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Magn. Reson. Discuss., referee comment RC1
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Comment on mr-2022-14

Anonymous Referee #1

Referee comment on "Fine optimization of a dissolution dynamic nuclear polarization experimental setting for ^{13}C NMR of metabolic samples" by Arnab Dey et al., Magn. Reson. Discuss., https://doi.org/10.5194/mr-2022-14-RC1, 2022

The manuscript submitted to Magnetic Resonance by Dey et al. represents a valuable piece of work that with some work on the manuscript will serve a good basis for development of robust workflow for metabolomic studies, will allow other laboratories to get started with dDNP-NMR based metabolomics and will provide a data basis for cross laboratory studies with different hyperpolarization methods.

I am very impressed with the systematic and voluminous work gone into the manuscript.

I do however find that the manuscript is written a bit hasty and that the value of the presented improvements could be enhanced by sorting out some of the more spurious findings and supporting other findings with additional data and reasoning as well as discussion. I will below suggest the most pressing things to work with and at the same time declare that I at this stage did not go into details.

High attention:

- In all figures the "alanine" chemical shift is given as 173 ppm in D2O. The pH is not stated but judged relative to the chemical shifts for the other metabolites this is not correct. It should be closer to 177 ppm. Pyruvate (n.a.) will show two chemical shifts in the carbonyl region and the 173 ppm fits well with C1 for pyruvate. Did the authors assign the spectra correct? Looking at the spectra given in figure 8.a then the red spectrum "optimized with D2O" shows pyruvate C2 at 208 ppm, TSP-d4 at 189 ppm, acetate at 184 ppm, alanine (I suggest) at 177 ppm and pyruvate C1 at 173 ppm. In methanol (green spectrum) there are one additional signal and some very small signals (could be relevant). Did the authors measure the metabolites individually with thermal NMR in methanol to verify chemical shifts?
- Most figures have an odd y-axis unit. I suggest to leaving out "x10-10" with the a.u being sufficient, and to standardize the axis units (ex. fig. 4b). It is important that what

should be comparable can be compared. Ex. Should figure 3a not be comparable to fig.4a? e.g. the red stables in fig.4a for 1H DNP (uw50%) are not comparable to the red (50mM) 1H DNP (uw 50%)?

Also, in the solid-state figures I do not understand how to interpret the "thermal signal" (in fig.3a I guess it is as in fig4.a the 1H signal without microwaves on. But how can this signal be on the same scale as the 1H DNP signal? In that case it looks as though the polarization is very low.

Make sure to give all the important information in the figure legends eg. tempol concentration in figure 4.

- Figure 6 should include protonated carbons (shown in fig.8). As should Table 2.
- Why is the longer "relax" times for "TSP 0 ppm" (blue and green) in figure 5b not comparable to similar signal quantifications in figure 2b? Is it not TSPd4 in all experiments? (should be stated).
- The high variability of the "TSP 0 ppm" signal in methanol (fig. 7b and Table 2) should be discussed in relation to the use of this standard for relative quantification or absolute quantification and as chemical shift reference.
- To be able to discuss the impact of the different optimized parameters it would be valuable to measure the T1 of the different carbons in the included metabolites and for the TSP standard. This can be done straightforwardly by increasing the concentration of the metabolites to 50 or 100 mM in a simulated sample (50 mM PA in 6:3:1 glycerol:D2O:H2O, total vol. 200 ul dissolved in 5 ml methanol) and run 2 inversion recovery experiments -one for carbonyl carbons and one for aliphatic carbons. It would be interesting to also perform these experiments without the added tempol radical.
- Since this is a hyperpolarization method optimization paper it is relevant to measure the polarization in a liquid state sample. To save time this is most easily done using a condition matched external standard with an exact concentration, ex. use 1-13C-acetate which can be made reliably in high concentration. The measurement will not be decimal exact but this is not important.

Other points:

p.8 section on "B.4 Vitrification parameters":

It is natural when working with complicated methods that experimental routines are implemented that has little theoretical meaning. Several points in this section refer to such experimental routines based on non-investigated observations. If rate of vitrification is important it should be shown. If it matters in which order the metabolites are dissolved (water first or water:glycerol mix or glycerol) it is a matter of solubility and should be investigated. Then also sample temperature may be an issue as well as total dissolution volume. I suggest you separate out parameters that you have identified as possibly

important for later study/optimization from the parameters that you have investigated and can conclude on and discuss.

Discussion:

The discussion is generally kept to stating the findings with a comment. The results are rarely discussed.

Ex.:

The authors have previously published (also nicely referenced in the manuscript) significant contributions to the use of dDNP NMR for allowing 13C direct detect natural abundance mixture analysis. Significant findings in those reports are not discussed relative to the results presented in this manuscript (e.g. use of Hellmanex and a suited internal standard for quantification). Please discuss the alternative choices in this manuscript and how they have improved previous results or was not part of the purpose.

The results are summarized stating that the main contribution to the significant method improvement is the transfer time. It would be interesting with a discussion about the consequences of the improvements. Especially the important choice of dissolving in methanol could be strengthen with a discussion on chemical shift changes in methanol (lack of database, temperature and concentration influences).

I just noticed:

Spelling error in Figure 1: ‘magentic’ should be ‘magnetic’

Example of unprecise language: l.148 ‘to trace the amount’ - maybe to weigh?

Please explain how the factor 2900 difference in sensitivity between 13C and 1H is calculated