Double Nuclear Coherence Transfer (DONUT)-HYSCORE: A New Tool for the Assignment of Nuclear Frequencies in Pulsed EPR Experiments

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Abstract: A two-dimensional experiment, termed DONUT-HYSCORE (*do*uble *nu*clear coherence *t*ransfer *hy*perfine *s*ublevel *cor*relation) designed to obtain correlations between nuclear frequencies belonging to the same electron spin manifold is presented. The sequence employed is $\pi/2-\tau_1-\pi/2-t_1-\pi-\tau_2-\pi-t_2-\pi/2-\tau_1-$echo, and the echo is measured as a function of t_1 and t_2 whereas τ_1 and τ_2 are held constant. It is complementary to the standard HYSCORE experiment which generates correlations between nuclear frequencies belonging to different M_S manifolds and is particularly useful for ^{14}N nuclei. The experiment is first demonstrated on a single crystal of copper-doped *l*-histidine hydrochloride monohydrate where the modulations are induced by a single ^{14}N nucleus, the remote nitrogen in the imidazole group. HYSCORE and DONUT-HYSCORE experiments were carried out on two crystal orientations. In the first, one Cu^{2+} site contributes to the echo and all six nuclear frequencies together with the expected correlation were observed. In the second, 12 frequencies corresponding to two Cu^{2+} ions at different crystallographic sites appeared and all expected correlations were detected as well. This rather trivial example demonstrates that the DONUT-HYSCORE pulse sequence indeed generates correlations within the M_S manifolds. The value of the DONUT-HYSCORE experiment is demonstrated on a frozen solution of a vanadyl complex with a bis-hydroxamate ion binder (VO-RL515). The modulations in this complex arise from the two ^{14}N nuclei in the hydroxamate groups, and orientation-selective three-pulse ESEEM (*e*lectron *s*pin−*e*cho *e*nvelope *m*odulation) spectra showed a number of well-resolved peaks. An unambiguous assignment of all peaks and their orientation dependences could not be achieved through HYSCORE alone because at certain orientations frequencies of one of the M_S manifolds were absent or overlapped with those of the other manifold. The application of the DONUT-HYSCORE experiment provided new correlations that led to the complete assignment of the ESEEM frequencies, thus paving the way for future systematic spectral simulations for the determination of the best-fit Hamiltonian parameters. This example shows that, in the case that the HYSCORE experiment cannot distinguish between two sets of frequencies belonging to the same M_S manifold in different centers (or orientations) because signals from the other manifold are missing or overlapping, the DONUT-HYSCORE becomes most valuable.

Introduction

Electron spin−echo envelope modulation (ESEEM) spectroscopy is a well-established method for measuring weak hyperfine interactions in solids.[1−3] It is currently routinely applied to investigate both orientationally ordered and disordered systems of interest in chemistry, physics, and biology. A number of pulse sequences can generate ESEEM waveforms, the most common of which are the two-, three-, and four-pulse experiments[4−6] where a series of pulses creates an echo and the echo intensity is typically measured as a function of one of the time intervals, yielding a modulated echo decay. Fourier transformation of the decay provides the ESEEM frequencies which correspond to nuclear transition frequencies of weakly coupled nuclei and their combinations. The ESEEM spectrum of a spin system with an electron spin $S = {}^1/_2$ coupled to one nucleus with spin $I = {}^1/_2$, such as ^{1}H or ^{15}N, consists of only two basic frequencies, ν^α and ν^β, one for each M_S manifold. For a nuclear spin $I = 1$ with a substantial nuclear quadrupole interaction, such as ^{14}N, there are six ESEEM frequencies, three in each M_S manifold, as shown in Figure 1. Two of the frequencies, $\nu_{sq1}^{\alpha,\beta}$ and $\nu_{sq2}^{\alpha,\beta}$, correspond to single-quantum transitions ($\Delta M_I = \pm1$), whereas the third, $\nu_{dq}^{\alpha,\beta}$, is related to the double-quantum transition ($\Delta M_I = \pm2$). Combination frequencies of the type ($\nu_i^\alpha \pm \nu_j^\beta$) are usually generated by the two- and four-pulse sequences,[4,6] but they are absent in the three-pulse experiment.[5] However, when several nuclei contribute

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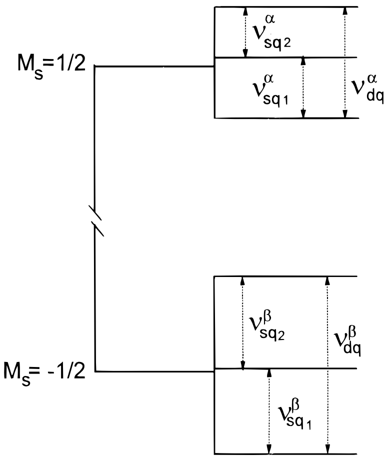

Figure 1. Schematic energy level diagram for an electron spin $S = {}^1/_2$ coupled to a nucleus with spin $I = 1$.

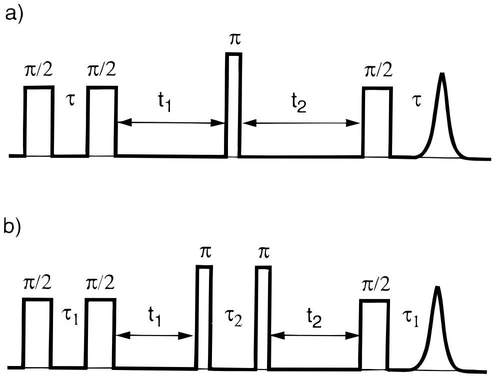

Figure 2. (a) HYSCORE pulse sequence and (b) DONUT-HYSCORE pulse sequence.

to the modulation pattern, combination frequencies of the types $(\nu_i^{\alpha}(k) \pm \nu_j^{\alpha}(l))$ and $(\nu_i^{\beta}(k) \pm \nu_j^{\beta}(l))$, where the indices k, and l represent the different nuclei, may appear in all three experiments, provided that the modulation depth is substantial.[4,7,8] The appearance of the latter is a very important feature of the ESEEM experiment because it provides information on the number of magnetically equivalent nuclei.[9]

The assignment of the ESEEM frequencies is often not straightforward, particularly when several nuclei with $I > {}^1/_2$ are involved and both basic frequencies and their combinations are observed. A major improvement in the resolution of the ESEEM frequencies and in their assignment has been achieved with the introduction of the two-dimensional (2D) *hy*perfine *su*blevel *cor*relation (HYSCORE) experiment.[10] In the HYSCORE experiment, shown in Figure 2a, the echo intensity is measured as a function of t_1 and t_2 and a 2D Fourier transformation yields a 2D spectrum with cross-peaks of the type $(\nu_i^{\alpha}, \nu_j^{\beta})$, (and $(\nu_j^{\beta}, \nu_i^{\alpha})$), which correlate ESEEM frequencies of *different* M_S manifolds. Accordingly, in the case of one $I = 1$ nucleus, a total of 18 cross-peaks are expected.[11] Additional assistance in peak assignment can be obtained from an experiment which leads to correlations of nuclear frequencies *within* each M_S manifold, rather than between the different M_S manifolds.

Recently, the five-pulse experiment, $\pi/2-\tau_1-\pi/2-t_1-\pi-\tau_2-\pi-t_2-\pi/2-\tau_1-$echo, shown in Figure 2b, with variable times t_1 and t_2, has been introduced to measure primary nuclear spin−echoes and their phase memory times.[12] A variant of this sequence with the variable times t_1 and $t_2 = \tau_2$, used to detect sum cross-peaks of the type $(\nu^{\alpha}, \nu^{\alpha} + \nu^{\beta})$ and $(\nu^{\beta}, \nu^{\alpha} + \nu^{\beta})$, was applied as well.[13] The original five-pulse sequence with the coherence mixer $(\pi-\tau_2-\pi)$ can also be used to correlate nuclear frequencies belonging to the *same* M_S manifold. Such a *do*uble *nu*clear coherence *t*ransfer (DONUT) is manifested as cross-peaks in a 2D spectrum obtained by Fourier transformation with respect to t_1 and t_2. In this work we demonstrate that this approach, termed DONUT-HYSCORE, can successfully be applied to unravel and assign nuclear frequencies spectra in systems with ^{14}N nuclei in both a single crystal and a frozen solution.

Two examples are presented. The first is a single crystal where all ESEEM frequencies are well resolved and all expected correlations appear in both the HYSCORE and DONUT-HYSCORE spectrum. In such cases the information provided by the DONUT-HYSCORE is redundant. The second example is more complicated and involves a frozen solution of a vanadyl complex. It represent typical problems encountered in peak assignment in orientationally disordered systems due to low resolution and demonstrates the effectiveness of the HYSCORE/DONUT-HYSCORE combination.

The single crystal used was Cu-doped *l*-histidine hydrochloride monohydrate (Cu-*l*-His·HCl·H_2O), where the Cu(II) is directly coordinated to the amine nitrogen of one histidine molecule, to the N_{ϵ} of the imidazole group of a second molecule, and to the carbonyl of a third molecule, the other ligands are two chloride ions and a water molecule.[14] The modulations observed are due to the remote nitrogen, N_{δ}, of the imidazole group.[11,15] In this crystal six ESEEM frequencies are observed for each crystallographic site. The two directly coordinated nitrogens have large hyperfine couplings that can be observed by ENDOR spectroscopy.[16] Orientation-dependent ESEEM and HYSCORE measurements were reported for this system, and the hyperfine and quadrupole tensors of the $^{14}N_{\delta}$ were determined.[11,15] The nitrogen hyperfine coupling is relatively small ($A_{xx} = 1.274$ MHz, $A_{yy} = 1.061$ MHz, $A_{zz} = 1.741$ MHz) and close to the values of the remote nitrogen in histidine residues in copper proteins.[17−20] At X-band frequencies (~9 GHz) this hyperfine coupling falls within a range that fulfills the cancellation condition, which is met when the hyperfine coupling is about twice the nuclear Larmor frequency ($|A| \approx 2\nu_I$), thus leading to a zero effective field in one of the electron spin

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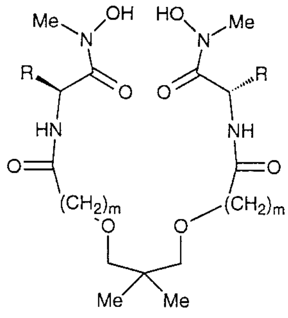

RL515 (R=Me, m=2)

Figure 3. Schematic representation of VO-RL515.

manifolds.[21,22] Throughout this work, the M_S manifold with the lower effective field will be referred to as the low effective field manifold (LEF) and the other as the high effective field manifold (HEF). At exact cancellation, the ESEEM frequencies in the LEF manifold correspond to the nuclear quadrupole resonance (NQR) frequencies $\nu_{sq1} = \nu_{-}$, $\nu_{sq2} = \nu_0$, and $\nu_{dq} = \nu_{+}$ and exhibit no orientation dependence.[21,22] Therefore, orientationally disordered systems, such as frozen solutions, often exhibit relatively highly resolved spectra.

The orientationally disordered system chosen is a frozen methanol solution of a complex of VO^{2+} with the bis-hydroxamate ion binder RL515 shown in Figure 3. This family of ion binders has been shown to have Insulineomimetic properties.[23] In this system the modulations are caused by two nitrogens in the hydroxamate group.[24] A series of VO^{2+} and Cu^{2+} analogous complexes have previously been studied by orientation-selective ESEEM and HYSCORE, showing that also in these systems the hyperfine couplings of the ^{14}N fall within the cancellation condition at ∼9 GHz.[24,25] Unlike the previous ESEEM spectra, the three-pulse ESEEM measurements of this analogue showed a larger number of highly resolved peaks and difficulties were encountered in their assignments.[26] It will be demonstrated that the application of the DONUT-HYSCORE experiment provided new correlations which were paramount for the assignment of the observed frequencies.

Experimental Section

Spectroscopic Measurements. The two-dimensional pulse EPR measurements were performed on a Bruker ESP 380E spectrometer operating at 9.7 GHz. Echo-detected (ED) EPR spectra were obtained with the two-pulse echo sequence, $\pi/2-\tau-\pi-\tau-$echo, where the echo intensity is recorded as a function of the magnetic field, while τ is held constant. HYSCORE spectra were recorded using the sequence shown in Figure 2a, and the unwanted echoes were removed using an eight-step phase cycle.[6] The lengths of the $\pi/2$ pulses were 48 and 24 ns for the Cu-*l*-His·HCl·H_2O and VO-RL515 measurements, respectively, and that of the π pulse was 16 ns for both samples. The dwell time in the t_1 and t_2 dimensions was 32 ns for Cu-*l*-His·HCl·H_2O and 24 ns for VO-RL515. The initial value of t_1 and t_2 was 0.296 μs for the Cu-*l*-His·HCl·H_2O measurements, and 48 ns for VO-RL515. The size of the data matrix was 500 × 500 for Cu-*l*-His·HCl·H_2O and 250 × 250 for VO-RL515. The same conditions were used for the DONUT-HYSCORE experiment, shown in Figure 2b. All of the measurements of the single crystal were carried out at 10 K with a repetition rate of 500 Hz. Those of VO-RL515 were done at 30 K with a repetition rate of 1 kHz.

The 2D data were treated with the Bruker WINEPR software. The background decay was removed using a polynomial or linear fit, and single-crystal data were convoluted with a sin-bell function, whereas a Hamming window was used for the frozen solution data. In all cases the spectra are presented in the absolute-value mode.

The orientation-selective ESEEM spectra were recorded at 10 K on a home-built pulse EPR spectrometer operating at ∼9 GHz.[27] The three-pulse sequence, $\pi/2-\tau-\pi/2-T-\pi/2-\tau-$echo, was employed with a four-step phase cycle to remove the unwanted echoes.[28] Prior to Fourier transformation, the missing data points due to spectrometer dead time ($\tau + 60$ ns) were reconstructed using linear prediction[29] and the spectra are presented in the absorption mode. Continuous-wave EPR spectra were recorded on a Varian E-12 spectrometer.

Sample Preparation. *l*-His·HCl·H_2O was purchased from Merck, and single crystals of Cu-doped *l*-His·HCl·H_2O were obtained as reported in the literature.[30] The bis-hydroxamate ion binder synthesis and the preparation of the VO^{2+} complex were as described earlier.[24] The solvent used was methanol, and the final concentrations of VO^{2+} and the ligand were 2.75 and 3.45 mM, respectively.

Results

The DONUT-HYSCORE Pulse Sequence. The DONUT-HYSCORE sequence is shown in Figure 2b. The first two $\pi/2$ pulses, separated by the time interval τ_1 generate nuclear coherences, whereas the first π pulse mixes the nuclear coherences of the two different M_S manifolds, as in the HYSCORE sequence. Then, after a free evolution of time τ_2, a second π pulse, that mixes again the nuclear coherences of the two M_S manifolds, is applied. This double nuclear coherence transfer causes nuclear spins evolving during the t_1 period with one of the nuclear frequencies of the α (or β) manifold, to evolve with either the same or any other nuclear frequency within the *same* manifold during the t_2 period. The last $\pi/2$ pulse transfers the nuclear coherences into electron coherences that are refocused to a stimulated echo after τ_1. The two-dimensional spectrum is obtained by a 2D Fourier transformation of the echo recorded as a function of the time intervals t_1 and t_2 with constant values for τ_1 and τ_2. The phase cycle used to eliminate unwanted echoes is the same as the one described earlier.[13] A detailed analytical description of the DONUT-HYSCORE approach will be presented elsewhere.

Single Crystal of Cu-*l*-His·HCl·H$_2$O. The HYSCORE spectrum recorded at a magnetic field of 364.1 mT, where only one crystallographic site contributes to the echo, is presented in Figure 4a. Each frequency in one manifold is correlated with all three frequencies of the other manifold, and vice versa, and cross-peaks appear in both (+, +) and (−, +) quadrants as expected for the case of intermediate coupling.[6,31,32−33] Table 1 lists the six observed ESEEM frequencies and their assign-

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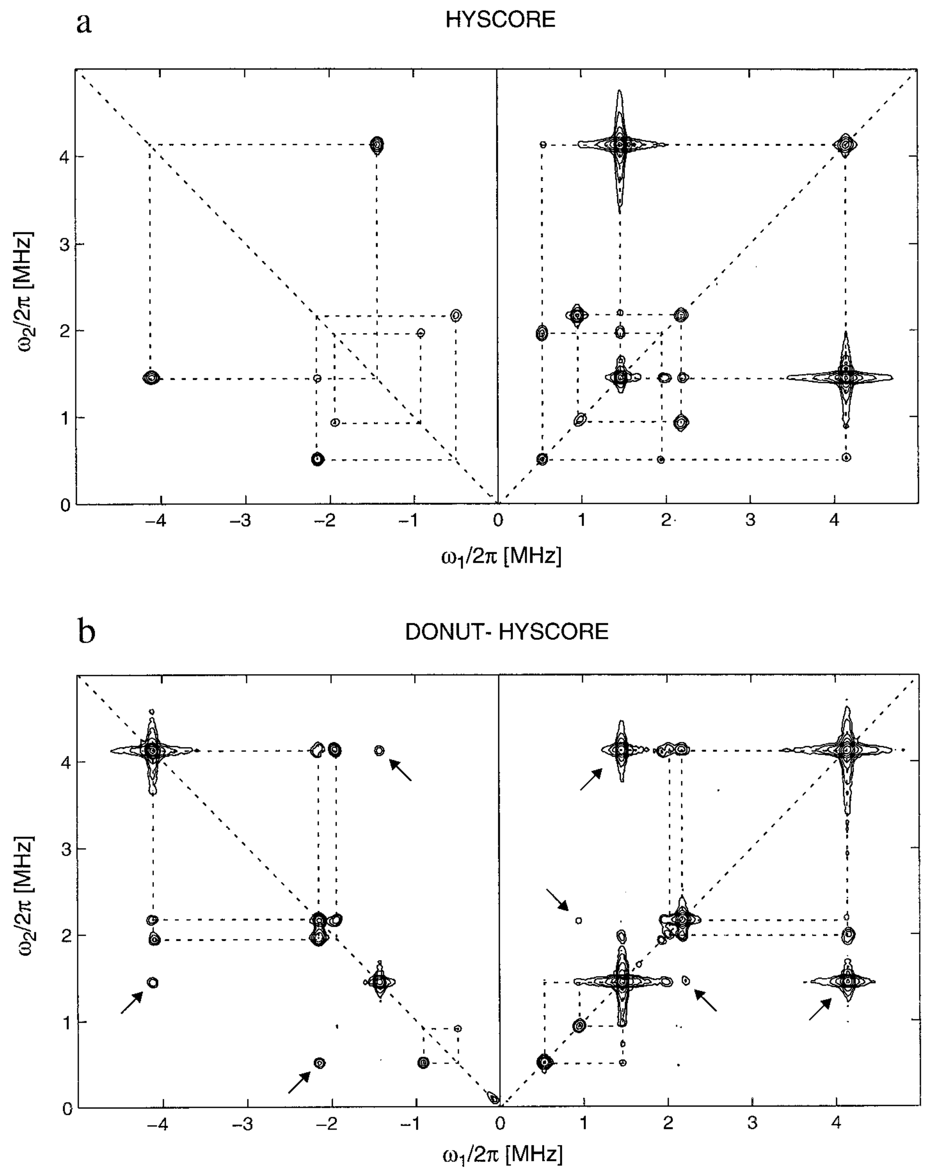

Figure 4. (a) HYSCORE and (b) DONUT-HYSCORE spectra of a single crystal of Cu-*l*-His•HCl•H_2O (arbitrary crystal orientation, observer field $B_0 = 364.1$ mT, and temperature 10 K). The HYSCORE spectrum was obtained with $\tau = 0.328$ μs and the DONUT-HYSCORE spectrum with $\tau_1 = 0.328$ μs and $\tau_2 = 0.400$ μs. Arrows in the DONUT-HYSCORE spectrum mark residual HYSCORE cross-peaks.

Table 1. ESEEM Frequencies of a Single Crystal of Cu-*l*-His•HCl•H_2O as Determined from the HYSCORE and DONUT-HYSCORE Spectra Shown in Figure 4

manifold	label	$\omega/2\pi$ (±0.03 MHz)	manifold	label	$\omega/2\pi$ (±0.03 MHz)
α^a	1	0.52	β	1′	1.96
	2	0.93		2′	2.18
	3	1.45		3′	4.14

[a] Assuming $a_{iso} > 0$.

ments as obtained from the HYSCORE spectrum. Both the HYSCORE and DONUT-HYSCORE have cross-peaks at symmetric positions with respect to the diagonal, namely peaks appear at (ν_i, ν_j) as well as at (ν_j, ν_i). Therefore, for the sake of brevity, only one of the pairs will be considered throughout this work.

The DONUT-HYSCORE spectrum, recorded under the same conditions, is shown in Figure 4b. It shows two types of cross-peaks, those corresponding to correlations within the α and β manifolds (two for each frequency, connectivities are shown with dashed lines) and a number of peaks marked with arrows, which are attributed to residual HYSCORE peaks. These peaks are caused by the nonideal behavior of the two mixing π pulses.[12] The intensity of residual HYSCORE peaks can be minimized by using π pulses considerably shorter than the $\pi/2$ pulses. While the intensities of the diagonal peaks are low in the HYSCORE spectrum, as expected, the DONUT-HYSCORE spectrum exhibits rather intense peaks on the diagonal of the (−, +) quadrant. These peaks are due to nuclear coherences that dephase during time t_1 and refocus during time t_2 on the same nuclear transition, i.e., $\nu_i^{\alpha} \rightarrow \nu_x^{\beta} \rightarrow \nu_{\mathrm{i}}^{\alpha}$.

Another example of a DONUT-HYSCORE spectrum is given in Figure 5, where the crystal was oriented such that two the crystallographic sites contribute to the echo. Since in the HYSCORE and DONUT-HYSCORE spectra cross-peaks occur

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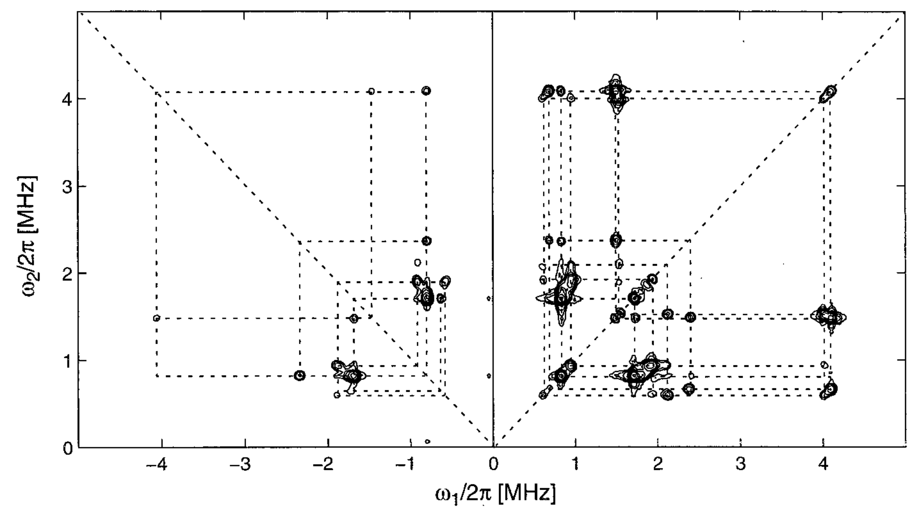
a HYSCORE



b DONUT- HYSCORE

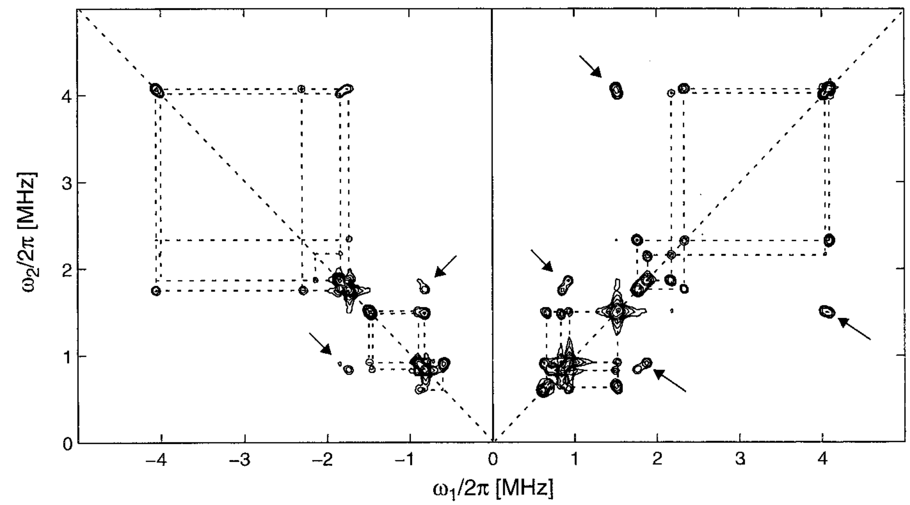


Figure 5. (a) HYSCORE and (b) DONUT-HYSCORE spectra of a single crystal of Cu-*l*-His·HCl·H_2O [arbitrary crystal orientation, different from Figure 4, observer field $B_0 = 364.1$ mT, and temperature 10 K]. The HYSCORE spectrum was obtained with $\tau = 0.328$ μs, and for the DONUT-HYSCORE spectrum, $\tau_1 = 0.328$ μs and $\tau_2 = 0.400$ μs. Arrows in the DONUT-HYSCORE spectrum mark residual HYSCORE cross-peaks.

Table 2. ESEEM Frequencies of the Two Cu(II) Sites in a Single Crystal of Cu-*l*-His·HCl·H_2O as Determined from the HYSCORE and DONUT-HYSCORE Spectra Shown in Figure 5

manifold		$\omega/2\pi$ (±0.03 MHz)	manifold	label	$\omega/2\pi$ (±0.03 MHz)
α[a] site a	1a	0.59	α, site b	1b	0.66
	2a	0.94		2b	0.82
	3a	1.53		3b	1.48
β, site a	1a′	1.86	*β*, site b	1b′	1.78
	2a′	2.14		2b′	2.32
	3a′	4.00		3b′	4.10

[a] Assuming $a_{\mathrm{iso}} > 0$.

only between frequencies of the same center, the six ESEEM frequencies of each site could be easily assigned as listed in Table 2.

Frozen Solution of VO-RL515. The low-temperature EPR spectrum of VO-RL515, presented in Figure 6, is a characteristic powder pattern of a system with anisotropic *g* and ^{51}V ($I = {}^{7}/_{2}$) hyperfine interactions. It shows that one VO^{2+} complex was present. The EPR parameters extracted from simulations were g_{xx} 1.985, $g_{yy} = 1.976$, $g_{zz} = 1.951$, $|A_{xx}(^{51}\mathrm{V})| = 49 \times 10^{-4}$ cm^{-1}, $|A_{yy}(^{51}\mathrm{V})| = 38.7 \times 10^{-4}$ cm^{-1}, and $|A_{zz}(^{51}\mathrm{V})| = 146.8 \times 10^{-4}$ cm^{-1}.[26] Owing to the large anisotropic character of the EPR spectrum of VO^{2+} and the limited bandwidths of the microwave pulses, ESEEM experiments carried out at different

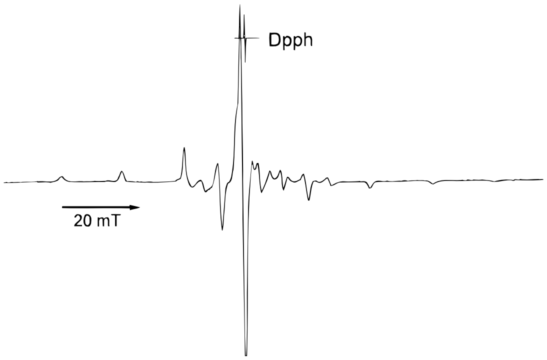


Figure 6. EPR spectrum of VO-RL515 recorded at 140 K and a spectrometer frequency of 9.32 GHz.

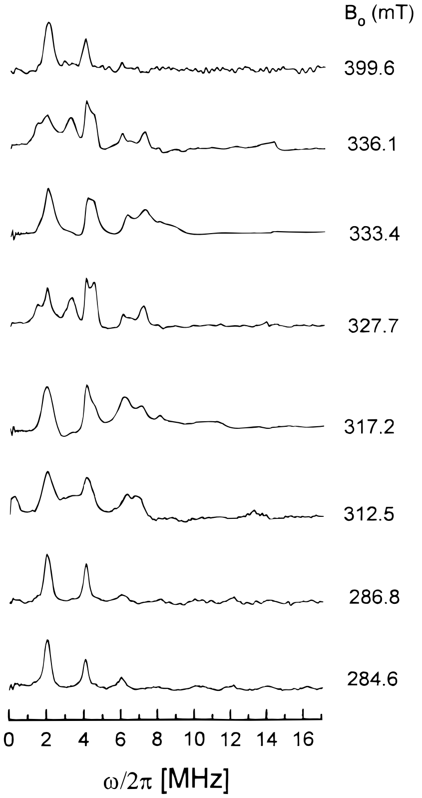


Figure 7. Orientation-selective three-pulse ESEEM spectra of VO-RL515 (temperature 10 K, $\tau = 0.200$ μs, and spectrometer frequency of $\nu_{MW} = 9.34$ GHz). The spectra recorded at 284.6 and 336.1 mT were obtained with $\tau = 0.22$ μs. The length of the $\pi/2$ pulse was 20 ns.

fields result in the selection of complexes with specific orientations.[34] Figure 7 shows a set of three-pulse ESEEM spectra recorded at different magnetic field positions within the EPR spectrum, commonly referred to as orientation-selective ESEEM spectra. Three important field settings within this series are at 284.6, 399.6, and 333.4 mT. The first two correspond to "quasi-single-crystal" positions ($A_{\|}$(^{51}V) for the $M_I = \pm^7/_2$), where complexes within a narrow range of orientations ($\theta_0 \sim 0-12°$, $\phi_0 = 0-180°$) are selected. At the "isotropic" position, 333.4 mT, all possible values of θ_0 and ϕ_0 ($M_I = -^1/_2$) contribute to the echo. The modulation frequencies observed in Figure 7 are attributed to the two nitrogens of the hydroxamate groups in the binding site.[24] In addition, peaks due to weakly coupled protons appear at the ^{1}H Larmor frequency. These are of no interest in this work and will not be discussed any further.

The ESEEM spectra recorded at 284.6 and 399.1 mT exhibit three rather narrow lines at 2.0, 4.0, and 6.0 MHz. Although the peaks at 2.0 and 4.0 MHz are apparent throughout the whole field range, they broaden and additional features around 1.5, 3.2, 4.4, and 7.0 MHz appear as fields within the center of the EPR spectrum are approached. A similar general behavior was observed for other τ values. The absence of a field dependence for the 2.0 and 4.0 MHz peaks suggests that the cancellation condition is satisfied and these peaks correspond to NQR frequencies, where $\nu_0 = \nu_- = 2.0$ MHz, $\nu_+ = 4.0$ MHz, and $\nu_0 + \nu_- = \nu_+$. From these, a value of 4.0 MHz is obtained for the quadrupole coupling constant (e^2qQ/h), 1.0 for the asymmetry parameter, η, and the hyperfine coupling should be around 2 MHz. These are in good agreement with earlier results on VO^{2+} and Cu^{2+} complexes with other bis-hydroxamates.[24,25] The possible assignment $\nu_0 = 2.0$ MHz, $\nu_- = 4.0$ MHz, and $\nu_+ = 6.0$ MHz is ruled out because it yields a quadrupole coupling constant of 6.6 MHz which is considerably higher than the value obtained for other VO^{2+} bis-hydroxamate complexes (3.9 MHz).[24] It is not clear, however, whether the peak at 6.0 MHz is a double-quantum transition, ν_{dq}, of the HEF manifold or if it is a combination peak ($\nu_{0,-} + \nu_+$) due to the presence of two nitrogens. Moreover, the origin of the set of peaks around 1.5, 3.2, and 4.4 MHz is not well understood. There are three possibilities to explain these features: (i) the peaks represent singularities in the powder patterns of single-quantum frequencies of the HEF manifold, (ii) they comprise a second set of NQR lines from a different nitrogen, i.e., the two nitrogens are inequivalent, or (iii) the two nitrogens are equivalent and the peaks are attributed to a set of LEF frequencies, close to the NQR frequencies, arising from a set of orientations different than those contributing to the 2.0- and 4.0-MHz peaks.

To resolve some of these ambiguities HYSCORE experiments were carried out at two different magnetic fields. The spectrum obtained at 300.0 mT, which at a spectrometer frequency of 9.7 GHz is close to $A_{\|}$ (^{51}V, $M_I = -^7/_2$), is shown in Figure 8a. The spectrum exhibits only two peaks on the diagonal at (1.9, 1.9) MHz and (4.1, 4.1) MHz and no cross-peaks, which is in agreement with our initial assignment of the 2.0- and 4.0-MHz peaks. Spectra recorded with other τ values showed the same results. The absence of cross-peaks is not attributed to spectrometer misadjustment since the same conditions were used to record the other HYSCORE spectra. It is not clear why frequencies belonging to the HEF manifold do not appear at this position. It is probably related to the relative orientation of tensors involved and the angular dependence of the ESEEM amplitudes. Although the measurements were carried out at the field position close to $A_{\|}$ (^{51}V, $M_I = -^7/_2$), some orientation distribution is still expected. This distribution can result in significant broadening if the ^{14}N quadrupole and hyperfine tensors are not collinear with the ^{51}V hyperfine tensor.

Unfortunately, due to the absence of cross-peaks, the 6.0-MHz line cannot unambiguously be assigned, although it suggests that it is a combination rather than a double-quantum peak. This ambiguity is solved by the DONUT-HYSCORE spectrum, shown in Figure 8b. The majority of the signals appear in the (−, +) quadrant with cross-peaks at (−1.9, 4.1), and (−1.9, 6.0). A weak peak at (−6.0, 4.1) MHz appears as well; its counterpart, however, does not show at the contour level shown. The appearance of the (−1.9, 4.1) correlation confirms the assignment to ($\nu_{0,-}$, ν_+), whereas the presence of the cross-peaks ($\nu_{0,-}$, 6.0) and (ν_+, 6.0) indicates that the feature at 6.0 MHz is a combination peak, ($\nu_{0,-} + \nu_+$). The two nitrogens have therefore the same ESEEM frequencies, at least when the magnetic field is parallel to the local symmetry axis of the VO^{2+} binding site. The peak at 8.0 MHz on the diagonal of the (−, +) quadrant is assigned to $2\nu_+$.

The HYSCORE spectrum of VO-RL515 recorded at 344.1 mT (corresponding to the isotropic position at 9.7 GHz) is shown in Figure 9a. Intense cross-peaks are observed at (1.5, 7.2), (±4.5, 7.2) and (−3, 7.0) MHz, along several rather intense ridges. We therefore assign the 1.5-, 3.0-, and 4.5-MHz frequencies to the LEF manifold and the 7.2-MHz frequency to a double-quantum transition of the HEF manifold. This indicates that the former do not correspond to single-quantum transitions in the HEF manifold. Nonetheless, the two sets of

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[34] Reijerse, E. J.; Shane, J. J.; de Boer E.; Collison D. In *Electron Magnetic Resonance of Disordered Systems II*; Yordanov, N. D., Ed.; World Scientific: Singapore, 1989; p 189.

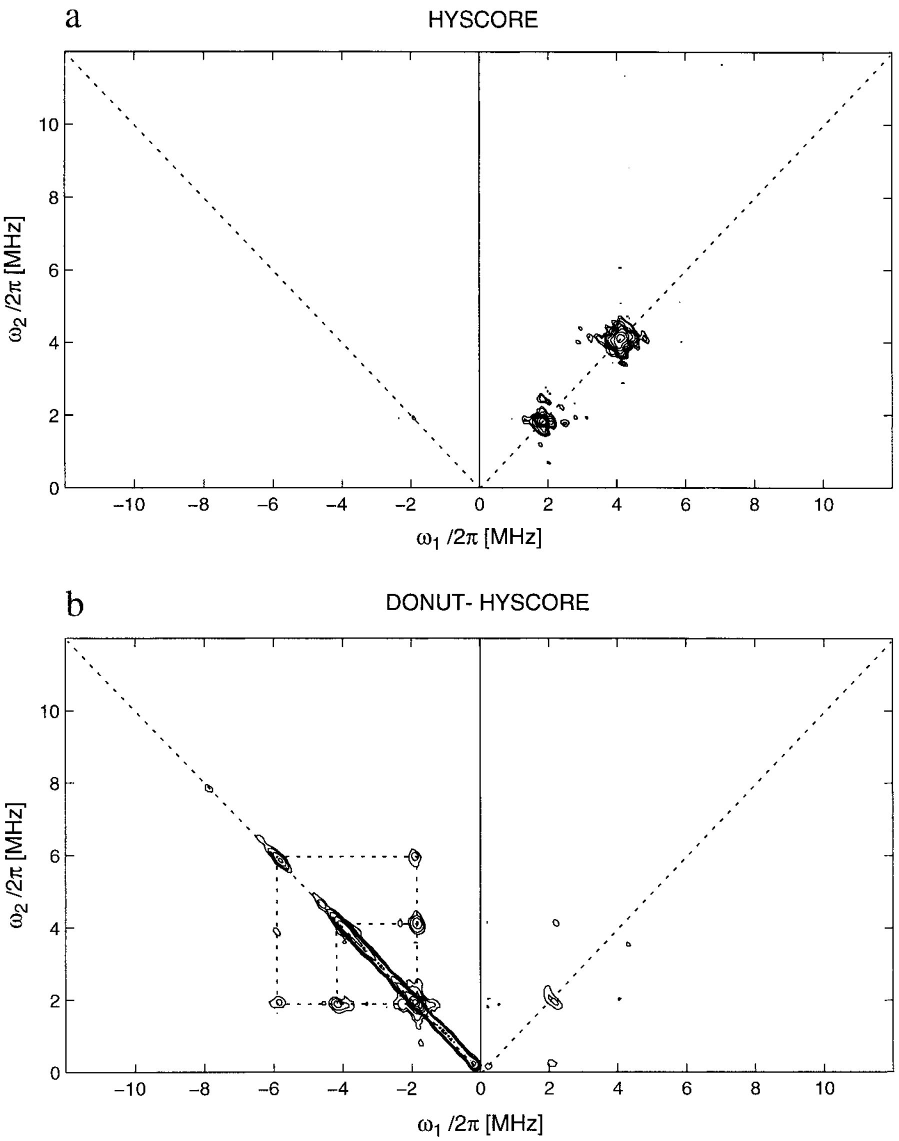

Figure 8. (a) HYSCORE and (b) DONUT-HYSCORE spectra of VO-RL515 (observed field $B_0 = 300.0$ mT). The HYSCORE spectrum was recorded with $\tau = 0.160$ μs and the DONUT-HYSCORE spectrum with $\tau_1 = 0.352$ μs and $\tau_2 = 0.400$ μs.

LEF frequencies, 2.0 and 4.0 MHz and 1.5, 3.0, and 4.5 MHz, can arise from two inequivalent nitrogens.

The corresponding DONUT-HYSCORE spectrum, shown in Figure 9b, is significantly different from the HYSCORE spectrum, and no residual HYSCORE peaks are detected. Correlations within the two sets of LEF frequencies, mentioned above, show up at (2.0, 4.0), (1.5, 3.0) and (1.5, 4.5) MHz, supporting their assignment to two sets of LEF manifolds. Cross-peaks at (1.5, 6.0), (4.5, 6.0), and (−3.0, 6.0) MHz appear as well, indicating that the peak at 6.0 MHz is a combination of the 1.5- and the 4.5-MHz frequencies. In principle, the 6.0-MHz peaks could also be assigned to a combination of the 2.0- and 4.0-MHz lines. However, the absence of correlations between the 2.0- and 4.0-MHz set and the 1.5-, 3.0-, and 4.5-MHz set makes the appearance of a correlation with any of their combinations very unlikely. Two additional sets of cross-peaks involving combination frequencies appear in the (−, +) quadrant at (−4.0, 8.0) and (−4.5, 9.0) MHz. They are attributed to $(-\nu_+, 2\nu_+)$ within each set of the LEF frequencies. These cross-peaks show that the two sets of the LEF frequencies belong to complexes with different orientations, rather than to two inequivalent nitrogens of the same molecule. The absence of correlations between the peaks at 4.0 or 4.5 MHz and their sum, 8.5 MHz, supports this assignment. The DONUT-HYSCORE result thus excludes possibility ii and leads to the following assignment: the 2.0- and 4.0-MHz set corresponds to singularities of the parallel orientation ($\theta_0 \sim 0°$), whereas the 1.5-, 3.0-, and 4.5-MHz set corresponds to the singularities in the region of the perpendicular orientation ($\theta_0 \sim 90°$). For convenience, we refer to these sets with the subscripts ∥ and ⊥, respectively. This orientation dependence also accounts for the slight shift of $\nu_0 = 1.9$ MHz and $\nu_+ = 4.1$ MHz from 2.0 and 4.0 MHz observed in the HYSCORE and DONUT-HYSCORE spectra obtained at 300.0 mT. At this field position the selected range of θ_0 is shifted somewhat from 0°. The assignments of the ESEEM frequencies of VO-RL515 are summarized in Table 3.

In the DONUT-HYSCORE experiment, the orientation dependence of the LEF frequencies is nicely displayed by the ridge observed in the (+, +) quadrant ranging from (2.0, 2.0) to (1.5, 3.0) MHz. The 2.0-MHz ($\nu_{0,-}$) peak splits, and ν_0 decreases to 1.5 MHz whereas ν_- increases to 3.0 MHz. The ν_+ frequency increases from 4.0 to 4.5 MHz such that the condition $\nu_- + \nu_0 = \nu_+$ is fulfilled for all orientations. Although the LEF frequencies are close to the NQR frequencies at all orientations, terming them by the notation of pure NQR

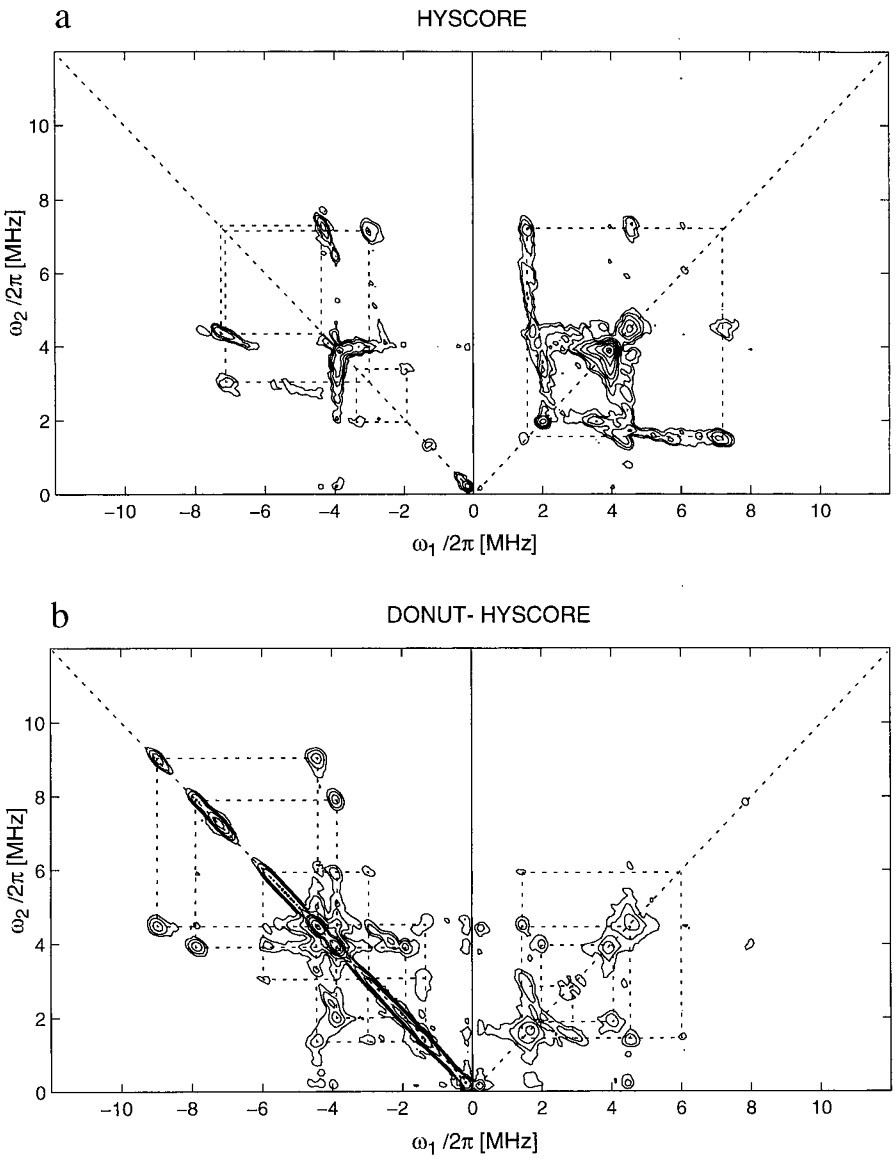

Figure 9. (a) HYSCORE and (b) DONUT-HYSCORE spectra of VO-RL515 (observer field B_0 = 344.1 mT). The HYSCORE spectrum was recorded with τ = 0.240 μs and the DONUT-HYSCORE spectrum with τ_1 = 0.240 μs and τ_2 = 0.400 μs.

Table 3. Assignment of the ESEEM Frequencies Observed in the HYSCORE and DONUT-HYSCORE Spectra of VO-RL515 Shown in Figures 8 and 9

manifold	label	$\omega/2\pi$ (±0.1 MHz)
α (300.0 mT)	$(\nu_{0,-})_{\parallel}$	1.9
	$(\nu_{+})_{\parallel}$	4.1
	$(\nu_{0,-} + \nu_{+})_{\parallel}$	6.0
α (344.1 mT)	$(\nu_{0,-})_{\parallel}$	2.0
	$(\nu_{0})_{\perp}$	1.5
	$(\nu_{-})_{\perp}$	3.0
	$(\nu_{+})_{\parallel}$	4.0
	$(\nu_{+})_{\perp}$	4.5
	$(\nu_{0} + \nu_{+})_{\parallel}$	6.0
	$(\nu_{0} + \nu_{+})_{\perp}$	6.0
	$(2\nu_{+})_{\parallel}$	8.0
	$(2\nu_{+})_{\perp}$	9.0
β (344.1 mT)	$(\nu_{dq})_{\perp}$	7.2

frequencies is somewhat inappropriate because the orientation dependence indicates that at least at some orientations they contain significant contributions from the hyperfine coupling and the Zeeman interaction. We kept this notation, rather than ν_{sq1}, ν_{sq2}, ν_{dq}, for the sake of convenience.

DONUT-HYSCORE spectra recorded with different τ_2 values are shown in Figure 10. All spectra show the ridge discussed above and the cross-peaks observed before, although with different intensities. The intensities depend on τ_1, like in standard HYSCORE, and in addition on τ_2. In all DONUT-HYSCORE spectra measured, an intense stretch on the (−, +) diagonal due to nuclear coherences that evolve on the same nuclear transitions during the time intervals t_1 and t_2 is observed. These also include the frequencies ν_{sq1}, ν_{sq2} which were not observed off the diagonal. This long stretch in Figure 8b, supports our earlier explanation regarding the possibility that ν_{sq1} and ν_{sq2} are inhomogeneously broadened also close to the "single-crystal position".

Discussion

The applicability of the DONUT-HYSCORE experiment and its efficiency as a complimentary experiment to HYSCORE for an electron spin $S = {}^{1}/_{2}$ coupled to one or more ^{14}N nuclei (I = 1) has been demonstrated on a single crystal and a frozen solution. In the case of a single crystal where all ESEEM frequencies can be observed, the information provided by the DONUT-HYSCORE experiment is redundant once the HYSCORE experiment has been applied. However, when

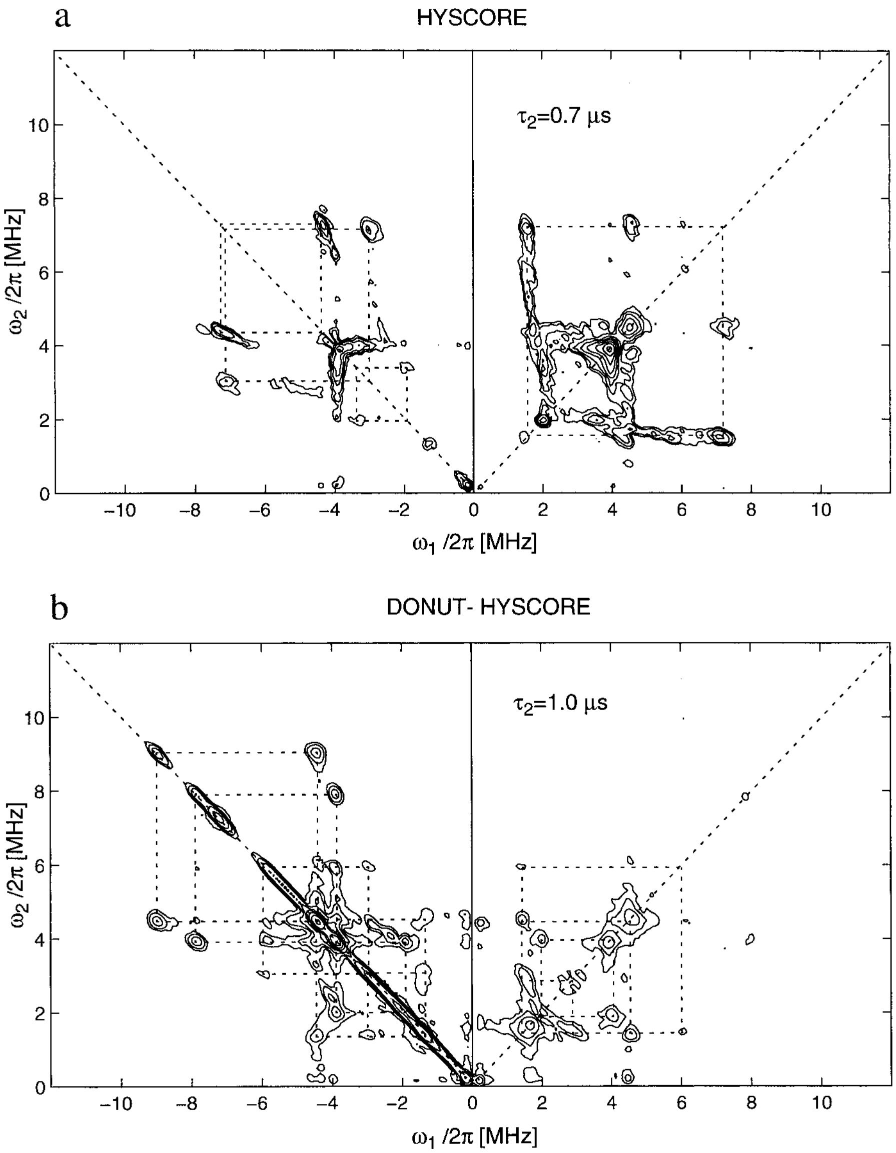

Figure 10. DONUT-HYSCORE spectra of VO-RL515 recorded with different values of τ_2: (a) $\tau_2 = 0.700$ μs, (b) $\tau_2 = 1.000$ μs. All other parameters are as in Figure 9.

frequencies of only one M_S manifold can be detected, DONUT-HYSCORE, rather than HYSCORE, is the experiment of choice. Such a situation was encountered in W-band (95-GHz) ESEEM measurements of a single crystals of the copper protein azurin where only frequencies of the LEF manifold of the directly bound nitrogen were observed.[35]

Unfortunately, many systems of interest in biology and materials science are not available in the form of single crystals. Therefore, the example of the orientationally disordered system has a significant practical value since such systems suffer from inhomogeneous broadening and the possibilities to determine the full ^{14}N hyperfine and quadrupolar tensors with the available techniques are often restrained. In disordered systems, part or all of the frequencies of one of the M_S manifolds may be missing from the spectra at certain selected orientations, thus limiting the value of HYSCORE. For example, HYSCORE cannot distinguish between two sets of frequencies belonging to the same M_S manifold in different centers (or different orientations) once signals from the other M_S manifolds are missing for one (or both) of the centers or those that are observed overlap. In contrast, the DONUT-HYSCORE experiment can identify the two sets because it provides correlations within such two sets of frequencies. Therefore, DONUT-HYSCORE should be a vital additional tool for the analysis of ESEEM frequencies in disordered systems.

In the case of VO-RL515 the DONUT-HYSCORE spectrum led to the unambiguous assignment of the 6.0-MHz combination line ($\nu_0 + \nu_+$) at the quasi-single-crystal orientation. In addition, the appearance of correlations involving combination lines of the type ($-\nu_+$, $2\nu_+$) and the diagonal peak at ($-2\nu_+$) in the spectrum obtained at the "isotropic" position led to the conclusion that the two sets of the LEF frequencies are due to singularities corresponding to different orientations. These results excluded the possibility that the two nitrogens are inequivalent. The complete assignment of the ESEEM frequencies together with the comprehension of their orientation dependence as obtained from the line shape of the DONUT-HYSCORE cross-peaks allows for future computer simulations targeted toward the determination of Hamiltonian parameters followed by their interpretation in terms of the binding site geometry. The process of simulating a series of orientation-selective ESEEM spectra is tedious and time-consuming because

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(35) Coremans, J. W. A.; Poluektov, O. G.; Groenen, E. J. J.; Canters, G. W.; Nar, H.; Messerschmidt, A. *J. Am. Chem. Soc.* **1997**, *119*, 4726.

of the relatively large number of parameters involved. Therefore, before attempting to simulate such a series, one should understand the orientation dependence of the frequencies and should have a good idea about the range of the parameters involved. Such a detailed quantitative analysis via computer is beyond the scope of this work, the objective of which is to introduces the DONUT-HYSCORE experiment.

Although the DONUT-HYSCORE experiment involves a larger number of pulses than HYSCORE, and as such is expected to have a lower signal-to-noise ratio, we found that if the echo intensity is sufficient for an HYSCORE experiment it will also be possible to record a DONUT-HYSCORE spectrum. This was well demonstrated in the DONUT-HYSCORE spectrum measured at the field position close to $A_{\|}(^{51}\mathrm{V}, M_I = -{}^7/_2)$, where the echo intensity is relatively low. Finally we note that, while correlations within the LEF manifold are well evident in the DONUT-HYSCORE spectra of VO-RL515, cross-peaks involving ESEEM frequencies of the HEF manifold are absent. This can be attributed to a strong orientation dependence of the latter. Nonetheless, some ridges due to cross-peaks of the type $(\nu_{dq}^{\alpha(\beta)}, \nu_{sq1,2}^{\alpha(\beta)})$ are still expected because they should be similar to ridges of the type $(\nu_{dq}^{\alpha}, \nu_{sq1,2}^{\beta})$ often observed in HYSCORE spectra.[31,32,36,37] Detailed information regarding the line shapes and relative intensities of cross-peaks and their dependence on the Hamiltonian parameter should be obtained by numerical simulations.

Conclusions

The two-dimensional DONUT-HYSCORE experiment correlates nuclear frequencies belonging to the same paramagnetic center and the same electron spin manifold and as such is complementary to HYSCORE. It is an important addition to the set of experiments designed to resolve and simplify the assignment of ESEEM frequencies for the case of an electron spin, $S = {}^1/_2$, interacting with a number of nuclear spins with $I \geq 1$ with a nuclear quadrupole interaction. It was demonstrated to be efficient for both single crystals and orientationally disordered systems, and it is particularly useful in cases where signals in one of the M_S manifolds are absent. Accordingly, it should be carried out in addition to HYSCORE to increase the information content of the pulse EPR experiment.

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