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Total correlation spectroscopy across all NMR-active nuclei by mixing at zero field

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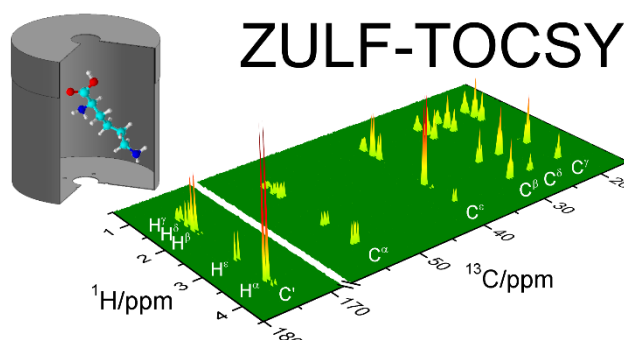
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Abstract

Multidimensional Nuclear Magnetic Resonance (NMR) is based on the combination of well-established building blocks for polarization transfer. These blocks are used to design correlation experiments through one or a few chemical bonds or through space. Here, we introduce a building block that enables polarization transfer across all NMR-active nuclei in a coupled network of spins: isotropic mixing at Zero and Ultra-Low Field (ZULF). Exploiting mixing under ZULF-NMR conditions, heteronuclear Total Correlation Spectroscopy (TOCSY) experiments were developed to highlight coupled spin networks. We demonstrate ¹H-¹³C and ¹H-¹⁵N correlations in ZULF-TOCSY spectra of labelled amino acids, which allow one to obtain cross-peaks among all hetero-nuclei belonging to the same coupled network, even when the direct interaction between them is negligible. We also demonstrate the interest of ZULF-TOCSY to analyze complex mixtures on a growth medium of isotope-labelled biomolecules. ZULF-TOCSY enables the quick identification of individual compounds in the mixture by their coupled spin networks. The ZULF-TOCSY method will lead to the development of a new toolbox of experiments to analyze complex mixtures by NMR.

I. Introduction

Two dimensional Nuclear Magnetic Resonance (2D-NMR)¹ has revolutionized NMR spectroscopy by introducing powerful methods to greatly increase spectral resolution, provide topological information to easily assign signals of complex molecules and structural constraints to refine the conformations of small and macro-molecules. As such, 2D NMR has had a deep impact in synthetic and materials chemistry, as well as structural biology. All 2D-NMR experiments are performed with the following steps (Figure 1a): preparation of the nuclear spin coherence of interest (step 1), evolution of this coherence under an interaction during the time t_1 (step 2), mixing (step 3), observation of the Free Induction Decay (FID) signal as a function of time t_2 (step 4). Repeating the experiment multiple times with variable time t_1 provides the signal intensity in two time dimensions, $S(t_1, t_2)$, which gives a spectrum in two dimensions after Fourier transformation.

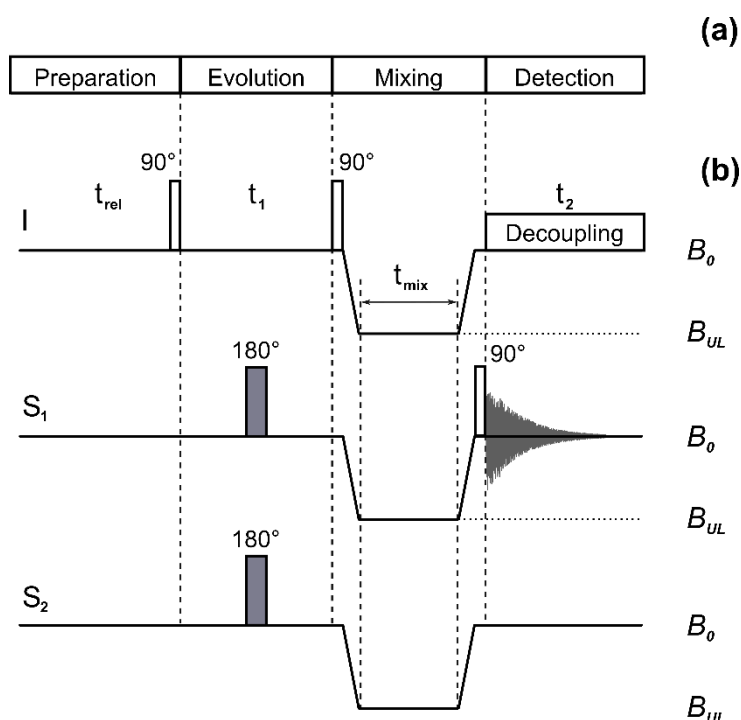


Figure 1. (a) Stages of a general 2D NMR experiment: preparation of an initial spin order; evolution during a variable time period t_1 ; mixing block converting spin order into observable NMR signal; detection of a time domain signal as a function of t_2 time. (b) Protocol of the heteronuclear ZULF-TOCSY experiment. A 90-degree pulse after a suitable relaxation delay t_{rel} generates transverse order of the I spins (here protons). This spin order evolves under chemical shift Hamiltonian during the period t_1 , mapping the indirect spectrum dimension. J-couplings IS_1 and IS_2 are refocused by simultaneous application of 180-degree pulses on the S_1 and S_2 channels, positioned in the middle of the evolution period. Here as S_1 and S_2 spins we used ^{13}C and ^{15}N nuclei; one of them was used for detection. At $t = t_1$ the transverse polarization of the I spins is converted back into longitudinal polarization. The mixing block consists of a field switch $B_0 \leftrightarrow B_{UL}$, lasting for $t_{sw} \approx 0.4$ s, and isotropic mixing under ZULF conditions for t_{mix} of several 10 ms. When the FID signal of S spins is acquired, i.e., the time domain signal in the direct dimension, composite pulse decoupling on the I -channel is applied.

The specific choice of the mixing block is central as it defines the nature of the correlation between the two frequency dimensions, *i.e.* the type of information that the 2D-NMR will provide. For instance, cross-peaks in COSY,² HSQC,³ HMQC,⁴ and HMBC⁵ experiments originate from scalar spin-spin interactions among homonuclei and heteronuclei, and so indicate topological connections through covalent bonds or hydrogen bonds,⁶⁻⁸ between atoms. On the other hand, nuclear Overhauser spectroscopy (NOESY) experiments reports on cross-relaxation between nuclear spins through space, which can be converted to distances between atoms.⁹ One of the most widely used 2D-NMR methods is TOtal Correlation SpectroscopY (TOCSY),¹⁰ which is designed to elucidate scalar-coupled spin networks. In the TOCSY case, the mixing is obtained by using a strong radiofrequency (RF) pulse, which gives rise to isotropic mixing among homonuclei. As a consequence, all spins belonging to the same coupling

network exhibit cross-peaks. The TOCSY method is very useful for assigning signals in molecules constituted of assembled units, such as proteins,¹¹ nucleic acids,¹² and polysaccharides.¹³ Similarly, the TOCSY experiment is extremely valuable for the investigation of complex mixtures of small molecules.¹⁴

Correlations between unlike spins across more than one bond in small molecules is often obtained from the HMBC experiment gives correlations of unlike spins through usually 2 to 3 bonds. Heteronuclear cross-polarization, also called heteronuclear Hartmann-Hahn (HEHAHA) provides correlations between two types of nuclei, with limitations on bandwidth¹⁵⁻¹⁷ and three-dimensional experiments such as TOCSY-HSQC¹⁸ and TOCSY-HMQC¹⁹ combine some features of TOCSY with correlations between proton-bound heteronuclei. Yet, there is, to date, no experiment that provides directly full correlations across network of coupled like and unlike nuclei. Here, we introduce a new concept in total correlation spectroscopy, providing correlations between all NMR-active nuclei in a coupled network. We achieve isotropic mixing across all nuclei by transferring the sample to Zero and Ultra-Low Field (ZULF) NMR²⁰ conditions using a sample shuttle,²¹⁻²⁴ while all chemical-shift evolutions are carried out at high field on a high-resolution NMR spectrometer (**Figure 1b**). Zero or ultra-low field TOCSY (ZULF-TOCSY) enables isotropic mixing among heteronuclei combined with sensitivity and excellent spectral resolution provided by modern high-field NMR methods. ~~We would like to note that the name ZULF-TOCSY bears a close similarity with that of the recently proposed zero field TOCSY technique,²⁵ in which all four key steps of the 2D-NMR experiment are carried out under ZULF conditions. Perhaps, in order to discriminate between the two methods, the technique proposed here can be named two-field-ZULF-TOCSY, or 2F-ZULF-TOCSY.~~ We illustrate the method on isotopically enriched amino acids, and demonstrate that ZULF-TOCSY allows the efficient assignment of signals of individual molecules in complex mixtures.

II. Methods

Experiments were carried out on aqueous solutions of uniformly ¹³C and ¹⁵N labelled amino acids, L-leucine (Sigma-Aldrich, product no. 608068; 100 mM, pH 5.9) and L-lysine (Sigma-Aldrich, product no. 608041; 100 mM, pH 5.7), as well as ISOGRO (MERCK; pH = 6.5) growth medium solubilized in deuterated water (Deutero GmbH). ISOGRO is a complex mixture of uniformly isotopically enriched ¹³C and ¹⁵N molecules: amino acids and small peptides (65%), glucose (2%), as well as salts (30%).²⁶ The pH of aqueous solution was measured by electrode calibrated in protonated water. All experiments were carried out at 300K.

NMR experiments were performed using a commercial 9.4 T NMR spectrometer (Avance III HD, Bruker) equipped by TXI and BBO probes. The spectrometer was augmented with an add-on device to shuttle the sample along the vertical axis between the detection point at 9.4 T and a magnetic shield with a magnetic field strength inside in the range 10 nT – 25 μ T.²⁷ To implement non-adiabatic field variation we used the same method as described before²⁷ with mechanical sample transport to an intermediate guiding field $B_{int} \approx 25 \mu$ T within 0.4 s, which is rapidly (within less than 100 μ s) reduced to a B_{UL} field of desired strength; the $B_{UL} \rightarrow B_0$ switch is done in the same way, $B_{UL} \rightarrow B_{int} \rightarrow B_0$.

Chemical shift referencing for proton spectra was done by referencing of residual water signal. The absolute water chemical shift was calculated using the formula $\delta(\text{HDO}) = 7.83 - T/96.9$,²⁸ where T is the absolute temperature; in the present case $\delta(\text{HDO})$ was 4.73 ppm. Referencing of the ¹³C and ¹⁵N resonance frequencies was done indirectly according to recommendations for biomolecular NMR²⁹ in the Mnova software (version 12).

III. Results and Discussion

The ZULF-TOCSY experiment was designed to achieve efficient isotropic mixing between heteronuclei and high-resolution chemical shift evolution in all dimensions. ZULF-TOCSY is somewhat related to the

two-field TOCSY (2F-TOCSY) experiment we have recently introduced. The 2F-TOCSY experiment takes advantage of mixing at a low magnetic field (*e.g.* 0.33 T) while all chemical shift evolutions occur at high field. {kaderavek 2017} Isotropic mixing at low field yields a very broadband experiment but the 2F-TOCSY experiment is limited to correlations between like spins. Here, we describe the ZULF-TOCSY pulse sequence in the case of a heteronuclear spin system comprising I spins and two S spins, termed S_1 and S_2 (Figure 1b). Hereafter, I spins are protons, while S_1 and S_2 spins are ^{13}C and ^{15}N or vice versa. High sensitivity is obtained by polarizing the sample at a high magnetic field B_0 . Chemical shift evolution for the I spins is obtained during the time t_1 , during which an inversion pulse on the S_1 and S_2 spins refocuses the evolution under heteronuclear couplings. Although detection is performed on the S_1 channel only, the S_2 spin are also present in the molecule. Hence, it is desirable to apply refocusing pulses on the S_2 channel as well in order to improve the quality of the spectrum in the indirect dimension, in particular, in the case where S_1 spins are ^{15}N nuclei and S_2 spins are ^{13}C nuclei. At $t = t_1$ transverse polarization is converted back to longitudinal polarization before a fast (non-adiabatic) field jump from the high-field B_0 to the ultralow field B_{UL} of about 100 nT for mixing (as explained above). Polarization transfer throughout all coupling networks is allowed at the ultra-low field during a time t_{mix} . The sample is transferred back to the B_0 field by a fast non-adiabatic field jump $B_{UL} \rightarrow B_0$ to detect at high field for optimal resolution on the S -channel. We would like to stress that despite the apparent similarity of the pulse sequence to heteronuclear NOESY, spin mixing works in a very different way and the mechanism of polarization transfer between heteronuclei is different (coherent polarization transfer rather than cross-relaxation).

The ZULF-TOCSY method relies on efficient isotropic mixing at the ultralow magnetic field B_{UL} . Here, we briefly explain the coherent mechanism for isotropic mixing. The Hamiltonian of the spin system at $B = B_{UL}$ is written as follows (in \hbar units):

$$\hat{\mathcal{H}} = \omega_I \sum_i \hat{I}_{iz} + \omega_S \sum_k \hat{S}_{kz} + 2\pi \sum_{i>j} J_{ij}^{II} (\hat{\mathbf{I}}_i \cdot \hat{\mathbf{I}}_j) + 2\pi \sum_{k>l} J_{kl}^{SS} (\hat{\mathbf{S}}_k \cdot \hat{\mathbf{S}}_l) + 2\pi \sum_{i,k} J_{ik}^{IS} (\hat{\mathbf{I}}_i \cdot \hat{\mathbf{S}}_k)$$

Here $\omega_{I,S} = -\gamma_{I,S} \times B_{UL}$ is the angular frequency of the Zeeman interaction of spins I , S with the gyromagnetic ratio $\gamma_{I,S}$. Here we neglect the chemical shifts of individual nuclei. J_{ij}^{II} , J_{kl}^{SS} , J_{ik}^{IS} are the homonuclear and heteronuclear scalar-coupling constants. In the situation where the difference in Larmor frequency $\{\omega_I - \omega_S\}/2\pi$ becomes much smaller than the heteronuclear coupling constants, J_{ik}^{IS} , there is no longer a difference between homo- and heteronuclear spin.³⁰⁻³⁵ The strong coupling conditions are identical to those in homonuclear TOCSY experiments,¹⁰ in which the chemical shift differences are quenched for homonuclei by applying a strong radiofrequency field. Thus, at $B = B_{UL}$ polarization transfer via an entire coupling network can be achieved. For $B_{UL} = 100$ nT the Larmor frequencies of ^1H , ^{13}C and ^{15}N are 4.26 Hz, 1.07 Hz and 0.43 Hz, respectively, whereas typical values of the direct scalar coupling constants are $^1J(^{13}\text{C}, ^1\text{H}) \sim 140$ Hz for a quaternary carbon and $^1J(^{15}\text{N}, ^1\text{H}) \sim 7$ Hz in the amino group. The homonuclear ^1H and ^{13}C subsystems are expected to be in the strong coupling regime during the field switching step at significantly higher fields, being in the mT range. Hence, at $B_{UL} = 100$ nT isotropic mixing of the entire spin network is expected.

To implement the ZULF-TOCSY method we used a 400 MHz NMR spectrometer equipped with a fast field-cycling device,²⁷ which allows one to perform magnetic field switches $B_0 \leftrightarrow B_{UL}$ in approximately 0.4 s; which is non-adiabatic for the heteronuclei.³⁶ Performing fast field jumps is a prerequisite for ZULF-TOCSY. Non-adiabatic field jumps turn the difference in the polarizations of the I spins and S spins into heteronuclear zero-quantum coherences (ZQCs), which mediate the polarization transfer; the ZQC evolution is driven by scalar spin-spin interactions.^{27, 37}

We have optimized the ZULF-TOCSY mixing time with a simple field-jump experiment (Figure 2a). Recovery was allowed for all nuclei (*e.g.* protons, ^{13}C , and ^{15}N) at high magnetic field, $B = B_0$ to reach

their equilibrium polarizations, which is different for different spin isotopes due to the difference in their gyromagnetic ratios. Subsequently, a non-adiabatic field jump to the ultralow field B_{UL} converts the difference in spin polarizations into ZQCs, which evolve during a variable time t_{mix} . The amount of polarization transfer was measured after a non-adiabatic field jump back to high field $B_{UL} \rightarrow B_0$, which converts ZQCs back into longitudinal polarizations, right before detection. Polarization transfer from ^1H to ^{13}C nuclei in uniformly ^{13}C , ^{15}N -labeled L-lysine is occurring on the timescale of around 10 ms (**Figure 2b**). The polarization transfer from protons leads to stronger signals of ^{13}C than those obtained at thermal equilibrium at $B = B_0$. The t_{mix} dependence of polarization contains fast oscillations, which are due to the evolution of the ZQCs, driven by J-couplings, confirming efficient coherent polarization transfer among hetero-nuclei at $B = B_{UL}$. Similar results were obtained for ^1H to ^{13}C polarization transfer in L-leucine, as well as for polarization transfer among protons and ^{15}N nuclei in Lysine (see **Supporting Information**). The t_{mix} value of 2 ms, corresponding to the first maximum in the oscillatory curves shown in **Figure 2b**, is usually sufficient for achieving polarization transfer at the B_{UL} field, i.e., for obtaining ZULF-TOCSY cross-peaks (experiments with longer t_{mix} times give similar results). In the case of ^1H - ^{15}N correlation longer t_{mix} , typically about 50 ms, are required.

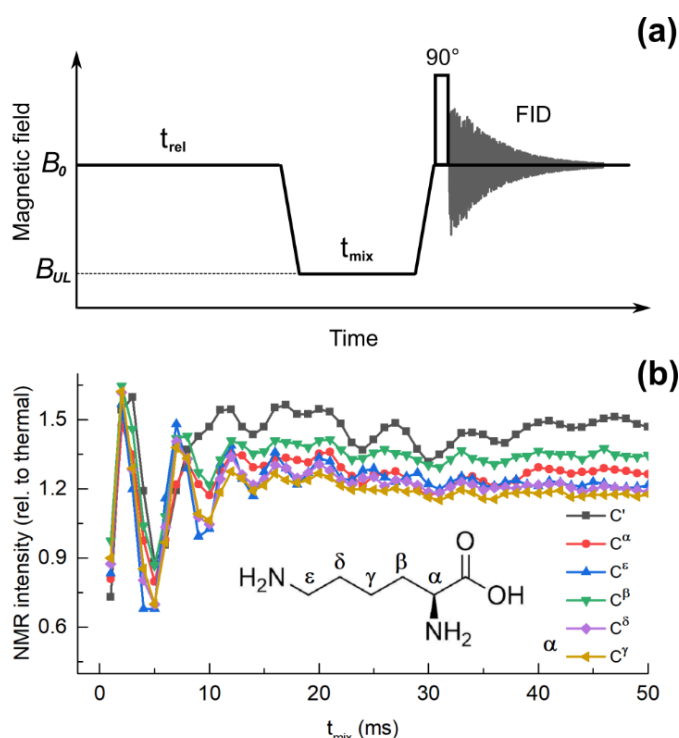


Figure 2. (a) Protocol of the ZULF polarization transfer experiment. Spin system with equilibrium magnetization is transferred non-adiabatically to an ultralow field B_{UL} . After evolution caused by difference in population of spins I and S during t_{mix} field is switched back to B_0 where FID of either spin I or spin S is detected. Varying t_{mix} one can obtain the kinetics of the polarization transfer between proton (I spins) and heteronuclei (S spin). (b) Dependence of ^{13}C NMR signal intensities of uniformly ^{13}C , ^{15}N -labeled L-lysine on t_{mix} obtained for $B_{UL} = 100$ nT. The resulting signals are normalized to the thermal signals at $B = B_0$.

The ZULF-TOCSY spectra of L-lysine display extensive ^1H - ^{13}C and ^1H - ^{15}N correlations (**Figure 3**). Polarization is transferred through the entire spin system, under isotropic mixing conditions at the ultralow field $B = B_{UL}$. In particular, the ^1H - ^{13}C spectrum shows that all proton peaks are correlated to all carbon peaks, since all of them belong to the same coupling network. For instance, the ϵ - CH_2 protons exhibit cross-peaks with all carbons, although sizeable direct coupling is expected only for the carbon nuclei in the ϵ and δ positions. At the same time, only the $\text{H}\epsilon$ - $\text{C}\epsilon$ correlation can be observed in the HSQC spectrum (see **Supporting Information**). It is also important to point out that the C' carbon, which does not exhibit any cross-peak in the HSQC spectrum (because of the absence of one-bond coupling to a proton), displays cross-peaks with all protons in the ZULF-TOCSY spectrum. In the ^1H - ^{15}N ZULF-TOCSY spectra one can also see cross-peaks of both ^{15}N nuclei with most protons, including remote protons

separated from the ^{15}N atoms by four bonds, which results in negligible direct couplings. By contrast, only two- and three-bond correlations can be observed in the HSQC spectrum with exceptionally long INEPT transfer times (**Supporting Information**). Interestingly, correlations with the signal of HOD demonstrates combined polarization transfer by isotropic mixing and chemical exchange of labile protons. The ZULF-TOCSY results for the uniformly labelled L-leucine, are qualitatively similar (**Supporting Information**). Detection of ZULF-TOCSY spectra on the X-channel provides excellent resolution in the direct ^{13}C or ^{15}N dimension.

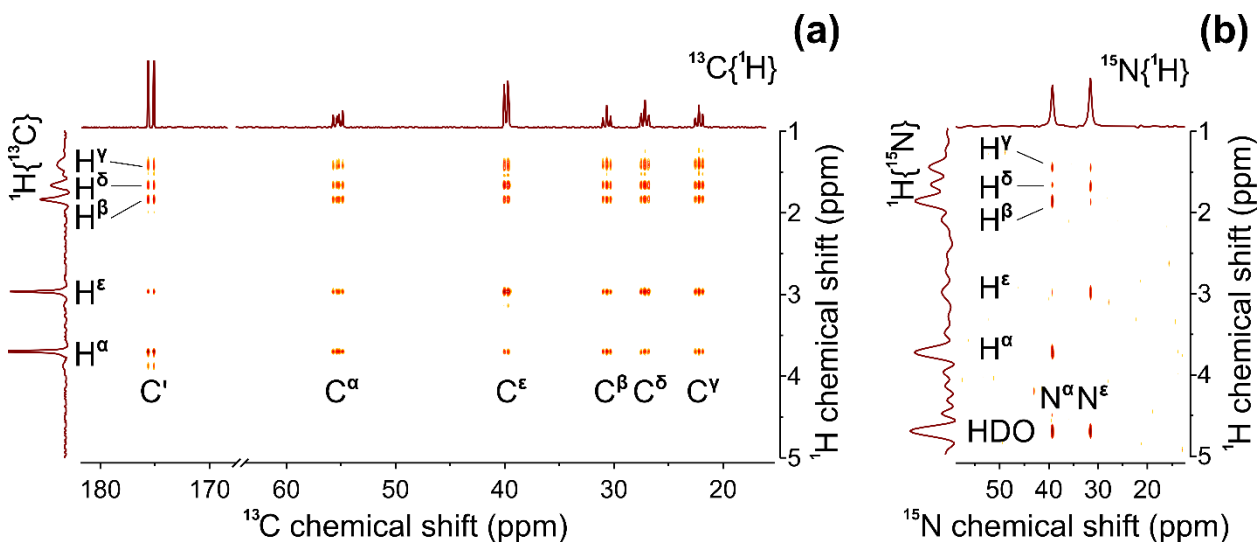


Figure 3. (a) ^{13}C - ^1H ZULF-TOCSY spectrum of ^{13}C , ^{15}N -labeled L-lysine. Experimental parameters: $B_{UL} = 100$ nT, $t_{mix} = 2$ ms, 128 transients in indirect direction, 4 scans per transient, relaxation delay 6 s, total experiment duration ca. 70 minutes. Linear prediction of the indirect dimension FID points 129-512 was applied using the Zhu-Bax³⁸ method. Acquired data multiplied by 90° shifted squared sinusoidal window function in both dimensions. Spectrum presented by 2048 \times 512 data points. (b) ^{15}N - ^1H ZULF-TOCSY spectrum of ^{13}C , ^{15}N labeled L-lysine. Experimental parameters: $B_{UL} = 100$ nT, $t_{mix} = 50$ ms, 64 transients in indirect direction, 128 scans per transient, relaxation delay 23 s, total experiment duration ca. 62 hours. Linear prediction of indirect dimension FID points 64-256 was applied using the Zhu-Bax³⁸ method. Acquired data multiplied by a 90° -shifted squared sinusoidal window function in both dimensions. The spectrum is given by 2048 \times 256 data points.

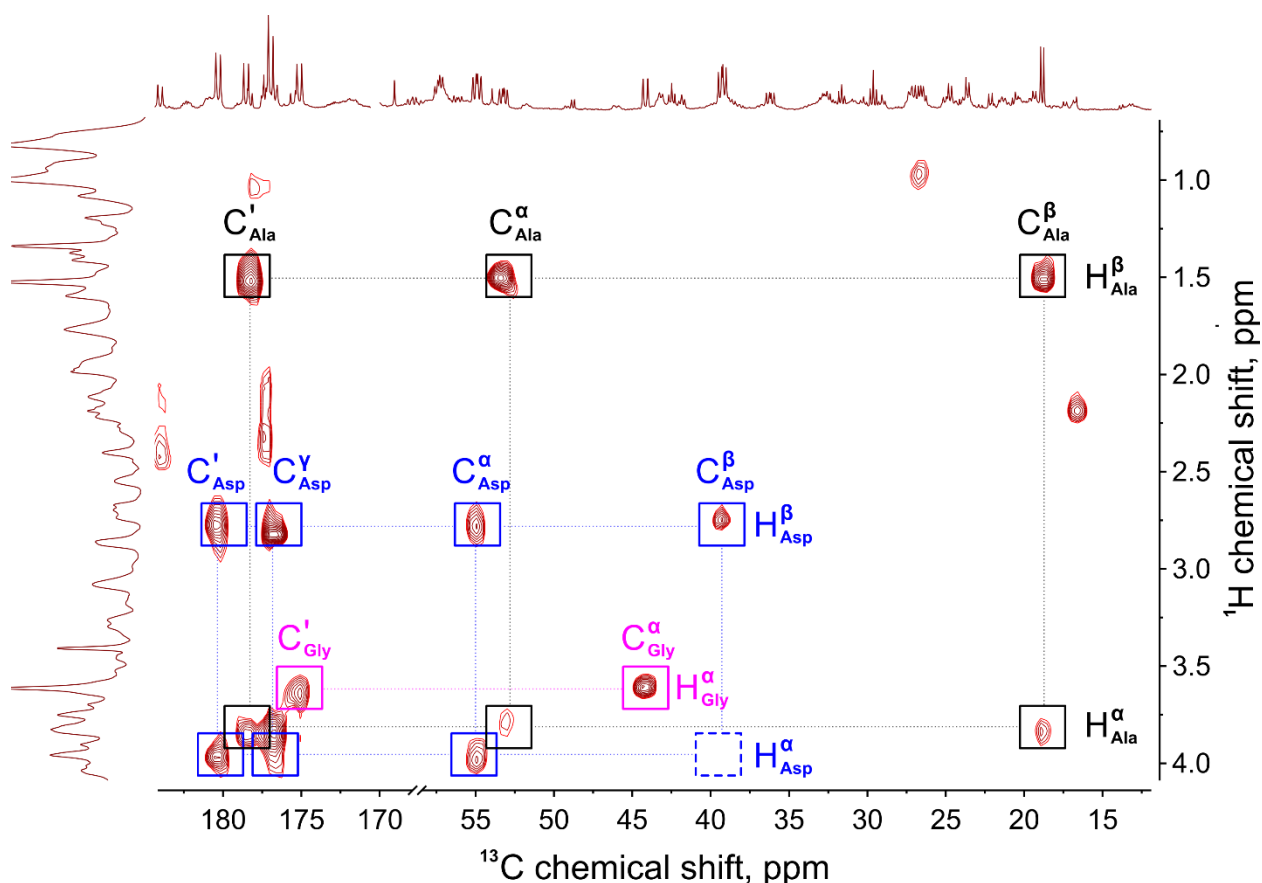


Figure 4. ZULF-TOCSY spectrum of the ISOGRO supernatant in D_2O (pH=6.5). Experimental parameters: $B_{UL} = 50$ nT, $t_{mix} = 40$ ms, 64 transients in indirect direction, 960 scans per transient, relaxation delay 3 s, total experiment duration ca. 69 hours.

ZULF-TOCSY spectra allow one to highlight heteronuclear coupling networks. A spectrum with all possible correlations provides invaluable information to identify individual molecules in complex mixtures. We have used ZULF-TOCSY to analyze a solution of the ISOGRO growth medium, which contains various isotopically labelled small biomolecules, e.g., amino acids. The 1H - ^{13}C ZULF-TOCSY spectrum of the ISOGRO sample (Figure 4) highlights the coupling networks in individual amino acids: signals of alanine, glycine and aspartate can be clearly identified. Hence, ZULF-TOCSY is a powerful method for the analysis of small molecules, whereas bigger molecules are expected to be subject to efficient relaxation during the field jumps $B_0 \rightarrow B_{UL}$ and $B_{UL} \rightarrow B_0$, which reduces the signals. We expect that when T_1 is shorter than or comparable to t_{sw} (which is the case for bigger molecules), ZULF-TOCSY peaks will be strongly reduced. This clear advantage of ZULF-TOCSY for the analysis of small molecules is also a limit of the method for its application to large-size molecules.

IV. Conclusion

In this work, we introduce the use of isotropic spin mixing under ZULF-NMR conditions to develop new high-resolution 2D-NMR experiments. Specifically, we propose to utilize coherent polarization transfer among heteronuclear spin systems to implement an analogue of the widely used TOCSY experiment, which is applied to homonuclear spin systems. Such a ZULF-TOCSY experiment allows one to identify coupling networks of all NMR active nuclei, displayed in 1H - ^{13}C or 1H - ^{15}N 2D correlations. We illustrate the features of ZULF-TOCSY on two isotopically enriched amino acids, leucine and lysine. Furthermore, we demonstrate the efficiency of ZULF-TOCSY as an analytical tool for complex mixture on a solution of ISOGRO growth medium, which is a complex mixture of various biomolecules. ZULF-TOCSY enables quick identification of individual amino acids in the mixture by their coupled intramolecular spin networks. A zero-field TOCSY experiment (ZF-TOCSY) was recently proposed{pines} that is fully carried out under

zero-field conditions, thus, it does not provide the convenient access to chemical shifts essential for the NMR analysis of molecules.

Mixing at ultralow field can be used as a building block in multi-dimensional experiments. The simultaneous correlation of all active nuclei makes ZULF-TOCSY particularly suited for parallel detection.³⁹ The method was demonstrated on isotopically labeled amino acids but can also be applied to molecules at natural abundance and is promising for the analysis of molecules containing NMR active nuclei, such as ¹⁹F, ²⁹Si or ³¹P. An advantage of the method is that the mixing time does not need to be set precisely to highlight specific spin-spin interactions: under ZULF-NMR conditions spin polarization is rapidly distributed over the entire coupled network. We anticipate that the proposed method can be used to assign NMR signals and to identify small molecules in complex mixtures as encountered in chemical synthesis, as well as mixtures of biomolecules and metabolites in biological fluids or lysed cells.

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