Reviewer 2

Specific comments

Materials & Methods:

Point 1.- The applied phase cycle has been referred in the text.

Point 2.- Information on the power of the ELDOR pulse and the process of sequence optimization has now been included in the text.

Point 3.- The reference was added to the text. Fuchs et al. 2002.

ELDOR-detected NMR

Point1.- The text was changed in the manuscript to correct the mistake.

Point2.- The corrections have been implemented in the text. We thank the reviewer for spotting these errors.

Point3.- Although the study was performed at W-band, Davies ENDOR was used in Schleicher et al 2021 to obtain the couplings of 13C-labeled flavins but the signal of 13C(4) nucleus was not detected. The success of EDNMR as compared to ENDOR might have to do with the transitions being partially forbidden. This clarification has been added to the text.

Point 4.- The detection frequency was placed off center in the resonator dip. The measurements of both samples were tuned as similarly as possible in order to minimize problems with comparison of the spectra. We do not think the signal at 11 MHz is (at least entirely) an artifact of the subtraction spectrum due to different acquisition conditions, which were kept as close as possible, since in the parameters of 13C(4) obtained from HYSCORE spectra also predict one of the nuclear frequencies to be about this magnitude for orientations close to the parallel orientation.

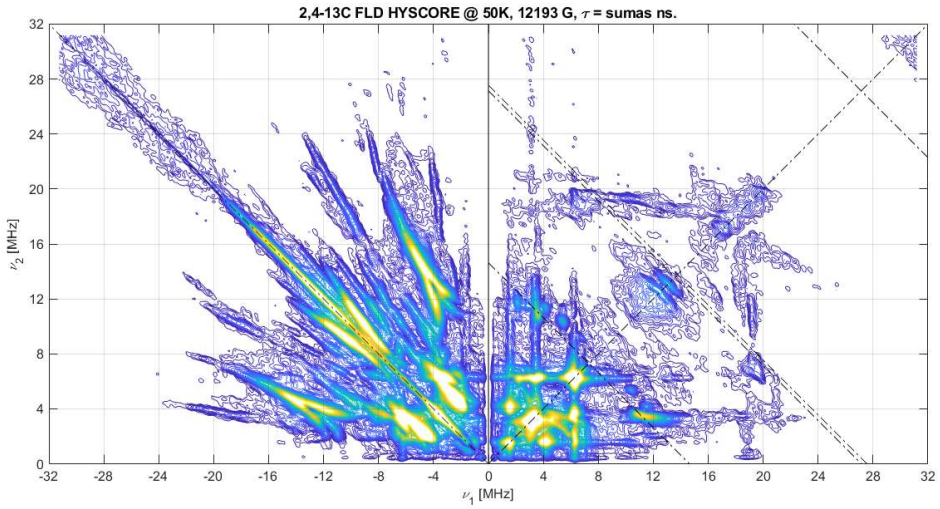
The referee is right about the labeling of the x-axis in the EDNMR spectra, we have changed it in the new version of the manuscript. We also have softened the statement about not having orientation selection at the center of the spectrum and we have detailed the spin Hamiltonian used for calculation of the orientation selection patterns. The patterns have been corrected since there was a mistake with the original excitation bandwidth, which is now explicitly mentioned.

Points 5, 6, 7, 8 and 10.- Description of the ^{13}C(4a) features in the EDNMR spectrum and analysis has been revised and rewritten following the reviewer's suggestions in order to make it more accurate and more clear.

Point 9.- Several HTA pulse lengths and powers were tried, as indicated now I the Materials & Methods section. However these trial spectra were performed in order to spot the best s/n ratios in order to optimize the HTA pulse. Unfortunately, no detailed comparison was performed on the signal widths.

13C HYSCORE

Point 1.- Here is one full spectrum for [13C(2,4a)-FMN]-FLd



Far from being devoid of signals, the (-+) quadrant is dominated by 14N nitrogen signals but there is no evident 13C signals observed in the (-+) quadrant. A careful comparison between spectra of [13C(2,4a)-FMN]-FLd and [13C(2)-FMN]-FLd does not bring any signals that could be attributed to 13C(4a) in the (-+) quadrant. Therefore, when we wanted to focus of the 13C signals we have only shown only the (++) quadrant.

Point 3.- We agree with reviewer that representation in squared frequency axes would give a first estimation of the hyperfine parameters. We considered that we could skip this step of the analysis in the manuscript in order to make it lighter, as we already obtained a first estimation of the parameters form our X-band CW-EPR and Q-band EDNMR results that are enough for the simulation refinement.

Point 4.- Typo in line 334 has been corrected.

Point 5.- We have added on page 18 a brief explanation on how the uncertainties in the parameters of the Spin Hamiltonian were estimated.

Point 6.- Specific references to figures in the supplementary material have been added at all points were the Supplementary Material was referred to.

Points 7-8.- Reviewer's suggestions on Figs. S1 and S2 have been followed. Note that although the approximate field position with respect to the EPR spectrum is the same in Figs 3a & S2, the exact field position is different due to differences in the microwave frequencies. EDNMR and HYSCORE were performed at different spectrometers at different times.

^{15}N and ^{14}N HSYCORE

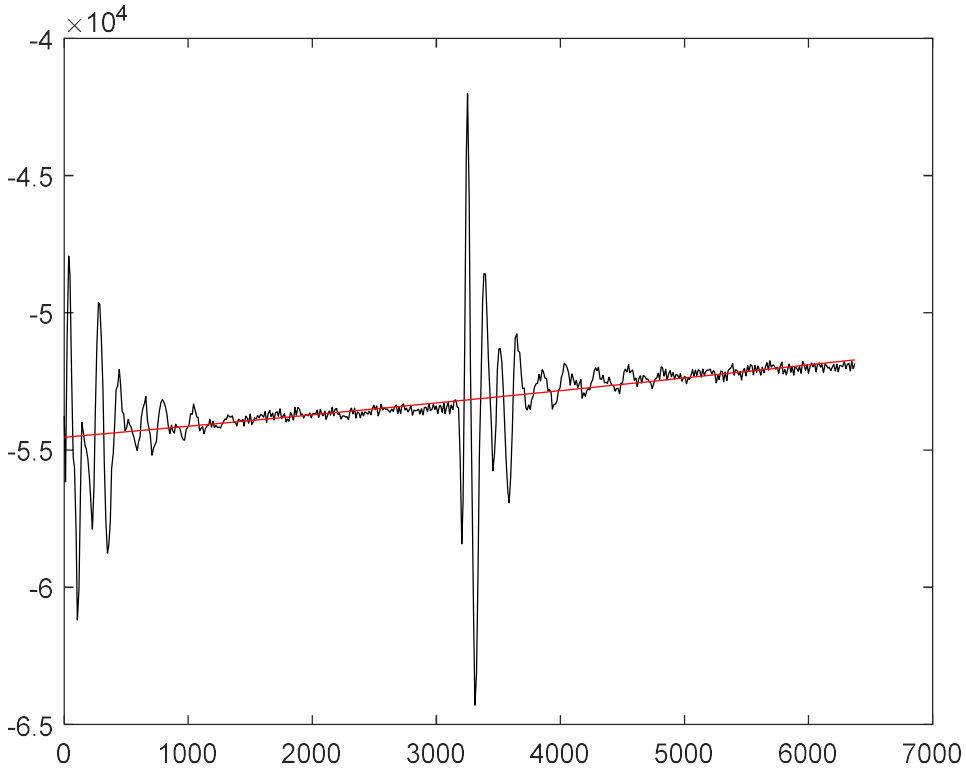
Points 1 & 2.-Figures 5 and 6. Due to the reviewer's comment, all field positions at which the experiments were performed were checked and, indeed, some inconsistencies together with flat-out errors were found. For example, the magnetic field values of the EDNMR experiments were swap, so we realized the tail experiments were actually performed at the high-field tail and not the low-field tail of the EPR spectrum. After careful verification, we believe the field positions and corresponding diagrams are now correct in all figures. About the reason to choose the high-field end: Since the dominating anisotropy in the EPR spectrum is due to the axial hyperfine couplings of N(5), N(10), which are much larger in the direction perpendicular

to the isoaloxacine plane, positioning the magnetic field at any of those ends would select the "parallel" orientation. The high-field end was chosen because there is a small g-anisotropy, which at Qband gives a slightly better orientation selection for the high-field end.

Point 3.- In order to have the best possible orientation selection, we tried to do the experiments at the highest possible magnetic field that gives a good signal. For 14N signals this was possible at a quite high field. On the other hand, in the ^{15}N labeled sample such a field position did not provide usable modulations and we had to move to a lower field. This is most probably related to the quadrupole coupling providing a way to mix the nuclear levels and yielding forbidden transitions that give good modulation of the echo. For ^{15}N, the quadrupole interaction is missing and for an orientation very close to the perpendicular to the plane, which is an eigenaxis of the hyperfine coupling, the transitions are allowed and no modulation is observed.

The difference in the field setting between the spectra of the two samples is due to the difference in the microwave frequency of the two experiments.

Point 4.- The sum of the spectra was performed after Fourier transformation. Since the echo is strongly modulated, its intensity varies very much along the time trace. However, no significant difference in the envelope intensity is worth mentioning between tau = 96 ns and tau = 168 ns.



Point 5.- For the sake of clarity, in Figure 5 we have removed all antidiagonal lines except the one of ^{15}N.

Point 6.- The sample is not deuterated but since some unidentified signals appear to lie on this diagonal, the antidiagonal line was drawn in the analysis phase. We have removed this antidiagonal line in order not to generate confusion.

Point 7.- After the display of the hyperfine parameters in the text, we have introduced a small paragraph explaining how the uncertainties were estimated. Within the mentioned uncertainty (+- 0.3 MHz) the shape of the correlation ridges is not compatible with rhombicity above the mentioned uncertainty. On the (++) quadrant, two separate and well defined peaks were found assigned to the parallel features of each of the two nitrogen nuclei. If there was any moderate rhombicity these features would be smeared out into a ridge.

We thank the reviewer's thorough comments, which allowed to identify and correct the above mentioned mistakes and increase the quality of the article.

Technical corrections.- All typos spotted by the reviewer were corrected.