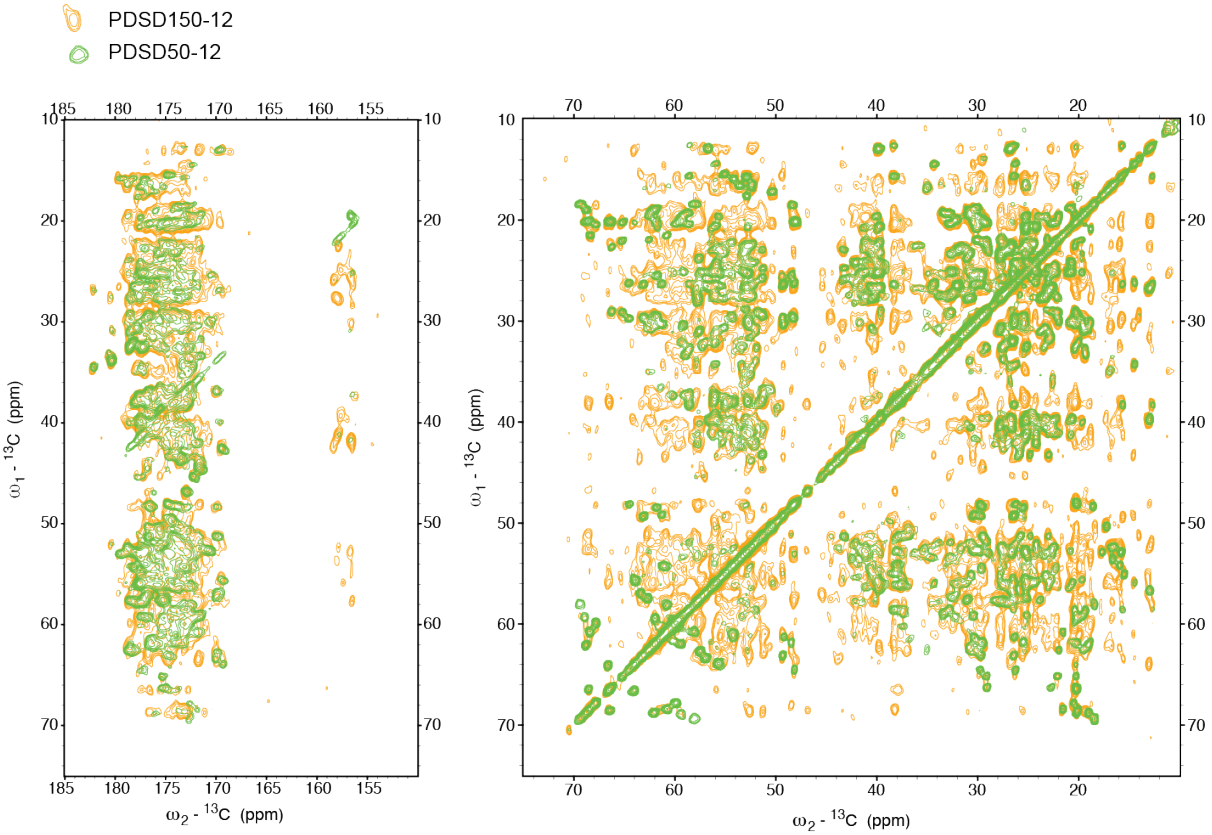
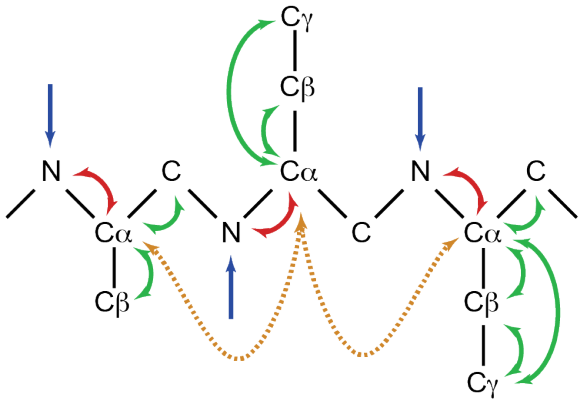


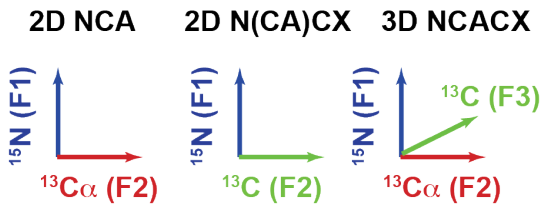
Figure 3. 2D and 3D ssNMR experiments

NCA/NCACX



Transfers :

1. CP HN (broadband)
2. SPECIFIC-CP NC
3. Mixing < 50 ms
3. Mixing > 150 ms



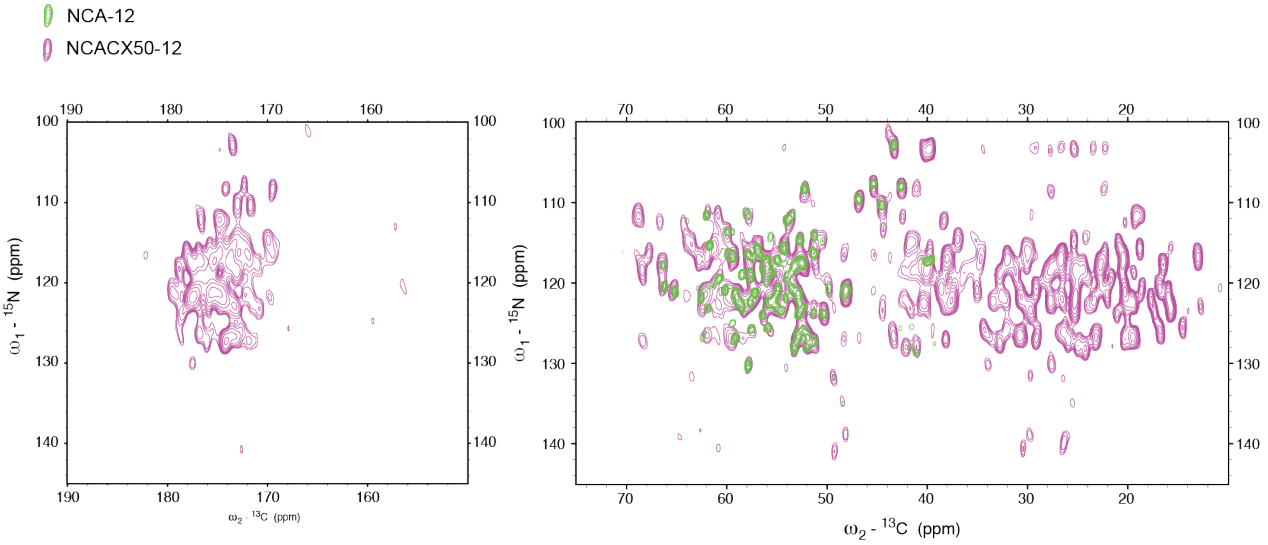
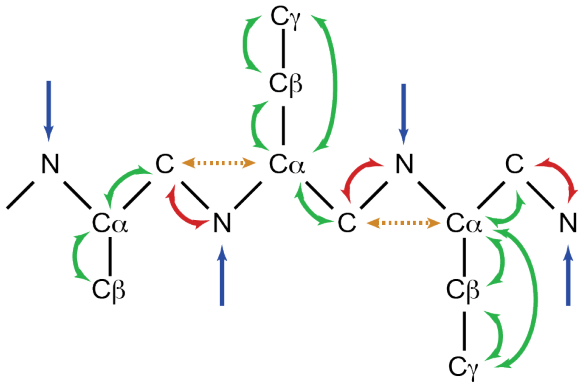


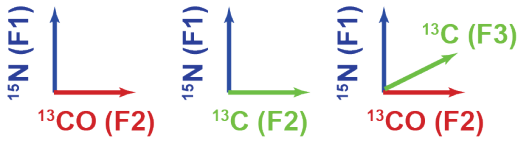
Figure 3. 2D and 3D ssNMR experiments

NCO/NCOCX



Transfers :

1. CP HN (broadband)
2. SPECIFIC-CP NC
3. Mixing < 50 ms
3. Mixing > 150 ms



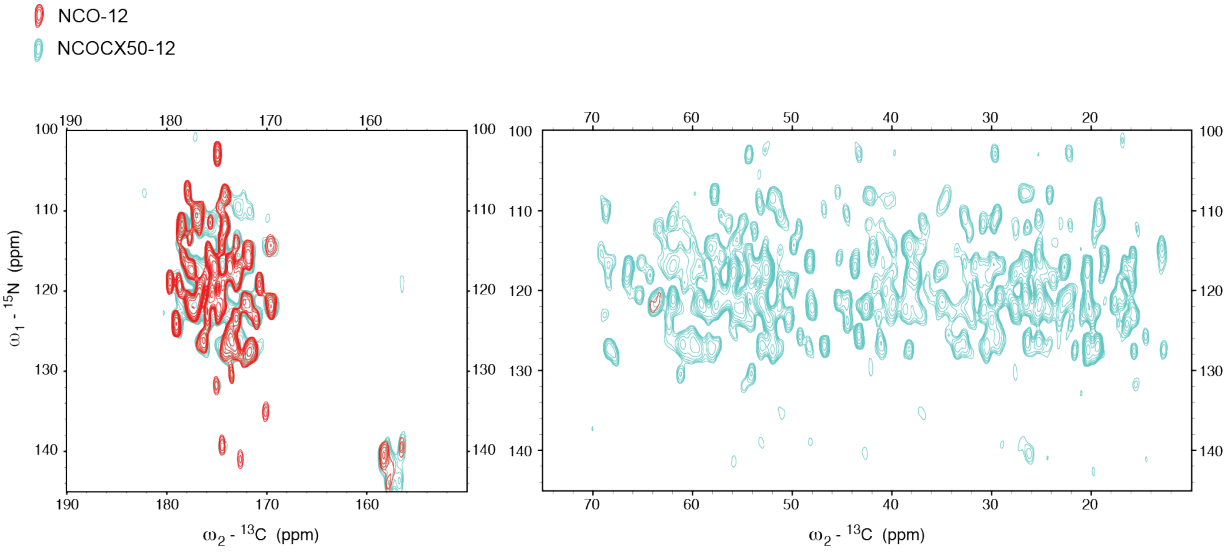


Figure 4. Characteristic intraresidue CC correlation patterns for the 20 amino acids

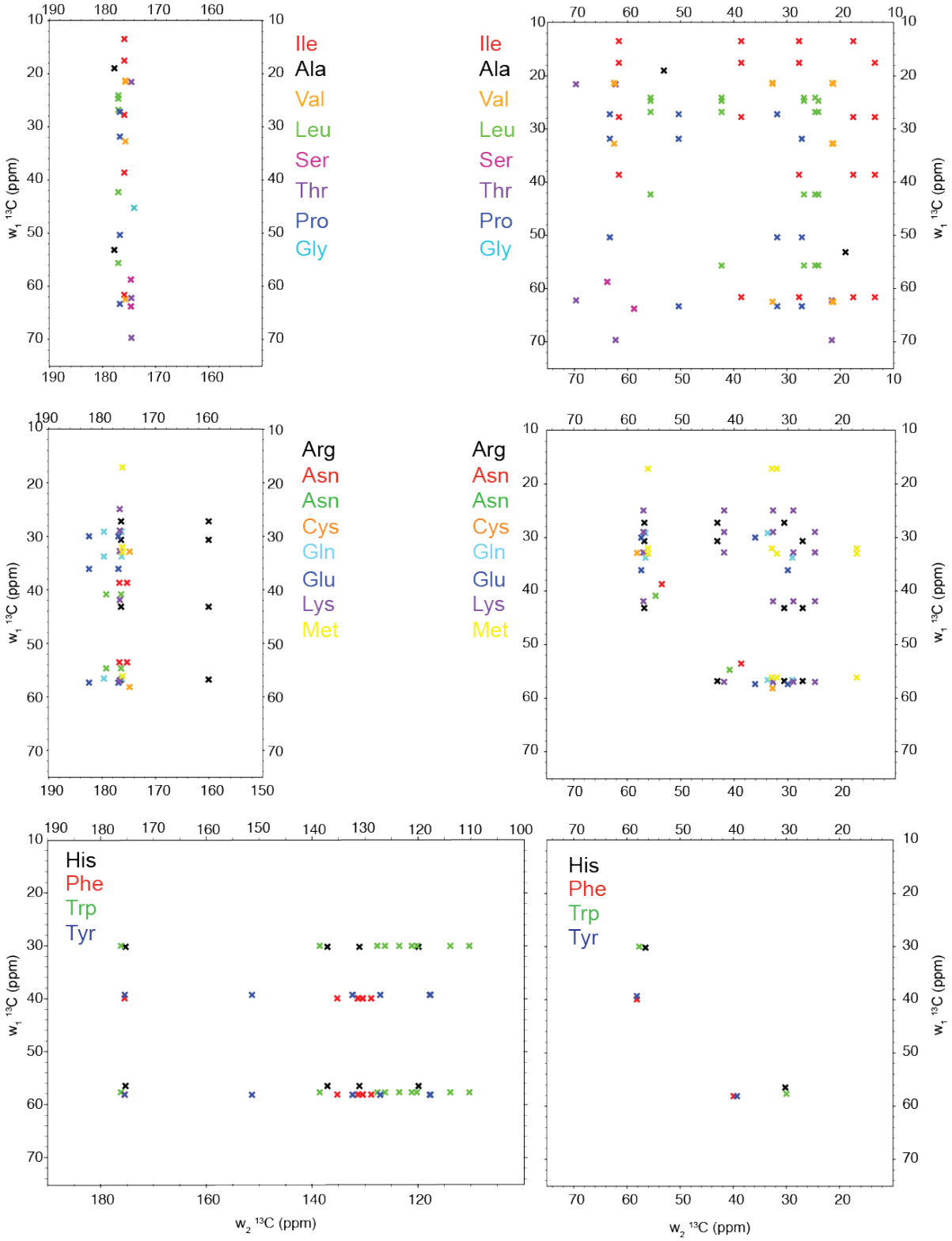
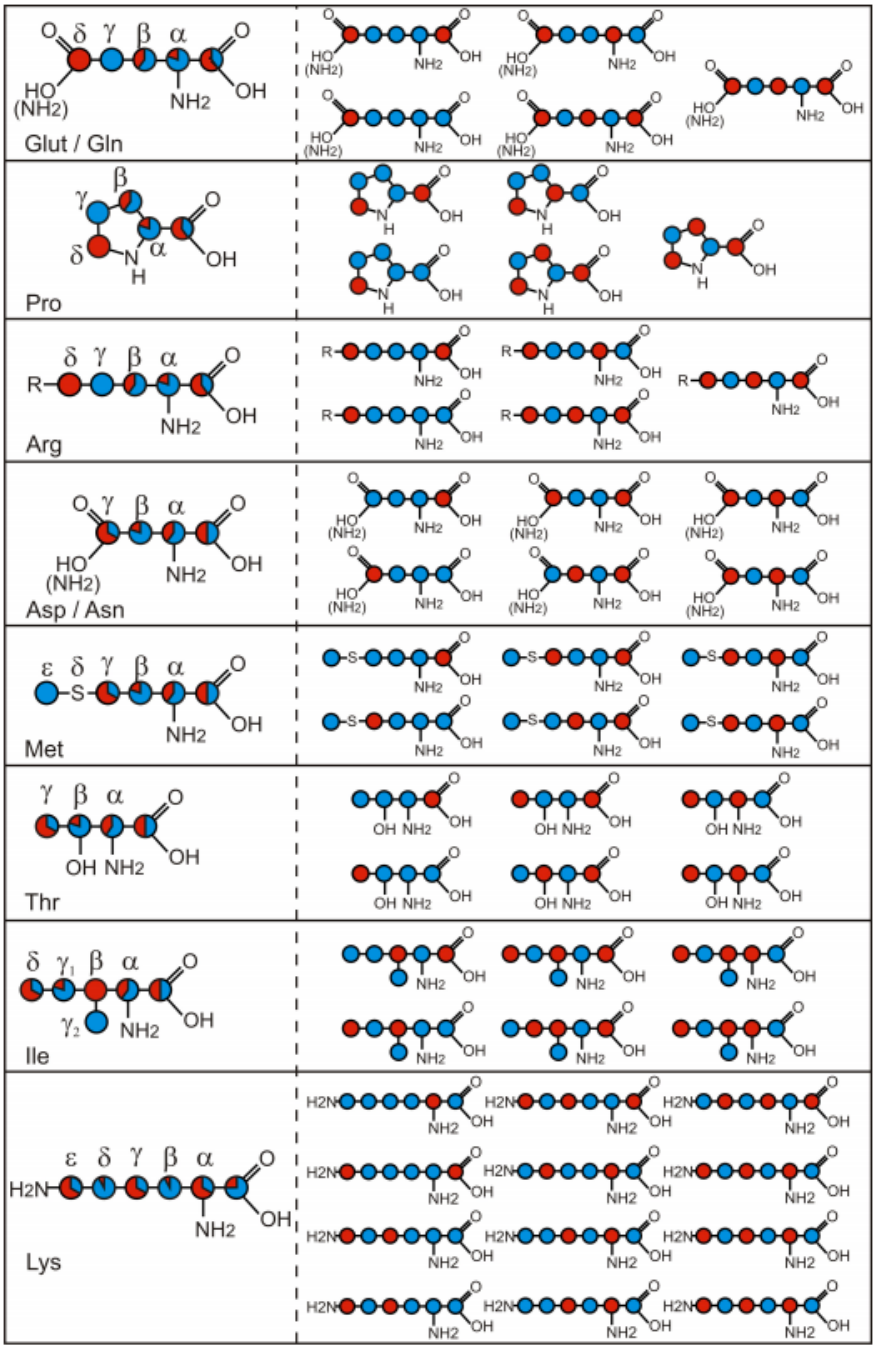


Figure 5. Other isotope labeling strategy used in ssNMR of proteins leading to fractional ^{13}C labeling schemes

Glycerol labelling pattern (isotopomers)

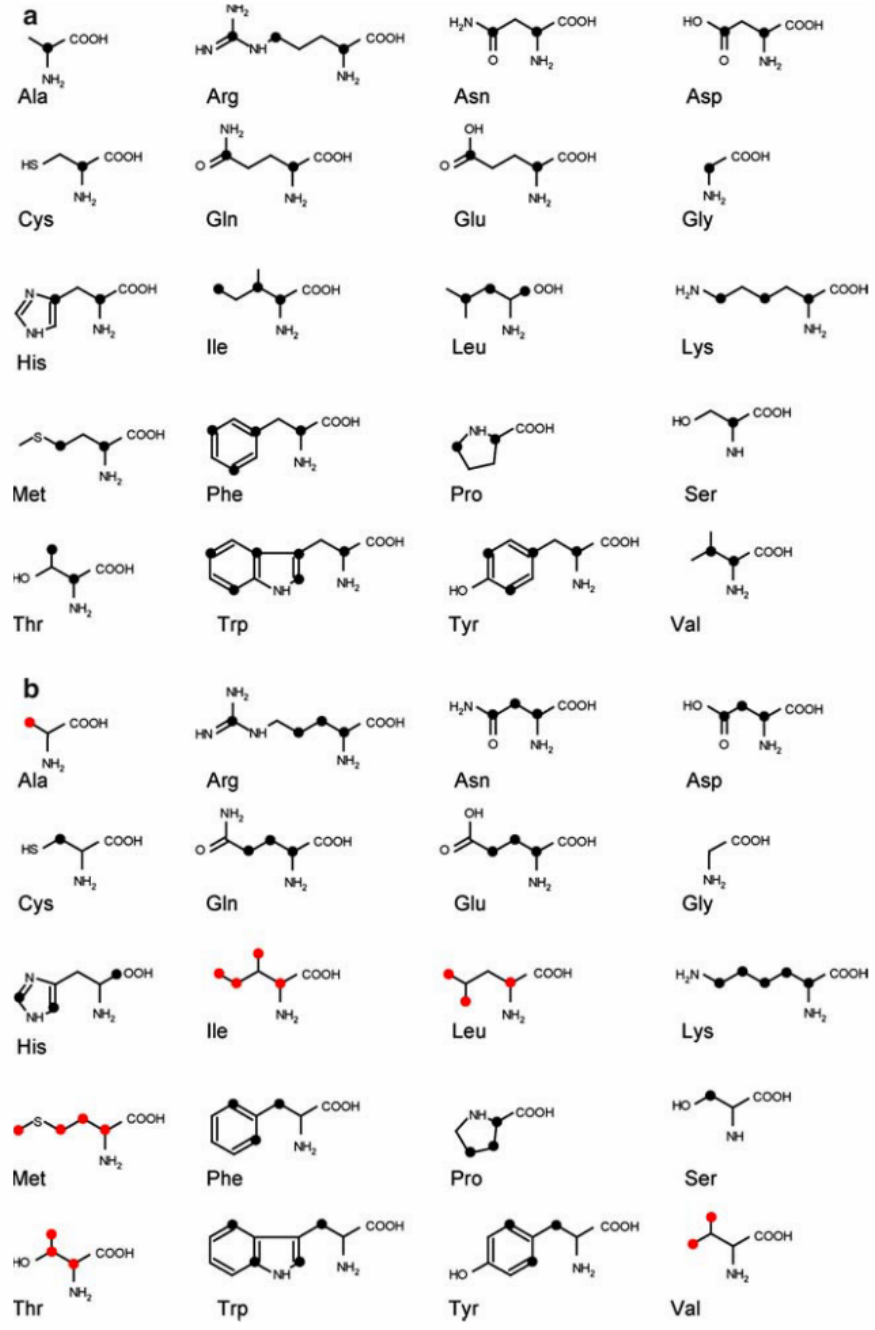


Blue: labelled in the 1,3-^{13}C glycerol sample
Red: labelled in the 2-^{13}C glycerol sample

Figure was taken from http://www.protein-nmr.org.uk/wp-content/uploads/2012/10/ccpnmr_analysis_sh3_tutorial_figures.pdf

Figure 5. Other isotope labeling strategy used in ssNMR of proteins leading to fractional ^{13}C labeling schemes

[2-^{13}C]-Glucose (a) and [1-^{13}C]-glucose (b) labelling patterns (isotopomers)



Circles indicate ^{13}C enriched positions. (a) Labeling patterns for all amino acids when [2-^{13}C]-Glc is used as the carbon source. The amino acids that are derived from TCA cycle intermediate may in principle be enriched at any position. For clarity we only show possible enriched positions that are compatible with simultaneous enrichment at Ca and illustrate the most complex of several labeling scenarios. (b) Labeling patterns for all residues when [1-^{13}C]- Glc is used as the carbon source. Enriched positions for residues with methyl side chains are highlighted in red. The most complex of several labeling scenarios is shown. ***Figure was taken from reference : Lundström, P. et al ; J. Biomol. NMR 38, 199–212 (2007)***.

Figure 6. Averaged chemical shift (in ppm) and standard deviation values (in parentheses) categorized according to secondary structure type

Amino acid	$^{13}C^{\alpha}$			$^{13}C^{\beta}$			$^{13}C'$		
	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix
Ala	50.86 (1.28)	52.67 (1.76)	54.86 (0.94)	**21.72** (1.77)	19.03 (1.27)	18.27 (1.08)	175.3 (1.61)	177.39 (1.45)	179.58 (1.39)
Arg	54.63 (1.50)	55.96 (1.94)	59.05 (1.21)	32.36 (1.82)	30.53 (1.77)	30.00 (0.83)	175.04 (1.18)	175.91 (1.27)	**178.11** (1.70)
Asn	52.48 (1.18)	52.94 (1.43)	55.67 (0.99)	40.43 (1.89)	**38.22** (1.47)	38.28 (1.12)	174.55 (1.28)	174.98 (1.38)	176.74 (1.66)
Asp	53.41 (1.15)	54.09 (1.59)	57.04 (1.00)	42.78 (1.75)	40.76 (1.34)	40.50 (1.12)	175.15 (1.54)	176.01 (1.45)	**178.07** (1.80)
Cys	57.64 (1.94)	**58.8** (2.06)	62.86 (1.85)	29.48 (1.97)	29.75 (1.86)[b]	26.99 (0.84)[b]	**173.86** (1.83)[a]	174.77 (1.38)[b]	177.42 (1.35)[b]
	54.19 (1.64)[a]	57.68 (1.43)[a,b]	58.57 (1.59)[a,b]	43.79 (4.04)[a]	38.38 (1.39)[a,b]	40.02 (1.78)[a,b]	172.73 (1.05)[a]	175.85 (1.58)[a,b]	176.84 (0.47)[a,b]
Gln	54.33 (1.39)	55.94 (1.83)	58.61 (1.04)	31.92 (1.74)	**28.67** (1.73)	28.33 (0.79)	174.58 (0.94)	175.88 (1.53)	178.35 (1.15)
Glu	**55.55** (1.45)	56.39 (1.84)	59.30 (1.05)	32.45 (1.96)	30.02 (1.62)	29.20 (0.77)	175.01 (1.24)	176.11 (1.47)	178.46 (1.34)
Gly	45.08 (1.20)	45.34 (1.17)	47.02 (0.90)				**173.01** (2.59)	174.30 (1.80)	**176.31** (1.50)
His	54.8 (1.75)	55.78 (2.02)	59.62 (1.57)	32.2 (2.52)	29.62 (1.99)	29.91 (1.67)	**173.80** (2.24)	174.88 (1.68)	176.83 (1.16)
Ile	60.00 (1.51)	60.64 (2.08)	64.68 (1.66)	40.09 (1.85)	38.26 (2.06)	37.59 (1.08)	174.79 (1.41)	175.46 (1.65)	**177.49** (1.62)
Leu	53.94 (1.19)	54.85 (1.79)	57.54 (0.98)	44.02 (1.99)	**41.87** (1.70)	41.40 (1.11)	**175.16** (1.31)	**176.61** (1.77)	178.42 (1.70)
Lys	55.01 (1.00)	56.40 (1.80)	59.11 (1.19)	34.86 (1.79)	32.57 (1.30)	32.31 (1.08)	174.93 (1.25)	176.15 (1.40)	177.79 (2.22)
Met	**54.10** (1.46)	**55.12** (1.79)	58.45 (1.66)	**34.34** (2.44)	32.93 (3.05)	**31.70** (1.72)	**174.64** (1.47)	175.93 (1.54)	177.76 (1.77)
Phe	56.33 (1.31)	**56.94** (1.98)	60.74 (1.63)	41.64 (1.65)	39.43 (1.93)	38.91 (1.49)	174.15 (1.93)	175.28 (1.88)	**176.42** (1.74)
Pro	62.79 (1.22)	63.53 (1.26)	65.52 (1.01)	32.45 (0.93)	31.87 (0.96)	31.08 (0.84)	**176.41** (1.50)	176.91 (1.72)	178.34 (1.53)
Ser	57.14 (1.11)	58.35 (1.78)	60.86 (1.27)	65.39 (1.48)	63.88 (1.24)	62.81 (0.58)	**173.52** (1.55)	174.33 (1.22)	**176.51** (1.40)
Thr	61.10 (1.71)	61.59 (2.04)	65.89 (1.55)	70.82 (2.11)	69.75 (1.29)	68.64 (0.98)	173.47 (1.39)	174.62 (1.45)	176.62 (1.24)
Trp	56.28 (1.52)	57.62 (1.25)	60.03 (1.94)	**31.78** (1.76)	29.27 (1.10)	28.74 (1.15)	175.10 (1.80)	175.91 (1.32)	**177.81** (1.62)
Tyr	56.56 (1.59)	57.72 (2.14)	61.07 (1.72)	40.79 (1.77)	38.71 (2.00)	38.38 (0.89)	174.65 (1.64)	175.32 (1.54)	177.05 (1.51)
Val	60.72 (1.59)	61.80 (2.25)	65.96 (1.39)	33.81 (1.79)	32.68 (1.76)	31.41 (0.74)	174.66 (1.36)	175.76 (1.63)	177.75 (1.49)

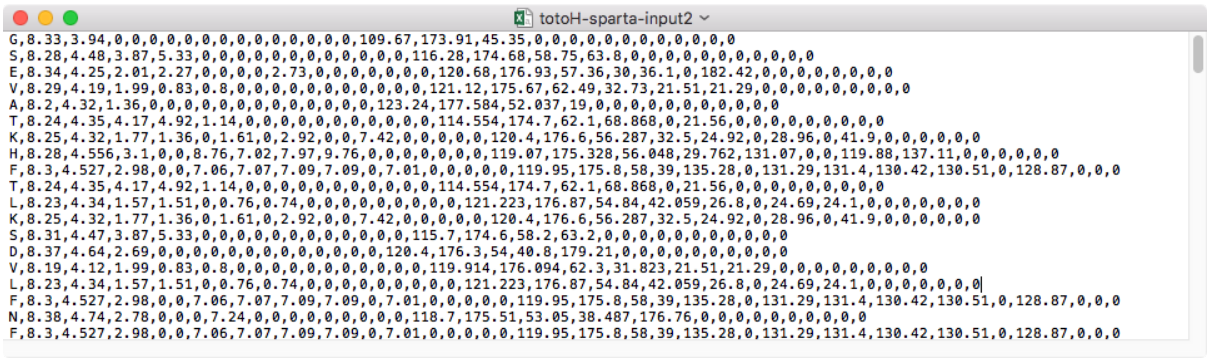
Amino acid	$^{1}H^{N}$			$^{1}C^{\alpha}$			^{15}N		
	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix
Ala	**8.59** (0.76)	8.11 (0.68)	7.99 (0.57)	**4.87** (0.46)	4.25 (0.35)	4.03 (0.31)	125.57 (4.80)	**132.52** (3.51)	121.65 (2.52)
Arg	8.57 (0.69)	8.17 (0.77)	8.03 (0.56)	**4.85** (0.47)	4.33 (0.37)	4.00 (0.33)	**122.60** (4.74)	**120.59** (4.42)	118.99 (2.56)
Asn	8.70 (0.55)	8.33 (0.72)	8.20 (0.66)	**5.26** (0.41)	4.60 (0.38)	4.45 (0.20)	**122.70** (4.18)	118.48 (4.58)	**117.60** (2.37)
Asp	8.56 (0.62)	8.39 (0.66)	8.05 (0.55)	5.01 (0.36)	4.64 (0.29)	4.44 (0.22)	123.82 (4.70)	120.69 (4.45)	119.90 (2.03)
Cys	9.00 (0.45)	7.81 (0.62)	8.22 (0.53)	5.18 (0.57)	4.63 (0.37)[b]	4.16 (0.25)[b]	123.27 (5.69)	117.01 (2.50)[b]	117.47 (3.04)[b]
	8.68 (0.98)[a]	8.53 (0.59)[a,b]	8.58 (0.48)[a,b]	5.21 (0.49)[a]	4.44 (0.29)[a,b]	4.53 (0.18)[a,b]	121.81 (4.34)[a]	118.62 (4.25)[a,b]	119.51 (2.44)[a,b]
Gln	8.51 (0.83)	8.25 (0.75)	8.11 (0.52)	4.97 (0.43)	4.26 (0.39)	4.03 (0.23)	123.14 (4.89)	119.73 (3.85)	118.59 (2.59)
Glu	8.66 (0.60)	8.29 (0.53)	8.32 (0.63)	4.76 (0.44)	4.28 (0.30)	3.99 (0.21)	123.52 (4.29)	120.87 (3.94)	119.89 (2.85)
Gly	8.27 (1.06)	8.34 (0.83)	8.23 (0.78)	4.09[c] (0.46)	3.95[c] (0.40)	3.84 (0.43)[c]	110.19 (4.20)	109.94 (4.09)	**107.34** (2.82)
His	**8.76** (0.79)	8.09 (0.83)	8.03 (0.68)	5.07 (0.50)	4.50 (0.51)	4.06 (0.54)	**121.65** (5.16)	**118.87** (4.98)	118.09 (3.17)
Ile	8.74 (0.66)	7.94 (0.66)	8.06 (0.56)	4.72 (0.42)	4.13 (0.36)	3.66 (0.30)	124.12 (4.93)	121.07 (5.17)	120.22 (2.75)
Leu	8.63 (0.67)	8.12 (0.72)	8.02 (0.56)	4.85 (0.43)	4.35 (0.36)	4.00 (0.27)	125.69 (4.14)	121.53 (4.30)	120.18 (2.46)
Lys	8.54 (0.63)	8.13 (0.66)	8.04 (0.61)	4.96 (0.46)	4.28 (0.31)	3.98 (0.26)	123.29 (4.76)	121.44 (4.19)	119.90 (2.93)
Met	8.43 (0.65)	8.37 (0.51)	8.05 (0.48)	4.94 (0.48)	4.55 (0.38)	4.03 (0.35)	121.67 (4.12)	120.19 (3.46)	118.69 (2.36)
Phe	8.80 (0.70)	7.95 (0.90)	8.21 (0.66)	5.17 (0.47)	4.62 (0.42)	4.11 (0.40)	121.95 (4.38)	119.41 (4.75)	119.12 (4.05)
Pro				4.72 (0.45)	4.41 (0.30)	4.13 (0.39)			
Ser	8.57 (0.65)	8.26 (0.74)	8.11 (0.50)	5.08 (0.48)	4.48 (0.35)	4.20 (0.19)	117.44 (4.19)	115.94 (4.13)	**114.78** (2.39)
Thr	8.50 (0.58)	8.22 (0.74)	8.10 (0.55)	4.81 (0.46)	4.33 (0.38)	4.02 (0.27)	**118.09** (4.86)	114.41 (5.70)	115.30 (3.72)
Trp	8.83 (0.73)	**7.59** (0.84)	8.24 (0.82)	**5.24** (0.41)	4.54 (0.24)	4.35 (0.40)	124.04 (5.43)	**120.57** (3.58)	120.48 (2.89)
Tyr	8.69 (0.73)	**7.90** (0.79)	8.10 (0.70)	5.00 (0.51)	4.55 (0.45)	4.14 (0.36)	122.55 (4.70)	120.05 (4.23)	119.67 (3.19)
Val	8.73 (0.61)	7.88 (0.75)	7.99 (0.63)	4.66 (0.42)	4.14 (0.40)	3.57 (0.34)	123.27 (5.05)	**119.66** (5.62)	119.53 (3.19)

[a] Cys in the oxidized form.
[b] Number of the chemical shifts used in the statistical analysis is less than 10.
[c] Averaged value fro Gly.

Table extracted from reference : Wang Y. and Jardetzky O, Protein Sci. 2002, 11, 852-61)

Figure 7. Procedure used for predicting backbone chemical shift from a crystal structure

1. Download the PDB file : toto.pdb
2. Create ^{1}H atoms (using the software Reduce)
   > in a terminal window, type : reduce –BUILD toto.pdb > totoH.pdb
3. Open the pdb file totoH.pdb and check that H atoms are present
4. Connect to the server of Sparta + (http://spin.niddk.nih.gov/bax/nmrserver/sparta/)
5. Follow the instructions :
   a. Select PDB Input file: totoH.pdb
   b. Chemical Shift Data for Comparison (optional) > NO [Optional input table of chemical shifts and sequence information. If this table is given, the output produced by SPARTA+ will compare the shifts in this input table and the corresponding predicted chemical shifts.
   c. Apply offset correction > NO
   d. Generate PDF graphic >NO
   e. Fill in the contact information (email)
   Submit
6. Check your email inbox, open the message from the Sparta+ server and save the 3 files : pred.tab ; struct.tab ; sparta.pdf
7. Generate a shift list (here, the FANDAS format <name>.txt was used) from the predicted chemical shifts indicated in the pred.tab file



NOTE :

8. Generate a shift list (here, the FANDAS format <name>.txt was used) from the solution NMR assignment table. (missing or unassigned resonances are labeled with a 0)

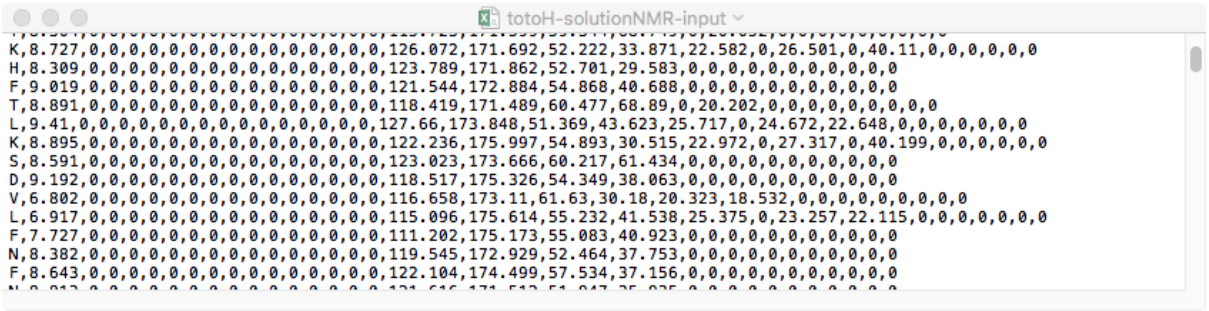


Figure 8. Procedure used to plot NC and CC correlation patterns from predicted or experimental chemical shifts

1. Download the shift list generated from the predicted chemical shifts (totoH-sparta-input)
2. Connect to the server FANDAS (http://tintin.science.uu.nl/services/FANDAS/html/main.php)
3. Follow the instructions for the Spectrum prediction
    a. Predict intra-residue ^{13}C-^{13}Cspectrum > PDSD50 & DARR50
        Save the peaklist with annotations as a <name>.peaks file
    b. Predict sequential ^{13}C-^{13}C spectrum > PDSD150
    c. Predict 2D ^{15}N-^{13}CA spectrum > NCA
    d. Predict 2D ^{15}N-^{13}CA-^{13}CX (intra) spectrum > NCACX50
    e. Predict 2D ^{15}N-^{13}CO spectrum > NCO
    f. Predict 2D ^{15}N-^{13}CO-^{13}CX (intra) spectrum > NCOCX50
4. Now, we have generated 6 peaklists in a Sparky format <name>.peaks from the backbone chemical shifts predicted from the crystal structure by using sequentially Sparta + and FANDAS servers.
5. Download the shift list generated from the solution NMR assignment table (totoH-solutionNMR-input)
6. Repeat the same procedure
7. Now, we have generated 6 peaklists in a Sparky format <name>.peaks from the solution NMR backbone and sidechain chemical shifts obtained on the soluble (U-^{13}C,^{15}N)-labeled protein.