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Real-time bilinear rotation decoupling in absorptive mode *J*-spectroscopy: Detecting low-intensity metabolite peak close to high-intensity metabolite peak with convenience

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ABSTRACT

"Pure shift" NMR spectra display singlet peak per chemical site. Thus, high resolution is offered at the cost of valuable *J*-coupling information. In the present work, real-time BIRD (BIlinear Rotation Decoupling) is applied to the absorptive-mode 2D *J*-spectroscopy to provide pure shift spectrum in the direct dimension and *J*-coupling information in the indirect dimension. Quite often in metabolomics, proton NMR spectra from complex bio-fluids display tremendous signal overlap. Although conventional *J*-spectroscopy in principle overcomes this problem by separating the multiplet information from chemical shift information, however, only magnitude mode of the experiment is practical, sacrificing much of the potential high resolution that could be achieved. Few *J*-spectroscopy methods have been reported so far that produce high-resolution pure shift spectrum along with *J*-coupling information for crowded spectral regions. In the present work, high-quality *J*-resolved spectrum from important metabolomic mixture such as tissue extract from rat cortex is demonstrated. Many low-intensity metabolite peaks which are obscured by the broad dispersive tails from high-intensity metabolite peaks in regular magnitude mode *J*-spectrum can be clearly identified in real-time BIRD *J*-resolved spectrum. The general practice of removing such spectral overlap is tedious and time-consuming as it involves repeated sample preparation to change the pH of the tissue extract sample and subsequent spectra recording.

1. Introduction

Enhancing sensitivity and resolution are two fundamental objectives in the area of NMR spectroscopy. The homonuclear scalar coupling between protons lowers resolution by splitting the signals into multiplets. The limited proton chemical shift dispersion along with the presence of *J*-multiplets leads to crowded spectra which often complicate analysis and assignment of ^{1}H signals. Spectral overlap forbids measurement of *J*-couplings essential for the determination of molecular structure and conformation. Particularly in metabolomics, which often relies on one-dimensional proton spectrum for identification and quantification of metabolites in complex bio-fluid, spectral congestion significantly hampers assignment. Spectral overlap can severely affect metabolomic analysis when biomarker signals are overlapped by the multiplets from other high abundant metabolites. Although, regular magnitude mode *J*-resolved spectroscopy partially alleviate this problem by dispersing the multiplet information in a second dimension, the dispersive phase twisted lineshape prevents absorption mode recording of the spectrum, thus, sacrificing much of the potential high resolution that could be achieved.

In the last few years, "pure shift" NMR has emerged as a successful method for producing singlet peak per chemical site, achieving substantial improvement in resolution [1–22]. Numerous pure shift methods and their applications have emerged based on BIRD [9–15], Zangger and Sterk (ZS) pulse-sequence elements [1–6], selective methods such as BASH (band-selective homonuclear) or HOBS (homonuclear band-selective) [8,17,22], and PSYCHE (Pure shift yielded by chirp excitation) [23,24]. Real-time pure shift methods are very interesting as they refocus the effect of *J*-couplings during data acquisition imparting great improvement in sensitivity per unit of measurement time [6,7,10,11,17,19]. Broadband approaches such as, BIRD, and ZS pulse-sequence elements, and band selective methods such as BASH or HOBS have been implemented in real-time [8,17,22] or directly detected dimension (F_2). PSYCHE [20,21] offers order of magnitude higher sensitivity than ZS or BIRD but is applicable only in indirect (F_1) dimensions of multidimensional approach. The BIRD scheme achieves refocusing of ^{1}H–^{1}H *J*-couplings by exploiting the large one bond heteronuclear coupling

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constants in sparse heteronuclei. As a result, the method is not applicable to uniformly ^{13}C-enriched samples. Real-time application of BIRD in HSQC leads to great improvement in sensitivity and resolution [11]. Refocusing of *J*-coupling in ZS scheme relies on spectrally selective pulses in the presence of field gradients. The major limitation of ZS is severe loss of sensitivity when coupled spins are closer in frequency space as it demands highly selective pulses to refocus *J*-evolutions. Highly selective pulses also extend the acquisition time in real-time mode resulting in line broadening and other artifacts [6].

The real time BASH/HOBS methods involve band-selective refocusing of *J*-coupling by repeated application of a band-selective and a nonselective 180° pulse pair during interrupted data acquisition [17,22]. The BASH/HOBS method has found applications in 2D NOESY, TOCSY, and ^{1}H–^{15}N HSQC experiments. BASH decoupling improves both sensitivity and resolution in a band selective manner in contrast to ZS and BIRD which achieve high resolution in a broadband manner at the cost of sensitivity.

The key limitation of pure shift spectrum is the lack of valuable *J*-coupling information. Although *J*-multiplets are important, their presence further complicate crowded 1D proton spectrum as they increase the ambiguity in signal assignment. 2D *J*-resolved NMR [23] is the only simple approach that produces pure shift spectrum in F_2 while keeping the *J*-coupling information along F_1 intact. It allows precise measurement of *J*-couplings, as the spin echo removes the static field inhomogeneity along the F_1 dimension. However, regular *J*-resolved spectroscopy displays poor resolution and severely distorted intensities in crowded spectral region as only absolute value mode of the experiment is reliable. Presence of broad dispersive lineshape prevents absorption mode *J*-resolved spectroscopy. Spectral overlap is further amplified in complex mixtures as the strong and broad dispersive tails from high abundant species obscure the peaks from low abundant species. Several approaches have been proposed for producing absorption mode 2D *J*-spectra. These include modified pulse sequences [24–26], combined data manipulation and pattern recognition [25], and purely data post-processing [27]. All these approaches have not found widespread use as either lower sensitivity or complicated post processing hampers their efficiency.

Absorption mode multiplet can be obtained in adiabatic *z*-filtered *J*-spectroscopy which utilizes a *z*-filter to eliminate the dispersive antiphase peaks [28,29]. However, this method produces full multiplet in F_2 requiring multiplet reduction algorithm to produce pure shift spectrum. Recently, we have replaced these multiplets by singlets in F_2 by applying real-time BASH decoupling improving resolution and sensitivity of the technique in a band selective manner [30]. Triple Spin Echo (TSE) PSYCHE 2D *J*-sequence is another approach reported very recently to produce absorption mode *J*-spectrum with pure shift spectrum in F_2 and *J*-coupling information along F_1 [31]. The method is based on Pell and Keeler method [24] and utilizes PSYCHE *J*-refocusing pulse element to reverse the sense of t_1 evolution in 2D *J*-spectroscopy. The method records two separate *J*-resolved experiments with t_1 evolution reversed in one. The two experiments when added up cancel out the dispersive contribution. If, strong coupling is present, the method needs many chirp pulses in the presence of long gradients to get cleaner spectrum. This results in loss of signal intensity, however, for small molecules this effect is not significant in magnitude. Higher sensitivity of TSE PSYCHE *J*-sequence is a great advantage.

In the present work, we report a sequence which is simple in design and implementation – the original *z*-filtered *J*-resolved sequence with real-time refocusing of *J*-modulations by BIRD produces broadband pure shift spectrum in F_2 with *J*-couplings in F_1 in a single experiment. The elegant BIRD sequence does not need any optimization. High-quality spectrum from complex metabolomic mixture such as tissue extract from rat cortex is reported. Application to amino acid mixture is also demonstrated.

2. Description of the pulse sequences

The BIRD pulse sequence element selectively inverts ^{12}C bound protons leaving ^{13}C bound proton unchanged. As a result, sensitivity of BIRD based pure shift spectrum is 1.1% of a regular proton spectrum. However, this signal intensity is enhanced by a factor of two or more due to collapse of the multiplets to singlets. The BIRD method offers a number of unique advantages such as – accurate inversion of passive spins, more compatible with real-time application as the decoupling pulse element is shorter in duration (6–10 ms depending upon ^{13}C or ^{15}N), no need of optimizing the BIRD block for improving decoupling performance in closely resonating spins. However, BIRD based method can suffer from strong coupling effect because the splitting of each directly bonded CH proton signal into two satellite resonances doubles the chances of partial degeneracy with a coupled proton. Contrary to this, those cases where the resonance of a CH proton is strongly coupled when it is attached to a ^{12}C, there is a good chance that it will become weakly coupled when it is directly attached to a ^{13}C. Thus, if a ZS spectrum shows strong coupling effect, the corresponding BIRD spectrum will often be clean. Several methods exist in literature such as CLIP/CLAP HSQC, and double spin echo methods that can eliminate the complications arising from strong coupling [32,33]. Another drawback of BIRD is that geminal *J*-coupling between diastereotopic protons is not refocused although coupling to third partner is refocused. Perfect BIRD and constant time BIRD were developed to address this problem which can achieve full broadband decoupling even in the case of diastereotopic methylene protons [34,12]. The lower sensitivity of BIRD method demands a large number of scans. However, the time spent in acquiring these scans can be utilized wisely in a 2D *J*-resolved sequence with BIRD implemented during data acquisition. This will produce the desired broadband pure shift spectrum in F_2 with the precious *J*-coupling information along F_1.

The pulse sequence for real-time BIRD 2D *J*-resolved spectroscopy is displayed in Fig. 1. This is achieved by the INEPT transfer [35,36] ['*c*' to '*e*'] to ^{13}C, back transfer to ^{1}H ['*f*' to '*g*'], and refocusing to in-phase proton magnetization at time point '*h*' where t_1 evolution starts. From time point '*h*' to '*i*' is the spin echo block of *J*-resolved pulse sequence. At the end of the t_1 period, the in-phase proton magnetization is stored as population by the 90° pulse between time points '*i*' and '*j*'. The dispersive antiphase terms are eliminated by the *z*-filter between time point '*j*' and '*k*'. The in-phase ^{1}H magnetization stored as population is brought back by the 90° pulse between time points '*k*' and '*l*'. In general, these in-phase terms produce the absorption mode *J*-spectrum but full multiplet is retained in F_2 reducing resolution. To convert these broad multiplets into narrow singlets, BIRD is applied during data acquisition to refocus *J*-couplings which also improves sensitivity. Therefore, the signal is acquired after time point '*m*' and the FID is interrupted by implementation of BIRD blocks between time points '*n*' and '*o*' and also between '*q*' and '*r*'. The 180° ^{13}C pulses during BIRD are replaced by composite ^{13}C pulses for more accurate inversion. ^{13}C decoupling is performed during signal acquisition but switched off during application of BIRD blocks. The whole acquisition block from time point '*m*' to '*t*' is repeated n times to cover the whole acquisition time. The initial double INEPT sequence aids in suppressing unwanted proton signals attached to ^{12}C.

3. Experimental section

All the one and two-dimensional experiments reported in this paper were carried out on a BRUKER AVANCE III 800 MHz NMR