# Rapid analysis of isotopically unmodified amino acids by high-resolution $^{14}$N-edited $^{1}$H–$^{13}$C correlation NMR spectroscopy†

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**A $^{14}$N-edited $^{1}$H–$^{13}$C NMR method is described for structural analysis under high-resolution of biomolecules without any enrichment.**

Most of nuclear magnetic resonance (NMR) structural analyses of biomolecules, such as proteins or nucleic acids, are currently performed in solution and are mainly based on the determination of contacts between the three most abundant atoms in these compounds: hydrogen, carbon and nitrogen. However, some biological samples (*e.g.* membrane and fibrous proteins) are not amenable to solution NMR techniques owing to insolubility or unfavorable relaxation properties. In this case, multidimensional solid-state NMR (SS-NMR) provides a good alternative and enables efficient characterization of large protein domains.[1,2] The current SS-NMR methods require the use of $^{13}$C,$^{15}$N-enriched samples. To avoid spectral overlap and gain resolution, it is often desirable to selectively observe $^{1}$H/$^{13}$C signals next to $^{14}$N. However, despite its high isotopic abundance (99.6%), $^{14}$N (spin $I = 1$) NMR spectroscopy has remained a difficult challenge until recently. The main difficulty in observing $^{14}$N arises from the large quadrupole interaction, which results in broad NMR lines for molecules in both liquid and solid states. In solution, the broadening results from very fast quadrupolar relaxation. In solid powder, NMR line-widths of several MHz are observed because of the large first-order quadrupole interaction. The $^{14}$N MAS study of powdered solids has therefore been restricted to samples with quadrupole coupling constants of $C_Q \sim 1$ MHz or less.[3–5] Tycko and Opella,[6] have demonstrated overtone NMR spectroscopy, where spectra are only influenced by the much smaller second-order quadrupole interaction, but the sensitivity is very poor. Recently, renewed interest in $^{14}$N NMR methodology led by Gan[7] as well as Cavadini *et al.*[8] has yielded a method for the indirect detection of $^{14}$N by transferring coherence *via* a combination of scalar *J*-couplings and residual dipolar splittings (RDS). Using these new experiments it is possible to obtain two-dimensional (2D) spectra where $^{14}$N spectra along the indirect dimension display large shifts from isotropic chemical shift positions and relatively small line-widths on the order of a few kHz. These shifts along with the line broadening are mainly the result of second-order quadrupolar interaction. More recently, Gan[9] has shown that the combination (called *D*-HMQC) of dipolar recoupling and HMQC allows the efficient indirect detection of $^{14}$N using heteronuclear dipolar couplings for the coherence transfers. As the dipolar coupling is much larger than both the *J*-coupling and RDS, Gan *et al.* obtained a 2D $^{1}$H–$^{14}$N *D*-HMQC correlation NMR spectrum of an amino acid in just a few minutes.[10] It has also been shown on a L-[U-$^{13}$C] histidine·HCl·$H_2O$ sample, that a 3D $^{1}$H–$^{13}$C–$^{14}$N correlation spectrum could also be obtained using $^{1}$H → $^{13}$C and $^{13}$C → $^{14}$N → $^{13}$C coherence transfers *via* the through-space dipolar interaction.[11] However, the efficiency of $^{13}$C → $^{14}$N → $^{13}$C transfer was very weak (4–5%) and the experiment lasted 85 h on a 18.8 T spectrometer. It was recently pointed out[12] that in case of small or medium sized molecules, peak assignment could be extracted from three 2D spectra instead of a much longer 3D experiment: (i) a $^{1}$H–$^{14}$N *D*-HMQC spectrum, (ii) a $^{1}$H–$^{13}$C CP-HETCOR spectrum and (iii) a $^{14}$N-edited $^{1}$H–$^{13}$C CP-HETCOR spectrum. $^{14}$N edition allows selecting cross-peaks resulting from $^{13}$C nuclei in proximity to a nitrogen atom. A method to obtain $^{14}$N-edited $^{1}$H–$^{13}$C spectra has already been proposed with the Saturation-Pulse Induced Dipolar Exchange with Recoupling (SPIDER).[13] However, SPIDER is mainly suitable for slow spinning rates ($\nu_R \approx$ 5 kHz), which are not sufficient to cancel the large CSA of $^{13}$C nuclei at high-field spectrometers. In ref. 12, the 2D $^{14}$N-edited $^{1}$H–$^{13}$C CP-HETCOR spectrum was obtained from a 2D version of the 3D $^{1}$H–$^{13}$C–$^{14}$N previous experiment that was improved by using a more robust heteronuclear dipolar recoupling[14] and $^{1}$H homonuclear dipolar decoupling.[15] Nonetheless, as stated before, the $^{13}$C–$^{14}$N–$^{13}$C HMQC filter transfer remains only ≈5% efficient and the $^{14}$N-edited $^{1}$H–$^{13}$C spectrum lasted 340 min at 18.8 T for $^{13}$C-labeled histidine. Such a performance is not sufficient to expect 2D spectra in a reasonable amount of time for non-enriched samples. The weak sensitivity of this HMQC-filter experiment is related to the use of the two $^{14}$N hard-pulses (Fig. 1a + b + c), which are too weak to irradiate correctly all atoms due to the large frequency spread (on the order of a few MHz) of powder pattern. Recently, Z. Gan managed to acquire

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† Electronic supplementary information (ESI) available: Fig. 3: L-glutamine. (a) Molecular structure with atom labelling matching IUPAC recommendations.[21] (b, c) CP-HETCOR spectra with: (b) all ($S_0$) or (c) $^{14}$N-edited ($S_0 - S_1$) $^{1}$H–$^{13}$C cross-peaks. $PMLG^{x\bar{x}}_{mm}$ decoupling scheme was applied during $t_1$ with $\nu_{1-1H}$ = 99 kHz and $\tau_p$ = 1.4 μs. $^{1}$H axis has been rescaled. See DOI: 10.1039/b816362f

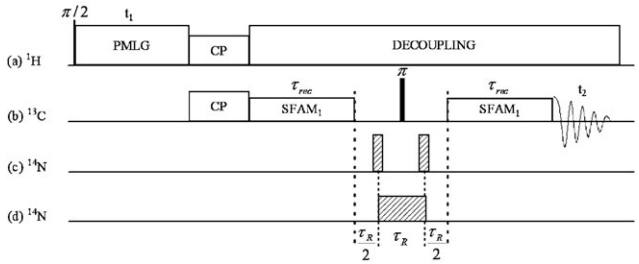


**Fig. 1** Pulse sequences for the $^{1}$H–$^{13}$C heteronuclear correlation NMR experiments implementing $^{14}$N-editing *via* dipolar driven *D*-HMQC sequence (a + b + c) or adiabatic $^{14}$N change of states (a + b + d). The usual $^{1}$H–$^{13}$C CP-HETCOR spectrum is obtained with no $^{14}$N rf irradiation (a + b; $S_0$). In order to enhance $^{1}$H resolution in the indirect dimension, homonuclear $PMLG^{x\bar{x}}_{mm}$ decoupling sequence has been used during $t_1$.[18] Recoupling of $^{13}$C–$^{14}$N dipolar interactions is achieved by $SFAM_1$ irradiations applied on $^{13}$C nuclei.[14,19] The phase cycling consists in varying the phase of the first $^{1}$H $\pi/2$ pulse from $+y$ to $-y$, while the receiver phase is changed from $+x$ to $-x$ concurrently. The phases of CP irradiations, $SFAM_1$ recoupling and $^{13}$C $\pi$ pulse are $+x$, while the phase of $PMLG^{x\bar{x}}_{mm}$ is given in ref. 18.

first-order $^{14}$N quadrupolar wide-line spectra with $^{13}$C detection under MAS using one strong pulse on $^{14}$N.[16] In this experiment, the pair of $^{14}$N short pulses of the HMQC sequence is replaced by a single pulse, which partly prevents the $^{13}$C–$^{14}$N dipolar refocusing. The first-order $^{14}$N quadrupolar spectra are acquired by plotting, in a REAPDOR fashion,[17] the difference of $^{13}$C signal intensities obtained with and without the $^{14}$N pulse. The whole spectra can only be recorded point by point by varying the frequency offset of the $^{14}$N pulse. We use here this pulse to detect carbons that are close to nitrogen atoms (Fig. 1a + b + d). In this case, the $^{14}$N pulse offset can be fixed. For optimal sensitivity, this offset should be in the $^{14}$N spectrum center, but its value is not critical (Fig. 1c in ref. 16). Furthermore, including a $t_1$ evolution period of $^{1}$H coherences allows identifying protons coupled to the selected $^{13}$C nuclei. According to Gan's communication, a $^{14}$N pulse longer than one rotor period can generate a $\nu_R$ modulation of $^{14}$N signal.[16] For $^{14}$N edition, we verified experimentally that the $^{14}$N pulse length does not affect much efficiency.

The 2D spectrum of Fig. 2d was acquired with a pulse duration of one rotor period, but similar results were obtained for other multiples of the rotor period. The $^{14}$N rf amplitude is the most significant parameter of the experiment as it alters the adiabaticity factor, $\nu_{1-14N}/(\nu_R C_Q)^{1/2}$, of this pulse.[16] Its value must be strong enough to introduce significant spin state changes during the very brief level-crossing related to the MAS modulation of the first-order quadrupolar coupling. A saturation occurs for very large rf-fields, but the value of optimal $^{14}$N rf amplitude exceeds most of the time the probe specifications.[16] Therefore, there is no parameter to optimize for the $^{14}$N pulse, as its amplitude has only to be fixed to its maximum accessible value, 50 kHz for our probe. Two $^{1}$H–$^{13}$C 2D experiments must be performed: one with the $^{14}$N irradiation (Fig. 1a + b + d: $S_1$) and the second without (Fig. 1a + b: $S_0$). $S_0$ produces a classical $^{1}$H–$^{13}$C dipolar HETCOR spectrum and $S_0 - S_1$ the $^{14}$N edited one. It should be noted

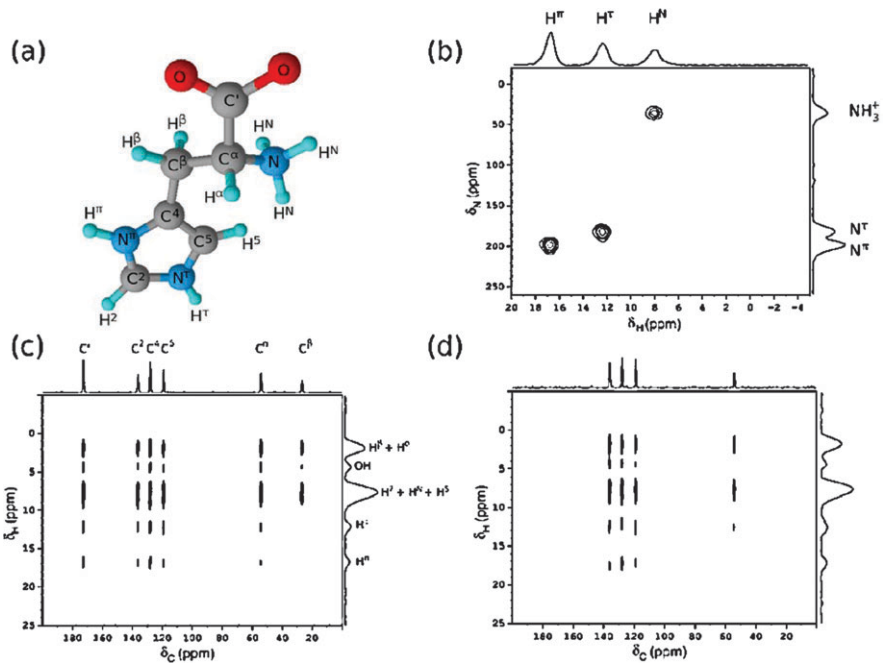


**Fig. 2** L-Histidine·HCl·$H_2O$. (a) Molecular structure with atom labelling matching IUPAC recommendations.[21] (b) 2D $^{1}$H–$^{14}$N *D*-HMQC spectrum.[11] $PMLG^{x\bar{x}}_{mm}$ decoupling scheme[18] was applied during $t_2$ with $\nu_{1-1H}$ = 99 kHz and $\tau_p$ = 1.4 μs. $^{1}$H axis has been rescaled. A $SAM_4$[15] decoupling with $\nu_{1-1H\text{-peak}}$ = 86 kHz was sent on protons during $t_1$ to enhance the $^{14}$N resolution. $B_0$ = 18.8 T, $\nu_R$ = 20 kHz, $\nu_{1-14N}$ = 44 kHz. The experimental time has been of 5 min. (c, d) CP-HETCOR spectra with: (c) all ($S_0$) or (d) $^{14}$N-edited ($S_0 - S_1$) $^{1}$H–$^{13}$C cross-peaks. $PMLG^{x\bar{x}}_{mm}$ decoupling scheme was applied during $t_1$ with $\nu_{1-1H}$ = 99 kHz and $\tau_p$ = 1.4 μs. $^{1}$H axis has been rescaled. Spectra are the result of averaging 112 transients for each of 120 $t_1$ increments with $\Delta t_1$ = 70 μs, with a recycle time of 2 s. The total experimental time for the two spectra has been of 15 h (112 × 120 × 2 × 2 s). The $SFAM_1$ scheme was used for $^{13}$C–$^{14}$N recoupling with $\tau_{rec}$ = 888 μs, $\nu_{1-13C}^{peak}$ = 41 kHz and $\Delta\nu_{0-13C}$ = 20 kHz. $^{1}$H–$^{13}$C CP-MAS: contact time = 2 ms, $\nu_{1-13C}$ = 54 kHz, the power of $^{1}$H is optimized with tangent-ramped shape. $B_0$ = 9.4 T, $\nu_R$ = 13.51 kHz, $\nu_{1-14N}$ = 50 kHz. $^{1}$H decoupling: $\nu_{1-1H}$ = 86 kHz. $^{13}$C $\pi$ and $\pi/2$ pulses: $\nu_{1-13C}$ = 50 kHz.

that this experiment does not require a perfectly adjusted magic angle or a perfectly stabilized spinning speed, as with previous $^{13}C$–$^{14}N$[9] and $^{1}H$–$^{14}N$[10] *D*-HMQC experiments. Indeed, the $^{14}N$ edition does not involve heteronuclear polarization transfer, the efficiency of which is generally weak, but relies on the incomplete refocusing of the $^{13}C$–$^{14}N$ dipolar couplings. The $^{1}H$–$^{13}C$ spectra have been recorded at 9.4 T with a Bruker AVANCE-II console, and a 4 mm rotor. During $t_1$, we have used the $PMLG^{x,\bar{x}}_{mm}$ sequence, which performs well at $\nu_R$ = 13 kHz to achieve a $^{1}H$ high-resolution spectrum,[18] with an rf amplitude of 99 kHz. The $^{13}C$–$^{14}N$ dipolar recoupling sequence must be sent on a spin-1/2 nucleus,[14] and thus on the $^{13}C$ channel in this experiment. We have used the simultaneous frequency and amplitude modulation ($SFAM_1$) recoupling sequence, which is one of the most efficient and robust heteronuclear recoupling method.[14] For $SFAM_1$, the carrier frequency, $\Delta\nu_{0-13C}(t)$, and the amplitude, $\nu_{1-13C}(t)$, of the rf field are modulated co-sinusoidally and sinusoidally, respectively, with a frequency equal to $\nu_R$.[14,19] $SFAM_1$ reintroduces both heteronuclear dipolar coupling and CSA. The two dipolar recoupling periods must be rotor-synchronized (see Fig. 1). The spin–echo segment in the middle of the sequence refocuses the $^{13}C$ CSA interaction. For comparison with the two previous experiments,[11,12] we have used histidine·HCl·$H_2O$, which has three different nitrogen sites all covalently bonded to at least one $^{1}H$ and one $^{13}C$. The ammonium nitrogen is bonded to three protons ($H^N$) and one carbon ($C^\alpha$), while $N^\pi$ and $N^\tau$ nitrogens of imidazole ring are bonded to one proton and two carbons: ($H^\pi$, $C^2$/$C^4$), and ($H^\tau$, $C^2$/$C^5$), respectively (Fig. 2a). We have quantified the efficiency of the $^{13}C$–$^{14}N$ dephasing process related to this pulse. Due to the moderate $^{14}N$ quadrupole interactions ($C_Q \approx 1.1$ MHz), we observed a large efficiency, $(S_0 - S_1)/S_0$, which was equal to 0.80 ($C^2$), 0.40 ($C^4$), 0.60 ($C^5$), and 0.56 ($C^\alpha$), for the four carbons that are connected to a nitrogen atom. The very large efficiency for $C^2$ may be related to the fact this atom is connected to two nitrogen atoms. The sensitivity of the present experiment is *ca.* 4–5 times larger than with the previous[12] *D*-HMQC method. Long adiabatic transfers are much less sensitive to the frequency offset than short hard pulses and this sensitivity gain would thus increase with $C_Q$ values, provided the $^{14}N$ rf field is sufficient. Moreover, it must be noted that we presently obtained both normal ($S_0$ in Fig. 2c) and $^{14}N$-edited ($S_0 - S_1$ in Fig. 2d) 2D spectra instead of only the $^{14}N$-edited one with the method described in ref. 12. $^{1}H$–$^{13}C$ spectra shown in Fig. 2c and d, have been recorded with a long CP contact time to enhance the S/N ratio, and therefore they do not display any selectivity with respect to protons. This selectivity can be obtained with shorter CP contact times, at the expense of experimental time. It is worth to mention that when we ran the experiment on a fully $^{13}C$ enriched histidine·HCl·$H_2O$ sample, S/N ratio was not proportional to the spin abundance, as $^{13}C$–$^{13}C$ couplings broadened peaks and affected $SFAM_1$ dynamics. This problem could have been partly overcome by replacing $SFAM_1$ by $SFAM_2$, which is less sensitive to homonuclear dipolar interactions.[14] We have also recorded the complementary $^{1}H$–$^{14}N$ *D*-HMQC spectrum of this sample. We have used a high-field spectrometer (18.8 T) and a smooth amplitude modulated ($SAM_4$)[15] homo- and hetero-nuclear decoupling on the proton channel during $t_1$, which lead to a very good resolution along $^{14}N$ (Fig. 2b). The three 2D high-resolution spectra allow to assign the resolved $^{1}H$, $^{13}C$ and $^{14}N$ NMR peaks of histidine·HCl·$H_2O$. In particular, the assignment of $^{1}H$ resonances totally agrees with the one performed from high-resolution 2D $^{1}H$ double-quantum spectrum.[20] It must be reminded that the three spectra have been recorded: (i) with an isotopically unmodified sample, (ii) on a moderate field (9.4 T) spectrometer for $^{1}H$–$^{13}C$ spectra, and (iii) in a reasonable time (15 hrs altogether). We have also recorded the $^{14}N$-edited $^{1}H$–$^{13}C$ spectrum of another isotopically unmodified amino acid, L-glutamine, within an experimental time of 25 h on a 9.4 T spectrometer (see ESI†). We believe that this new method can be extended to larger biomolecules, especially by using high-field spectrometers to enhance the $^{1}H$ and $^{13}C$ resolution. It can also be extended by performing another experiment with a $^{14}N$ off-resonance irradiation of *ca.* 1.5 MHz. This second experiment will provide $^{14}N$-edited $^{1}H$–$^{13}C$ CP-HETCOR spectra, where only appear signal of carbon atoms close to $^{14}N$ nuclei submitted to large $C_Q$ values.[16]

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