Spatially Encoded Transfer of ^{1}H polarization to ^{13}C for uniform $^{1}J_{CH}$-Response in HSQC

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Abstract:

Two dimensional (2D) NMR display better resolution than one-dimensional (1D) ^{1}H NMR. However, 2D NMR does not display a straightforward quantitative aspect due to *J*-dependent polarization/coherence transfer. 1D ^{1}H NMR is versatile for quantification; however, it displays significant spectral overlap in biological or organic complex mixtures, which forbids quantification of a large number of signals in 1D ^{1}H NMR. The significant variations in ^{1}H-^{13}C scalar couplings, T_1, T_2, and pulse imperfections are the main problems. Although T_1, T_2 can be suitably chosen to minimize their adverse effect on quantification, the large variations in ^{1}H -^{13}C couplings lead to variations in cross peak intensity, which is more influenced by the amount of polarization transfer rather than the quantity of metabolites or amount of analytes in a complex mixture. In the present work, we show that spatial encoding of the polarization transfer periods can be executed in ^{1}H-^{13}C HSQC using sweep frequency pulses in the presence of a magnetic field gradient. As a result, uniform transfer of polarization from ^{1}H to ^{13}C over a range of ^{1}H -^{13}C couplings can be performed, subsequently improving the quantitative aspect of HSQC or improve the intensity of cross-peaks, which are mistuned in regular HSQC.

Keywords: HSQC; Spatial Encoding

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Introduction:

Investigation of biological complex mixtures or complex organic mixture has gained significant importance in recent years. The study of the biological complex mixture is the focus of metabolomics studies, where quantity of metabolites is correlated to the pathophysiological state of a biological system.[1] The integration values of the resolved 1D ^{1}H NMR signals are directly correlated to the number of ^{1}H spins in the metabolomics or organic mixtures for quantification of metabolites.[2, 3] Signal overlap in 1D ^{1}H NMR hampers assignment as well as quantification.[4] 2D NMR displays a better resolution of signals; however, the quantification aspect of 2D NMR is not straightforward.[5] For instance, 2D ^{1}H -^{13}C HSQC[6] spectra of complex mixtures display much higher resolution; however, quantification using cross peak intensities becomes ambiguous. The cross-peaks display a resonance-specific signal modulation due to the influence of large variations in $^{1}J_{CH}$, relaxation, imperfect pulses, and even ^{1}H-^{1}H *J*-modulations.[7, 8] The site-specific signal correction factors (prior estimated $^{1}J_{CH}$, T_1, T_2)[10] and calibration curves of known concentration of metabolites are used to calibrate 2D peaks intensities, which are time-consuming approaches.[4] Another approach termed as extrapolated time-zero ^{13}C HSQC (HSQC0)[9] can avoid prior determination of correction factors using a series of HSQC spectra recorded with incremented repetition time; this approach also demands recording a series of HSQC spectra. Another approach proposes the utilization of suitably selected 2Δ values (from iterative optimization), particularly four vales of 2Δ (2.94, 2.94, 2.94, and 5.92 ms), to suppress the $^{1}J_{CH}$ dependence to less than 2%, which again requires multiple scans at the top of the existing minimum scans determined by phase cycling.[9] Thus, an approach that does not require multiple scans or multiple HSQC spectra or priory estimated correction factors has not been realized to date. We show that the spatial encoding strategy akin to ultrafast multidimensional NMR[11-15] offers a strategy to impart variable *J*-evolution per scan resulting in a uniform polarization transfer over a range of *J*-values in the ^{1}H -^{13}C HSQC experiment. In ultrafast NMR, sweep frequency pulses and magnetic field gradient are simultaneously applied to encode entire indirect time domain evolutions as a spatially dependent phase or amplitude modulation within a single scan. We show that a spatial encoding of heteronuclear scalar coupling evolution can be performed during the polarization transfer period (INEPT) using two opposite sweep frequency refocusing pulses in the presence of a magnetic field gradient. Thus, a variable *J*-evolution period can be imparted within a single scan so that all $^{1}J_{CH}$ couplings can contribute equally to cross-peaks, which results in quantitative HSQC spectrum similar to the 1D ^{1}H spectrum.

Results and Discussion:

Figure 1A displays the Spatially Encoded Polarization Transfer HSQC (SEPT-HSQC) pulse sequence. In a regular HSQC, the INEPT transfer delay from a to e is tuned to a given $^1J_{CH}$ coupling. For this delay, the antiphase coherence becomes maximum for a given ^{1}H-^{13}C site in a molecule so that maximum polarization transfer takes place for that site via the *J*-antiphase state. However, various sites in a molecule or in a mixture of molecules, in general, have a range of couplings, and hence maximum antiphase coherence cannot be obtained for all sites simultaneously. As a result, the regular HSQC experiment displays weak intensity cross-peaks for the mistuned couplings, which vary a lot from the average tuned couplings. Hence regular HSQC cannot be applied directly for quantitative measurement. In an earlier effort in this direction, the Q-HSQC utilizes a combination of suitable 2Δ values in 4 scans so that all $^1J_{CH}$ couplings are sampled equally. The present work utilizes the concept of spatial encoding. In ultrafast NMR, sweep frequency pulses are used along with gradient to encode the entire indirect time domain spin evolution as amplitude modulation or phase modulation within a single scan. In the present work, we execute a spatially encoded evolution of heteronuclear scalar couplings during the polarization transfer period, as shown in the SEPT-HSQC pulse sequence. The two sweep frequency pulses, P11 and P12, each of duration 1ms applied in the presence of gradient during the INEPT period from time point b to time point d, executes the spatially encoded polarization transfer from ^{1}H to ^{13}C. The parameters are adjusted such that the first slice at z=0 shown in Fig 1B experiences zero net evolution of heteronuclear scalar couplings, and the last slice at z=L experiences maximum evolution of scalar couplings for a given $^1J_{CH}$ value. Thus, a range of heteronuclear *J*-evolution periods can be used in a single scan so that all variations in *J*-couplings are sampled equally in each scan. Initially, Δ_1 needs to be optimized so that maximum evolution occurs for each $^1J_{CH}$ in a range, and a variable INEPT period 1-5 ms per scan was found to execute equal evolution of all $^1J_{CH}$ in the range 120-215 Hz. Due to the sampling of all coupling with a variable INEPT period, the sensitivity of this SEPT-HSQC is less than regular HSQC. Similarly, the reverse INEPT polarization transfer is also spatially encoded so that uniform refocusing occurs over a range of $^1J_{CH}$ values in each scan.

A very long recycle delay ($5T_1$=35 s) and short T_2 (each INEPT period of 5 ms maximum and t_1 acquisition time of 2.5 ms) was chosen to minimize the resonance specific attenuation effect of T_1 and T_2 relaxation. In HSQC, the error in quantification mainly arises from large variations in the magnitude of ^{1}H -^{13}C couplings, which lead to differential transfer for different ^{1}H -^{13}C pair. It can be shown that, for a 2Δ delay of 3.57 ms, the ^{1}H to ^{13}C transfer is 100 percent efficient for a $^1J_{CH}$ coupling of 140 Hz but only 51% efficient for coupling of 209 Hz.

To check this, we took the molecules with considerable variation in ^{1}H-^{13}C scalar couplings 212 Hz to 125 Hz, shown in table 1. We took molecules of $CHCl_3$, DCM, EA in the molar concentration ratio of

1:0.8:1(mixture 1). Therefore the normalized integration ratio for the same group of protons should follow this trend. For instance, if the equal molar concentration of $CHCl_3$ and EA are taken, integration values of CH in $CHCl_3$ and CH_2 and CH_3 in EA are 1:2:3 in proton NMR, which becomes 1:1:1 when normalized for one proton. It is quite well established that ^{1}H NMR integration values provide the most quantitative estimation of different species in metabolomics complex mixtures. Further, we consider that if x represents the integration value from ^{1}H NMR for a given peak, and y represents the integration value from HSQC for that cross-peak, then if a perfect fit to a model y=x, between the integration values for different peaks from the two experiment is obtained, then that HSQC experiment can be considered quantitative. Any deviations from y=x lead to an error in the estimation of concentration from the HSQC experiment. The relative concentrations determined from HSQC cross peak intensities can be converted to absolute concentrations by comparing them to an internal standard, synthesized electronic signal,[2,16] PULCON techniques[7,18] or receiver efficiency or solvent signal.[17] Normally, just one value for 1JCH-coupling is selected to obtain the spectrum, typically optimized for an average 1JCH-coupling of 145 Hz (Δ) 1/(21JCH)) 3.45 ms).

A regular HSQC was recorded with INEPT delay (3.57 ms) tuned to 140 Hz, which is typically used for routine set-up of this experiment-considered an average of the existing natural 1JCH range. When the INEPT delay is tuned to 140 Hz (a value close to CH_2 and methyl group of EA, Table 1), looking into the bar plot in Fig 2A, the intensity of $CHCl_3$ is 0.61 from HSQC while it is 1.02 from ^{1}H NMR integral. From the bar plot in Fig 2A, looking at 1D ^{1}H NMR integral ratios 1:1.6:2:3, the order follows gravimetric concentration ratios ($CHCl_3$:DCM:EA=1:0.8:1) when normalized for one equivalent proton taken. Thus, significant underestimation of $CHCl_3$ concentration takes place (0.61 vs. 1.02) in HSQC, although CH_2 in DCM (1.6 /1.47) and CH_2 in EA (2.0/2.21) and CH_3 (3/3) integration ratios remains close. The regression curve y=1.13x in Fig. 2A also reveals a departure from linearity, which implies CH (from 212 Hz) is underestimated in HSQC. For CH coupling of 212 Hz in $CHCl_3$, the ^{1}H to ^{13}C transfer becomes less efficient for INEPT delay tuned to 140 Hz.

Thus, with an average INEPT transfer delay tuned to 140 Hz ^{1}H-^{13}C coupling, it becomes difficult to have uniform polarization transfer efficiency for each type of CH couplings. Either different delay for different scans or site-specific correction factors for $^1J_{CH}$ couplings, T_1, T_2, or extrapolation to zero time using series of HSQC overcomes this issue, which are time-consuming approaches. A single scan solution to this problem has not been realized to date.

Fig 2B shows the bar plot and regression curve obtained from SEPT-HSQC with variable INEPT delay 1 to 5 ms per scan. The linear regression line does an excellent fit to y=1.02x; a good correspondence between ^{1}H 1D and SEPT-HSQC integrals are obtained for most of the integration values. The bar plot shows the intensity of all peaks in SEPT-HSQC remains close to ^{1}H NMR. In regular HSQC tuned to 140

Hz in Fig. 2A, bar plots in red colour, we see the ratios are 0.61:1.47:2.21 for $CH(CHCl_3)$:$CH_2(DCM)$:$CH_2(EA)$, which deviates a lot from 1D ^1H integral ratios (1.02:1.6:2). The bar plots from SEPT-HSQC in 2B the integration ratios 0.83:1.7:2.06 becomes much closer to that from 1H 1D i.e. 1.02:1.6:2. Thus, even the large coupling (212 Hz in $CHCl_3$), which is quite far from 140 Hz, does a better transfer in SEPT HSQC, 0.83 compared to 0.61 in regular HSQC, in addition to pushing the two CH_2 groups integration close to respective ^1H 1D NMR values. The small variations are due to J_{HH} evolutions, T_2, and diffusion losses.

In conclusion, we have demonstrated that spatial encoding in NMR improves the uniform transfer of ^1H polarization to ^{13}C over a wide range of $^1J_{CH}$ values, which leads to better quantitative values for the cross peak integrals.

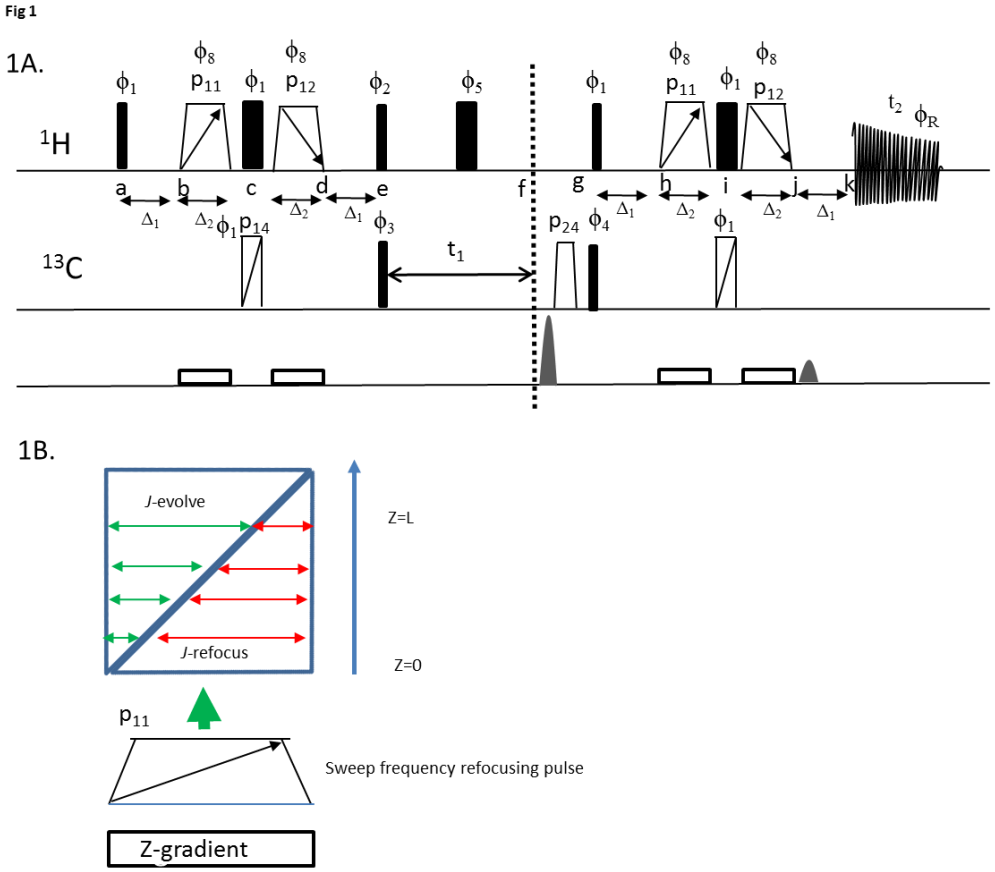

Fig. 1 The spatially encoded INEPT HSQC (SEPT-HSQC) is shown in Fig. 1 and, B is the visualization of the nature of encoding of heteronuclear couplings executed by the chirp refocusing pulses sweeping in opposite directions in the NMR sample of length L. The pulse element for spatial encoding of heteronuclear scalar couplings is implemented during the INEPT transfer period a to e in Fig 1A. The two trapezoids with diagonal arrows (P11 and P12) are two oppositely sweeping refocusing chirp pulses simultaneously applied with 10% z-gradient, which execute spatially encoded evolution of heteronuclear couplings. These two sweep frequency pulses are separated by the two simultaneously applied hard π pulses on ^1H and ^{13}C.

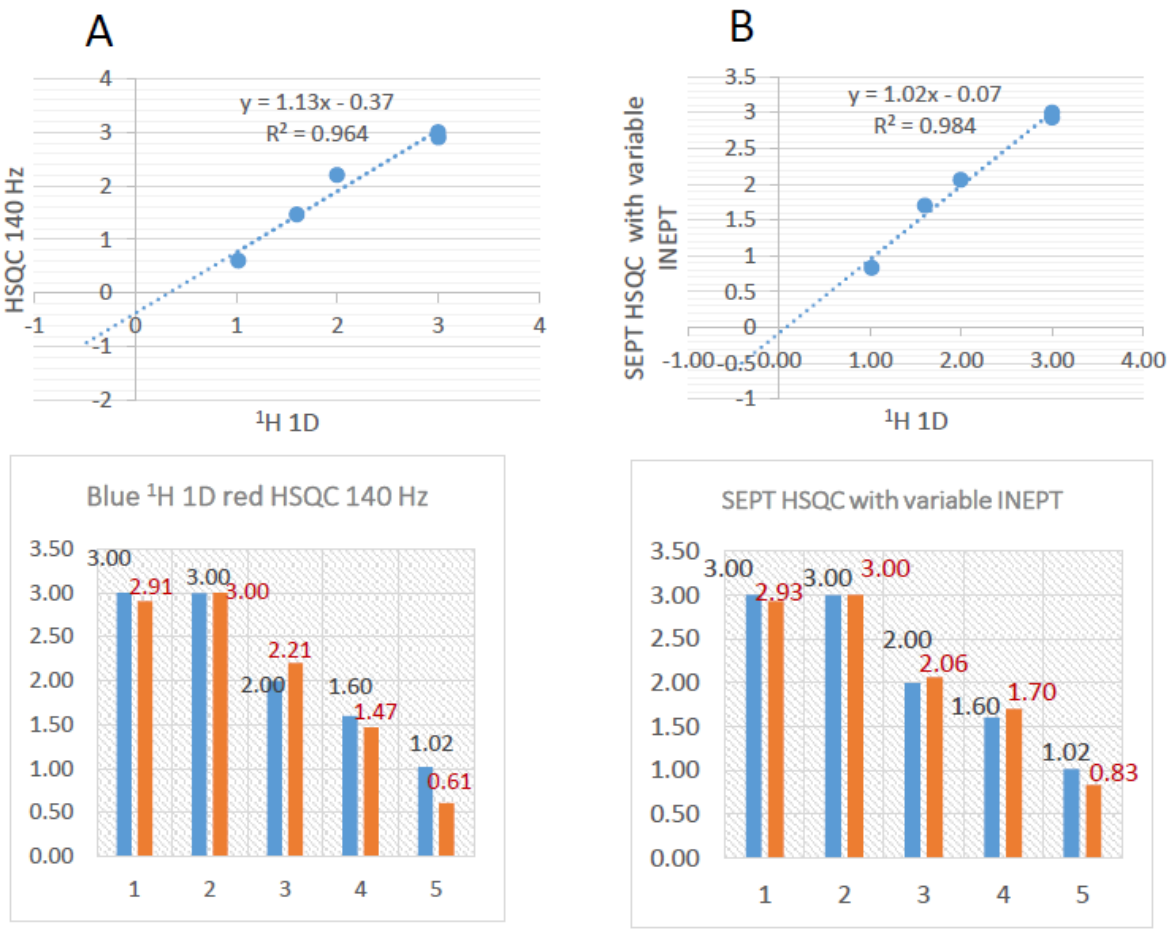

Fig. 2. A regression curve obtained from regular HSQC tuned to 140 Hz when plotted relative to the 1D ^{1}H NMR integrals. The methyl peaks in ^{1}H 1D and HSQC are normalized to 3, and subsequent integrals in HSQC and ^{1}H 1D are plotted. Fig B shows the regression curve obtained from SEPT-HSQC (with variable INEPT 1 to 5 ms per scan) vs. ^{1}H 1D. The best regression curve is obtained for SEPT-HSQC with variable INEPT period 1 to 5 ms per scan. The bar plots comparing the integrals of HSQC and ^{1}H 1D are also shown in each case. Numbers 1, 2, 3, 4, and 5 are CH_3(t, EA), CH_3(s EA), CH_2(q EA) CH_2(DCM), CH($CHCl_3$) respectively. More detail is provided in the main text. In the 1D 1H NMR bar plot (blue bars), the integration ratios are 1.02:1.6:2 for CH($CHCl_3$):CH_2(DCM):CH_2(EA), which is close to gravimetric concentration ratios taken 1:0.8:1 when normalized for one equivalent proton. In contrast, in regular HSQC tuned to 140 Hz in 2A, bar plots in the red colour we see the ratios are 0.61:1.47:2.21 for CH($CHCl_3$):CH_2(DCM):CH_2(EA), which are different from 1D ^{1}H integral ratios (1.02:1.6:2). In the bar plots from SEPT-HSQC in 2B, the integration ratios 0.83:1.7:2.06 become much closer to that from 1H 1D, i.e., 1.02:1.6:2. Thus, even the large coupling (212 Hz in $CHCl_3$), which is quite far from 140 Hz, does a better transfer in SEPT HSQC 0.83 compared to 0.61 in regular HSQC in addition to improving the two CH_2 groups integration.

Table 1. Molecules used for preparing the mixture in Fig. 2 and their $^{1}J_{CH}$ coupling variations

Molecule	Group	$^{1}J_{CH}$ (in Hz)
Ethyl Acetate (EA)	CH_3(singlet)	129
	CH_3(triplet)	127
	CH_2(quartet)	146
Dichloromethane (DCM)	CH_2(singlet)	177
Chloroform	CH (singlet)	209
57ul of $CHCl_3$, 32ul of CH_2Cl_2, and 100ul of EA were taken for preparing a stock solution, and 80ul was transferred to an NMR tube. $CDCl_3$ was chosen as the solvent.		

Methods:

All molecules used for preparing the mixture for which regression curves are shown in Fig 2 are displayed in Table 1. The mixture of $CHCl_3$, DCM, ethyl acetate (EA) was prepared from a stock solution followed by dilution as necessary for different sample types. All spectra are from 800 MHz NMR spectrometers (using CPTCI cryoprobe). For all experiments, acquisition and processing parameters, and optimization of adiabatic sweep frequency, ^{1}H pulses are given in Table 2.

Table 2: Experimental parameters

	TD(F_2/F_1)	DS/NS	SW(ppm)	AQ(F_2/F_1)	RG	O1P	D_1	SI(F_2/F_1)	WDW	LB(F_2/F_1)	
Fig 2 All 1H NMR (in each panel A-D)	98862	4/4	20	3s	7.12	6.175	35s	131072	EM	0.3	
Fig 2 All HSQC on the mixture in table 1	2812/70	8/2	8/80	220 ms/2.17ms	144	3.6/42	35	4k/1k	QSINE	1/0.3	SSB 2/2
The ^{13}C refocusing pulses during INEPT transfer P14 were 500 us long adiabatic shaped pulse Crp80,0.5,20.1. The ^{13}C refocusing pulse at the end of indirect dimensions was 2ms long adiabatic pulse Crp80comp.4. The two oppositely sweeping adiabatic π chirp pulses P11 and P12 were of duration 1ms each, P11 sweeping low to high, and P12 sweeping high to low were optimized for 10% gradient. Size of shape:1000, Total sweep-Width 42000 Hz, % to be smoothed 20%, Q=5											

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