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Simultaneous Characterization of a Mixture of Fluorochemicals Using Three-Dimensional $^{19}F-^{1}H$ Heteronuclear TOCSY Filtered/Edited NMR Experiments

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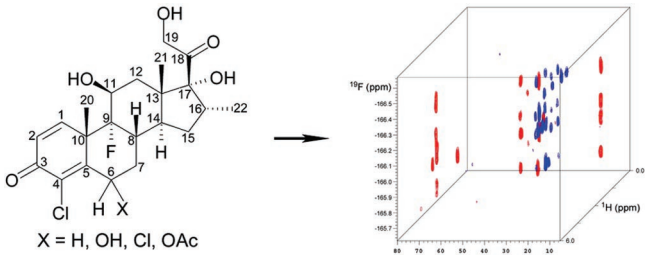

Heteronuclear $^{19}F-^{1}H$ cross-polarization can be used effectively as a tool for both spectral filtering and editing in the NMR analysis of the increasing number of fluorine-containing compounds encountered in drug discovery. Combined with LC−MS, three-dimensional $^{19}F-^{1}H$ heteronuclear TOCSY filtered experiments based on this approach have enabled the simultaneous identification of a mixture of closely related dexamethasone derivatives without the need for isolation.

The profiling of drug metabolites plays an important role in various aspects of pharmaceutical research. However, the analysis of drug molecules by NMR in the presence of complex biological matrices is often plagued by severe resonance overlap in the ^{1}H spectrum. Fortunately, an increasing number of molecules encountered in drug discovery also contain fluorine. In such cases, fluorine-19 NMR provides an attractive alternative. This is due to both the lack of endogenous fluorine-containing molecules in biomatrices and the high intrinsic sensitivity of fluorine-19, a spin-half with 100% natural abundance and a gyromagnetic ratio only slightly smaller than that of proton. In addition, the fluorine-19 chemical shift is highly sensitive to even subtle changes in the magnetic environment, making it an ideal probe for distinguishing closely related drug metabolites.

Clearly, however, the fluorine-19 chemical shift alone is far from sufficient for determining the structures of the metabolites. To this end, much more detailed spectral information will be essential. A large number of NMR experiments correlating fluorine to proton and/or carbon can be found in the literature.[1] Ultimately, however, knowledge of proton−proton and proton−carbon correlations will still be needed because of the high abundance of these nuclei. Such information can be achieved in part through specifically designed ^{19}F-filtered NMR experiments, such as those introduced by Castellino and Krishnamurthy.[2] In their approach, a refocused X-half-filter[3] is incorporated into the preparation period such that only those protons directly coupled to fluorine-19 will be retained, which are then subjected to appropriate manipulations to produce the desired information contents. Herein we demonstrate that significant improvement can be made using $^{19}F-^{1}H$ heteronuclear TOCSY[4] as an effective tool for both spectral filtering and editing in the profiling of fluorochemicals.

The timing diagrams of the pulse sequences proposed in this paper are shown in Figure 1. In our new approach, magnetization is first transferred from fluorine to protons within the same scalar coupling network, through heteronuclear cross-polarization in the transverse plane.[4] In the next step, the proton magnetization is manipulated accordingly to generate the specific spectral information of interest, such as proton−proton and proton−carbon correlations for the examples shown here.

Several features of the pulse sequences illustrated in Figure 1 are noteworthy. The initial step of $^{19}F-^{1}H$ cross-polarization serves as both a preparation and a filtration period, since it warrants that all subsequent magnetization must originate from the fluorine. Native proton magnetization not following this pathway is suppressed by the simple pulse train $-G_1-90°-G_2-90°-G_3-90°-G_4-$ as well as phase cycling. The sensitivity of these experiments can be enhanced considerably through the heteronuclear Overhauser effect by applying broad-band ^{1}H decoupling during the relaxation delay d_1. Highly efficient cross-polarization transfer can be achieved using popular mixing sequences for homonuclear TOCSY, such as DIPISI-2.[5] The short (~2 ms) trim pulses applied along the y-axis (labeled SL_y) are necessary to maintain the phase uniformity. The $^{19}F-^{1}H$ TOCSY filtered $^{1}H-^{1}H$ TOCSY pulse sequence of Figure 1a incorporates an efficient zero-quantum suppression technique proposed by Thrippleton and Keeler[6] into the mixing period for the homonuclear TOCSY to minimize distortions to the lineshapes. The $^{19}F-^{1}H$ TOCSY filtered, multiplicity edited $^{1}H-^{13}C$ CRISIS-HSQC sequence[7] of Figure 1b utilizes the broad-band inversion pulses (BIPs) developed by Shaka and co-workers[8] on both proton and carbon channels[9] to minimize sensitivity loss due to the extensive use of inversion pulses. Broad-band ^{19}F decoupling during acquisition is optional.

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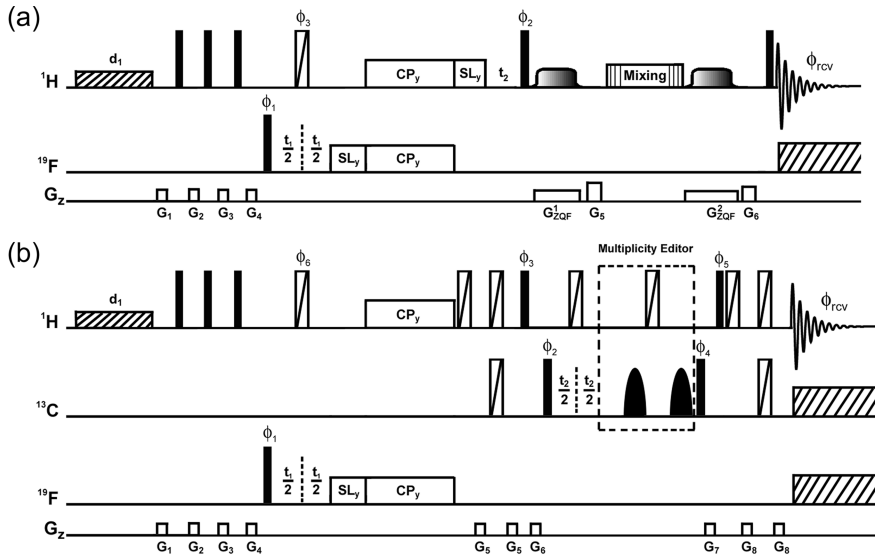

FIGURE 1. Timing diagrams of the pulse sequences used in this work. Hard 90° pulses are represented by solid bars and BIPs by scored icons. Frequency-swept inversion pulses used for zero-quantum suppression are indicated by the shaded icons and adiabatic inversion pulses used in the CRISIS-type multiplicity editor (indicated by the dashed box) by solid icons. Broad-band decoupling is designated by the hatched patterns. All pulses have phase *x* unless noted otherwise. All gradient pulses were applied along the *z*-axis without shaping. All shaped pulses other than the BIPs were generated using the program Pandora's Box available as part of the Varian NMR software. Quadrature detection in the F_1 and F_2 dimensions is achieved by phase shifting ϕ_1 and ϕ_2, respectively, together with ϕ_{rcv} using the States-TPPI method. (a) 3D ^{19}F−^{1}H heteronuclear TOCSY edited ^{1}H−^{1}H TOCSY. The basic phase cycling is $= x, -x$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(-x)$; and $\phi_{rcv} = x, -x, -x, x$. (b) 3D ^{19}F−^{1}H heteronuclear TOCSY edited, multiplicity edited ^{1}H−^{13}C CRISIS-HSQC. The appropriate phase cycling is $\phi_1 = x, -x$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(y), 4(-y)$; $\phi_4 = 8(x), 8(-x)$; $\phi_5 = 16(x), 16(-x)$; $\phi_6 = 32(x), 32(-x)$; and $\phi_{rcv} = \phi_1 + \phi_2 + \phi_3 + \phi_4 + \phi_5$.

The ^{19}F−^{1}H cross-polarization preparation offers several major advantages over the refocused X-half-filter approach.[2] First, the X-half-filter works well only if the heteronuclear coupling constant under consideration is known and much larger than the homonuclear coupling constants involved, e.g., the one-bond ^{1}H−^{15}N coupling in a nitrogen-15-enriched protein molecule vs the ^{1}H−^{1}H homonuclear couplings. In the case of ^{1}H−^{19}F couplings, these conditions are rarely met: usually no prior knowledge is available on the magnitudes of the ^{1}H−^{19}F coupling constants, and the ^{1}H−^{1}H couplings are often on the same order as the ^{1}H−^{19}F couplings, whose evolution during the X-half-filter can lead to severe phase distortions as well as signal loss. To complicate things further, the proton of interest can be coupled to multiple ^{19}F spins with drastically different coupling constants. As a result, it is often impossible to obtain pure in-phase magnetization and thus the highly desirable pure-absorptive lineshape using the refocused X-half-filter. In such cases the final spectrum has to be displayed in absolute value mode, where all useful information contained in the phase is lost. In contrast, our cross-polarization-based approach always generates pure in-phase magnetization and hence the highly desirable pure-absorptive lineshape regardless of the relative sizes of the ^{19}F−^{1}H and ^{1}H−^{1}H coupling constants, thus requiring no prior knowledge of the coupling network and preserving valuable information contained in the phase.

Second, in the X-half-filter approach, only a very small number of protons directly coupled to the fluorine are selectively retained. To extend the information content, an additional magnetization transfer step, often a homonuclear ^{1}H−^{1}H TOCSY,[10] is required. In our approach, this has become redundant, since both heteronuclear and homonuclear TOCSY transfer take place *simultaneously* during the cross-polarization sequence, resulting in much more extensive magnetization transfer and hence the obtainable information content. If desired, the degree of magnetization transfer can also be fine-tuned by altering the duration of the heteronuclear cross-polarization sequence according to specific experimental needs.

Moreover, with our method, an evolution period (t_1 in Figure 1) can be inserted immediately following the 90° ^{19}F excitation pulse, such that the ^{19}F spin can be frequency-labeled to allow spectral editing based on the ^{19}F chemical shifts of the analytes. Proton decoupling during t_1 is accomplished by applying a 180° proton pulse at the center of this evolution period. With the X-half-filter approach, expansion into 3D can be achieved by extending the X-half-filter into an HMQC sequence. However, the evolution of homonuclear proton−proton couplings during the t_1 period will both erode the resolution and introduce a phase error in the F_1 dimension.[11] The addition of a third dimension labeled with the ^{19}F chemical shifts enables the simultaneous identification of multiple fluorochemicals in a mixture without the need for separation, as demonstrated below.

One limitation the proposed method shares with the X-half-filter approach is its relatively low sensitivity, because all detectable magnetization has to originate often from a single fluorine nucleus. This can be particularly true for the ^{19}F−^{1}H TOCSY filtered, multiplicity edited ^{1}H−^{13}C CRISIS-HSQC experiment, where an extensive 64-step phase cycle has been used without unnecessarily lengthening the acquisition time. If practical, however, the phase cycle can be reduced to eight steps by eliminating the cycling of ϕ_4, ϕ_5, and ϕ_6.

The pulse sequences proposed here were tested on a mixture of four closely related dexamethasone derivatives (**2**−**5**) mim-

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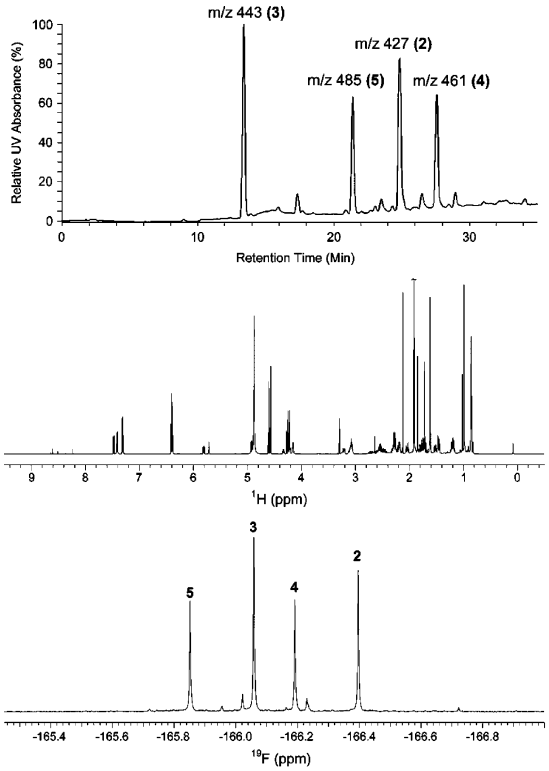
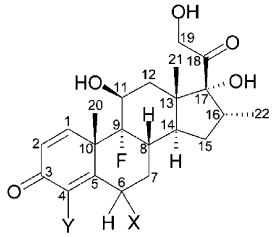


FIGURE 2. LC−UV trace (top panel), 1D ^{1}H NMR spectrum (middle panel), and 1D broad-band ^{1}H-decoupled ^{19}F NMR spectrum (bottom panel) of a mixture of four closely related dexamethasone derivatives (**2**−**5**).

CHART 1



1, X = H, Y = H; **2**, X = H, Y = Cl;
3, X = OH, Y = Cl; **4**, X = Cl, Y = Cl;
5, X = OAc, Y = Cl.

icking that of mammalian metabolites of dexamethasone (**1**) (Chart 1). The four derivatives **2**−**5** were prepared by reacting **1** with commercial bleach. For reference purposes, each component was isolated from the reaction mixture by HPLC and individually characterized by HRMS and NMR, before they were remixed together. The 1D ^{1}H and 1D broad-band ^{1}H-decoupled ^{19}F NMR spectra of the mixture, together with the LC−UV trace, are shown in Figure 2. The 1D ^{19}F spectrum, which contains essentially one peak for each species, is in stark contrast to the proton spectrum, where severe resonance overlap is observed throughout. In the presence of biological matrices, the complexity of the ^{1}H spectrum would increase exponentially, while little change is anticipated for the ^{19}F spectrum.

Figure 3 shows the 3D ^{19}F−^{1}H heteronuclear TOCSY edited ^{1}H−^{1}H TOCSY and the 3D ^{19}F−^{1}H heteronuclear TOCSY edited, multiplicity edited ^{1}H−^{13}C HSQC spectra of the mixture recorded with the pulse sequences of Figure 1. In the former experiment, a short mixing time (∼25 ms) was used for the

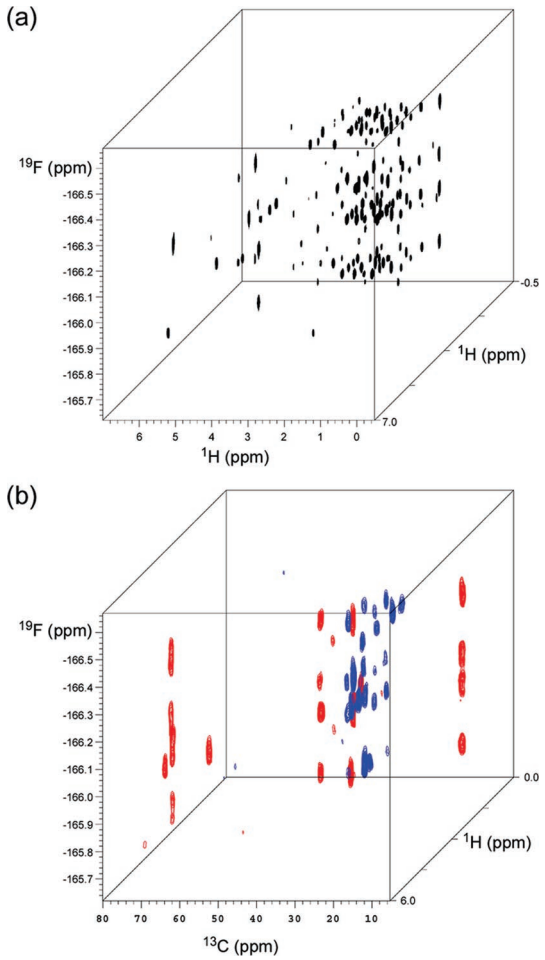


FIGURE 3. 3D ^{19}F−^{1}H heteronuclear TOCSY edited spectra of a mixture of four dexamethasone derivatives (**2**−**5**). Both spectra are displayed in phase-sensitive mode for all three dimensions. (a) 3D ^{19}F−^{1}H heteronuclear TOCSY edited ^{1}H−^{1}H TOCSY. (b) 3D ^{19}F−^{1}H heteronuclear TOCSY edited, multiplicity edited ^{1}H−^{13}C HSQC. Positive and negative contours are plotted in red and blue, respectively.

homonuclear TOCSY to limit the extent of magnetization transfer largely to directly coupled spins. In the latter spectrum, the highly valuable proton multiplicity information is explicitly defined by the phase of the cross-peaks; i.e., cross-peaks corresponding to methine and methyl groups are positive (plotted in red), and those corresponding to methylene groups are negative (plotted in blue). This is made possible by the excellent phase properties of the cross-polarization scheme, which always generates pure in-phase magnetization transfer and avoids phase distortions due to the evolution of homonuclear proton−proton couplings. Such information would be completely lost in the X-half-filter approach because of its unpredictable phase behavior.

To illustrate how these experiments, together with the MS data, can be used for identifying the structure of each individual component in the mixture, selected planes from the 3D spectra corresponding to $\delta(^{19}\mathrm{F}) = -166.06$ ppm are shown in Figure 4. Note the excellent quality of the homonuclear TOCSY spectrum (Figure 4a), which is almost completely free of artifacts. Most of the proton resonances can be assigned unequivocally on the basis of this spectrum and by referencing the parent compound **1**, except for H12′ and H14, which are perfectly overlapped with each other (indicated by the arrows

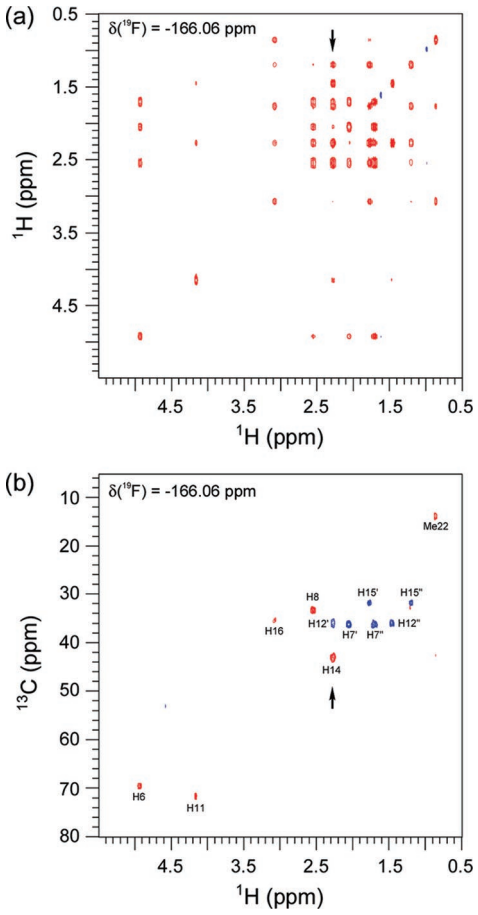


FIGURE 4. Selected 2D planes from the 3D spectra of Figure 3 corresponding to $\delta(^{19}F) = -166.06$ ppm. (a) 2D $^{1}H-^{1}H$ TOCSY plane. (b) 2D multiplicity edited $^{1}H-^{13}C$ HSQC plane.

in Figure 4). Such ambiguity is easily eliminated in the HSQC spectrum of Figure 4b, since the two carbon atoms C12 and C14 have distinct chemical shifts and proton multiplicities.

The HSQC spectrum also proves indispensable in determining the substituent at position 6. The unsubstituted 4-chlorodexamethasone (**2**; MF $C_{22}H_{28}O_5FCl$) should be readily distinguishable from the other three because of its different proton multiplicity at C6, which is reflected in the phase of the corresponding cross-peak in the multiplicity edited HSQC spectrum. In the example shown here, the chemical shift of C6 (69.5 ppm) is highly suggestive of substitution by a hydroxyl or acetoxy group but not chlorine, which is expected to give a more upfield chemical shift (50−55 ppm). The proton chemical shift of H6 (4.93 ppm) led us to assign this component to 4-chloro-6-hydroxydexamethasone (**3**; MF $C_{22}H_{28}O_6FCl$) instead of 4-chloro-6-acetoxydexamethasone (**5**; MF $C_{24}H_{30}O_7FCl$), which has a very similar ^{13}C chemical shift at C6 (70.2 ppm) but a considerably more downfield-shifted ^{1}H chemical shift for H6 (5.81 ppm). The fourth component is therefore assigned to 4,6-dichlorodexamethasone (**4**; MF $C_{22}H_{27}O_5FCl_2$), which indeed exhibits a ^{13}C chemical shift of 53.8 ppm at C6.

In summary, we have demonstrated that $^{19}F-^{1}H$ cross-polarization can be used successfully as a tool for both spectral filtering and editing in the analysis of fluorine-containing compounds by NMR. This strategy provides significant improvement over the X-half-filter method previously proposed. Three-dimensional $^{19}F-^{1}H$ heteronuclear TOCSY filtered/edited experiments based on this approach, combined with LC−MS data, allow simultaneous identification of a mixture of fluorochemicals even in the presence of complex biological matrices, without the need for physical separation. We expect these experiments to find widespread applications in the characterization of fluorine-containing drug molecules and metabolites.

Experimental Section

Preparation of Dexamethasone Derivatives. To an ice-cold solution of dexamethasone (76 mg) in glacial acetic acid (1.5 mL) was added dropwise 5.25% commercial sodium hypochlorite solution (1 mL). After the mixture was stirred for 1 h at ice-cold conditions, an additional aliquot of sodium hypochlorite solution (0.2 mL) was added, and the mixture was stirred for an additional 1 h. Following this, the reaction mixture was quenched with ice-cold saturated sodium bisulfite solution (1 mL), diluted with ice-cold water (15 mL), and extracted with ethyl acetate (20 mL). The ethyl acetate layer was washed with saturated sodium bicarbonate (20 mL) followed by water (20 mL) and dried over anhydrous magnesium sulfate. Solvent was removed, and the resulting extract was purified by reversed-phase chromatography using a 10 mM aqueous ammonium acetate and methanol gradient to yield dexamethasone derivatives **2**−**5**. The isolated yields were not determined. The structures of **2**−**5** were determined by the analysis of MS and conventional NMR data.

Data for dexamethasone derivative 2: HRESIMS *m/z* 427.1696 $(M + H)^+$ (calcd for $C_{22}H_{29}O_5FCl$, Δ + 0.8 mmu).

Data for dexamethasone derivative 3: HRESIMS *m/z* 443.1670 $(M + H)^+$ (calcd for $C_{22}H_{29}O_6FCl$, Δ + 3.3 mmu).

Data for dexamethasone derivative 4: HRESIMS *m/z* 461.1306 $(M + H)^+$ (calcd for $C_{22}H_{28}O_5FCl_2$, Δ + 0.8 mmu).

Data for dexamethasone derivative 5: HRESIMS *m/z* 485.1760 $(M + H)^+$ (calcd for $C_{24}H_{31}O_7FCl$, Δ + 1.8 mmu).

NMR Spectroscopy. All NMR spectra were recorded at 25.0 °C on a 600 MHz spectrometer equipped with a 3 mm $^{1}H-^{19}F\{^{1}H-^{19}F/^{13}C\}$ PFG triple-resonance probe and *z*-gradient accessory. The mixing time for the heteronuclear cross-polarization period was 116 ms using a 5 kHz DIPSI-2 sequence[5] for both 3D experiments.

For the 3D $^{1}H-^{19}F$ heteronuclear TOCSY edited $^{1}H-^{1}H$ TOCSY, a 25 ms mixing time with a 5 kHz DISPI-2 sequence was used for the homonuclear isotropic mixing. Each FID was acquired with 16 scans and 1024 complex points. The numbers of increments were 16 and 100 in F_1 and F_2, respectively. The resulting dataset was linear-predicted to 64 points in F_1 and 200 points in F_2 and subsequently zero-filled to generate a data matrix of the size 128 × 512 × 1024. Appropriate Gaussian window functions were applied in each dimension before Fourier transformation of the final dataset. The total acquisition time was 60 h.

For the 3D $^{1}H-^{19}F$ heteronuclear TOCSY edited $^{1}H-^{13}C$ HSQC, each FID was acquired with 128 transients and 1024 complex points. The numbers of increments were 16 and 32 for F_1 and F_2, respectively. The resulting dataset was linear-predicted to 32 points in F_1 and 128 points in F_2 and subsequently zero-filled to generate a data matrix of the size 64 × 256 × 1024. Appropriate Gaussian apodization was applied in each dimension before Fourier transformation of the final dataset. The total acquisition time was 100 h.

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