Electronic Supplementary Material (ESI) for Analytical Methods

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Optimization of heteronuclear ultrafast 2D NMR for the study of complex mixtures hyperpolarized by dynamic nuclear polarization

Clément Praud,[a] Victor Ribay,[a] Arnab Dey,[a] Benoît Charrier,[a] Joris Mandral,[a] Jonathan Farjon,[a] Jean-Nicolas Dumez[a] and Patrick Giraudeau [a*]

[a.] *Nantes Université, CNRS, CEISAM, UMR 6230, F-44000 Nantes, France*
*patrick.giraudeau@univ-nantes.fr

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1. Sample preparation

	200 mM Sample	**10 metabolite stock solutions at different concentrations**					**25 mM sample**
Sample ID	200 mM	LOQ1	LOQ2	LOQ3	LOQ4	LOQ5	25 mM
Lactate (mg)	170.0	45.9	35.6	22.3	16.1	5.0	7.1
[Lac] (mM)	201.9	165.3	126.0	79.3	54.9	17.8	24.98
Arginine (mg)	263.0	52.9	34.8	17.8	8.3	71.0	11.2
[Arg] (mM)	200.9	122.6	79.5	40.8	18.1	162.3	25.30
Succinic Acid (mg)	182.7	25.9	12.0	9.5	49.0	36.0	8.5
[Suc] (mM)	205.9	88.3	40.3	31.9	158.5	121.4	28.73
Alanine (mg)	147.7	26.5	18.2	8.8	4.1	35.8	5.7
[Ala] (mM)	220.6	119.9	81.1	39.3	17.6	160.0	25.08
Citric acid (mg)	289.9	19.7	10.0	83.9	57.2	38.5	294.7
[Cit] (mM)	200.8	41.4	20.7	174.0	113.9	79.7	605.50
Glycolic acid (mg)	114.1	15.0	7.4	5.5	32.8	24.2	6.8
[Gly] (mM)	199.6	79.3	38.4	28.5	164.8	126.4	35.14
Creatinine (mg)	169.2	11.3	6.3	45.1	34.0	22.9	8.4
[Crea] (mM)	199.0	40.2	22.2	158.7	114.7	80.6	29.14
Pyruvate (mg)	165.0	5.21	44.1	40.2	22.8	12.1	7.0
[Pyr] (mM)	199.5	19.1	159.3	145.5	79.0	43.6	25.08
Aminobutyric Acid (mg)	166.0	41.5	30.9	24.0	9.4	6.4	7.7
[Amino] (mM)	214.2	162.4	117.6	92.7	35.0	24.6	29.44
Acetate (mg)	125.2	4.8	32.8	24.7	16.7	9.3	6.0
[Ace] (mM)	203.1	23.8	158.6	120.1	77.8	45.3	28.73
Tempol (mg)	64.6	22.1	21.7	22.8	23.9	23.9	23.4
[TEMPOL] (mM)	49.9	51.8	50.0	52.6	53.0	55.1	53.63

Table S1: Composition of stock solutions, with the weighted mass (in mg) and concentration (in mM and in orange) of each metabolite once dissolved in the DNP juice (glycerol-d_8:D_2O:MilliQ water – 60:30:10 v/v%). The 200 mM sample was prepared with 200 mM of each metabolite, then five stock solutions were made with metabolite concentrations ranging from 160 to 20 mM and finally a 25 mM sample was studied with 25 mM of each metabolite (except for Citric acid with a concentration of 600 mM).

2. Hellmanex® Treatment

We first prepared an Hellmanex® solution by adding 2% by volume Hellmanex® III solution from Hellma Analytics (https://www.hellma.com/en/laboratory-supplies/cuvettes/hellmanex/) in deionized water. Then, NMR tubes were filled with this solution, and placed in a water bath at 40 °C for 40 min. After that, tubes were emptied and rinsed with deionized water twice. Finally, NMR tubes were dried in an oven at 40 °C overnight. A freshly treated NMR tube was used with each dissolution.

3. 10-metabolites mixture

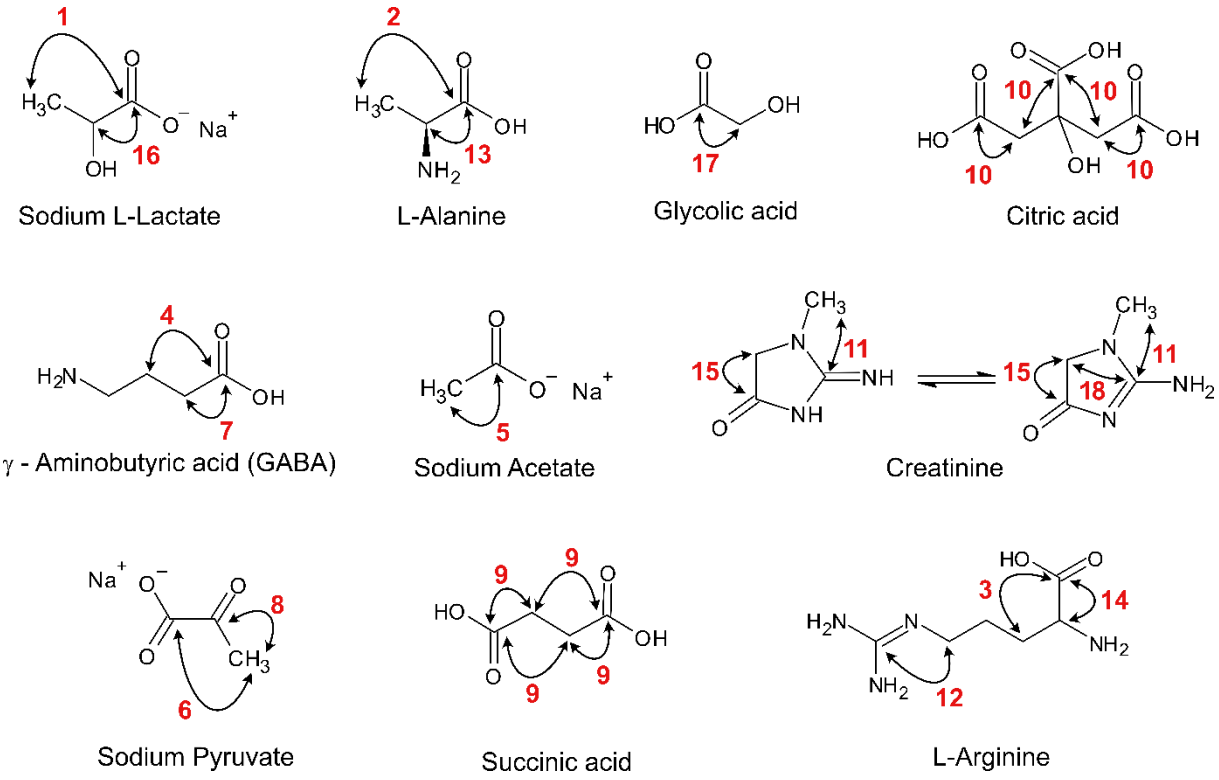

Fig. S1: Illustration of the 10 metabolites studied in this feature article, with the corresponding J_{CH} correlations detailed in the Table S2.

4. Chemical shifts assignment

		Reference conventional HMBC spectrum (D_2O)		Reference conventional HMBC spectrum (MeOD)	
Labels	**Correlations**	**δ^1H (ppm)**	**δ^{13}C (ppm)**	**δ^1H (ppm)**	**δ^{13}C (ppm)**
1	Lactate ($^3J_{CH}$)	1.195	183.57	1.193	183.13
2	Alanine ($^3J_{CH}$)	1.326	177.58	1.327	177.57
3	Arg ($^3J_{CH}$)	1.758	176.18	N/A	N/A
4	GABA ($^3J_{CH}$)	1.767	181.45	1.761	180.71
5	Ace ($^2J_{CH}$)	1.883	180.10	1.840	178.83
6	Pyr ($^3J_{CH}$)	2.200	206.83	2.162	206.31
7	GABA ($^2J_{CH}$)	2.236	181.46	2.231	180.68
8	Pyr ($^2J_{CH}$)	2.201	171.88	2.161	172.50
9	Suc ($^2J_{CH}$ and $^3J_{CH}$)	2.421	180.83	2.391	179.93
10	Cit ($^2J_{CH}$ and $^3J_{CH}$)	2.563	181.33	2.525	182.18
		2.563	177.76	2.525	178.41
		2.677	181.33	2.638	182.18
		2.677	177.76	2.638	178.41
11	Crea ($^3J_{CH}$)	2.967	161.65	2.932	169.12
12	Arg ($^3J_{CH}$)	3.086	158.66	3.070	160.03
13	Ala ($^2J_{CH}$)	3.631	177.57	3.526	177.81
14	Arg ($^2J_{CH}$)	3.615	176.22	3.525	175.95
15	Crea ($^2J_{CH}$)	3.837	181.07	3.895	185.25
16	Lac ($^2J_{CH}$)	4.005	183.56	3.943	183.13
17	Gly ($^2J_{CH}$)	4.075	170.14	3.792	180.88
18	Crea ($^3J_{CH}$)	4.076	161.65	3.893	169.06

Table S2: Chemical shift assignments for all reported peaks on the d-DNP UF 2D experiments after dissolutions in D_2O and MeOD. Peaks were assigned thanks to conventional HMBC experiments and the use of internal standards (data not shown).

5. IMPACT-HMBC experiment

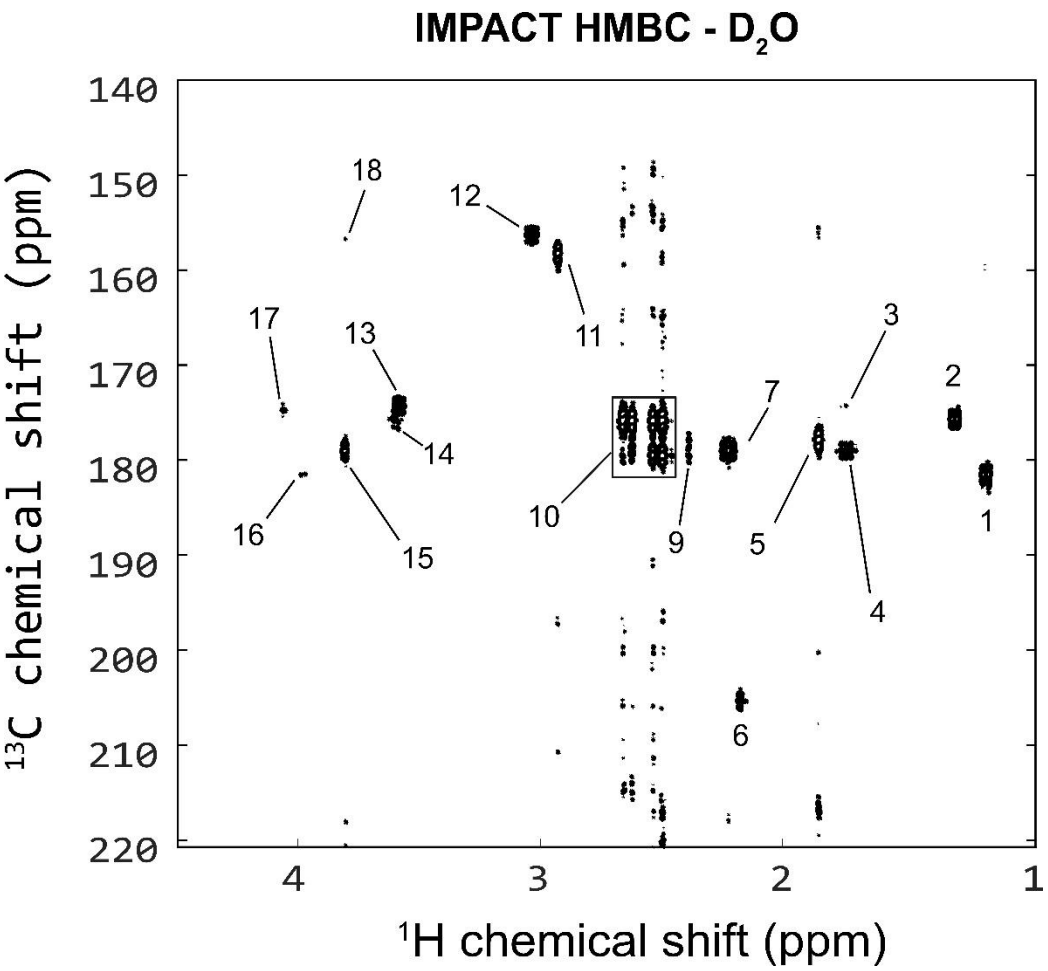

Fig. S2: IMPACT-HMBC experiment of a 200 mM 10-metabolite mixture in D_2O, recorded in 20 minutes and 34 seconds on a 400 MHz spectrometer equipped with a nitrogen-cooled cryoprobe and at 298 K. The pulse sequence included a 40 ms DIPSI-2 scheme during the recycling time to allow a short inter-scan delay of 0.25 s, and an Ernst angle pulse at 120° was used for the first pulse applied on the ^{1}H channel. The spectrum was recorded with 25% non-uniform sampling, 128 t_1 increments and 54 scans per increment, and an acquisition time of 0.3 seconds. The spectral widths were 15.6 x 90 ppm in the ^{1}H and ^{13}C dimensions, centred at 2.5 and 175 ppm, respectively. GARP decoupling was applied on the ^{13}C channel during ^{1}H detection, using a band selective Reburp.1000 shaped pulse of 650 µs applied on quaternary carbons. A long-range coupling delay of 33 ms was used, as for the UF HETCOR experiment. Compared to the original IMPACT-HMBC (Ref. 59 in the main article) experiment, the pulse sequence was not constant-time and did not include a $^{1}J_{CH}$ filter, since those elements led to a decrease in sensitivity for the peaks of interest.

6. Reference HMBC in MeOD

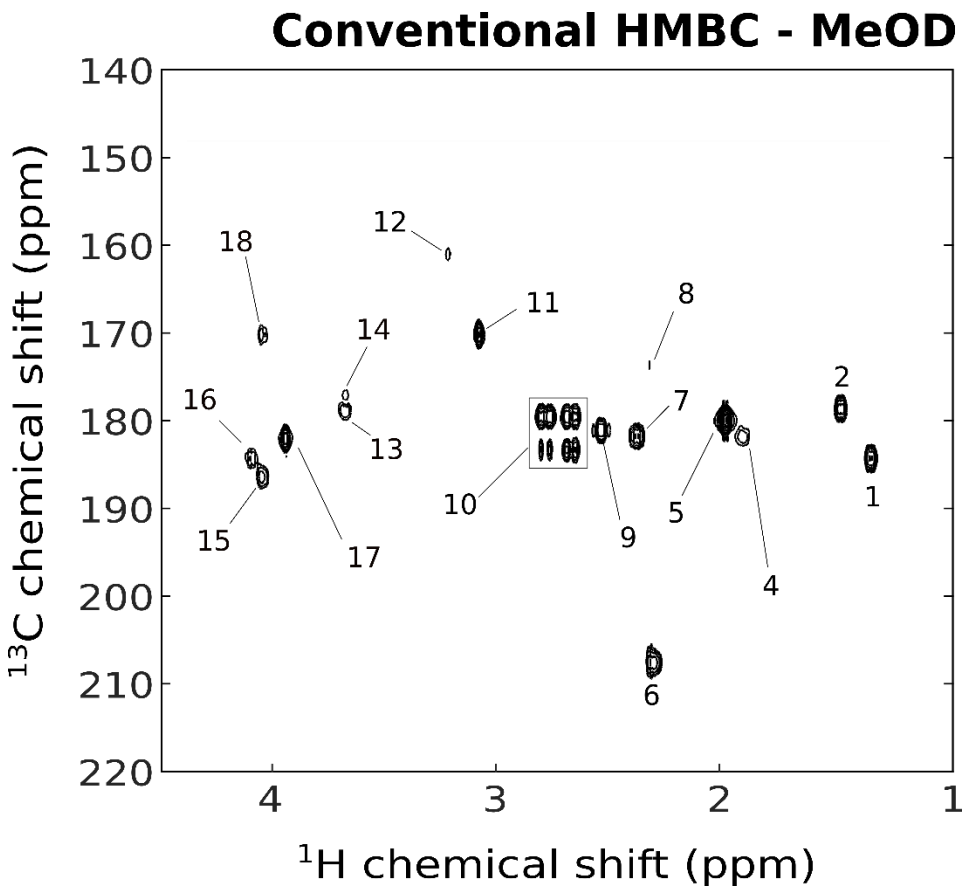

Fig. S3: Reference spectrum acquired at 400 MHz with a conventional HMBC pulse sequence in 3 hours with 4 scans and 512 t_1 increments on 10-metabolites mixture sample dissolved in MeOD. Individual metabolite concentrations are at 100 mM. Labels are detailed in Table S2.

7. Stabilization effect in dissolution in D_2O

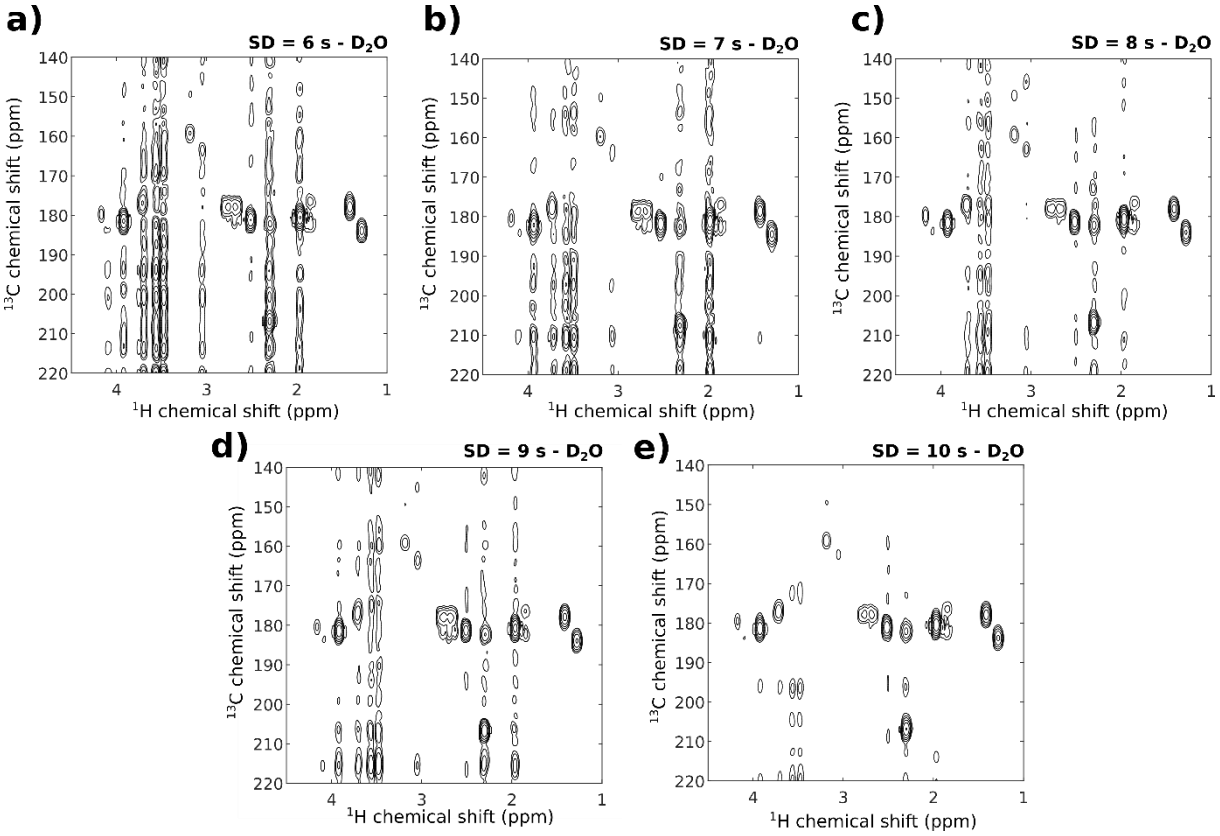

Fig. S4: UF long-range HETCOR spectra recorded after 21 minutes of hyperpolarization, 3.2 seconds of transfer and 6 to 10 seconds of stabilization delay respectively (from a to e). Each experiment has been recorded after a dissolution in D_2O solvent, on a 400 MHz equipped with a BBO prodigy probe at 298 K.

8. Triplicate experiments comparison

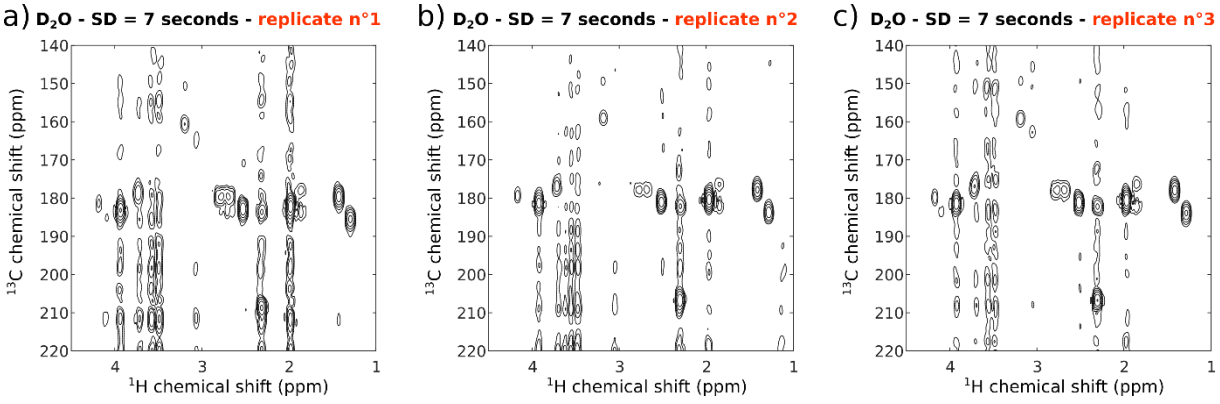

Fig. S5: UF long-range HETCOR spectra recorded after 21 minutes of hyperpolarization, 3.2 seconds of transfer and 7 seconds of stabilization delay (SD) respectively on the 10-metabolites mixture at 200 mM (input). These triplicate experiments were recorded after D_2O dissolution, on a 400 MHz equipped with a BBO prodigy probe at 298 K.

9. Stabilization effect in dissolution in MeOD

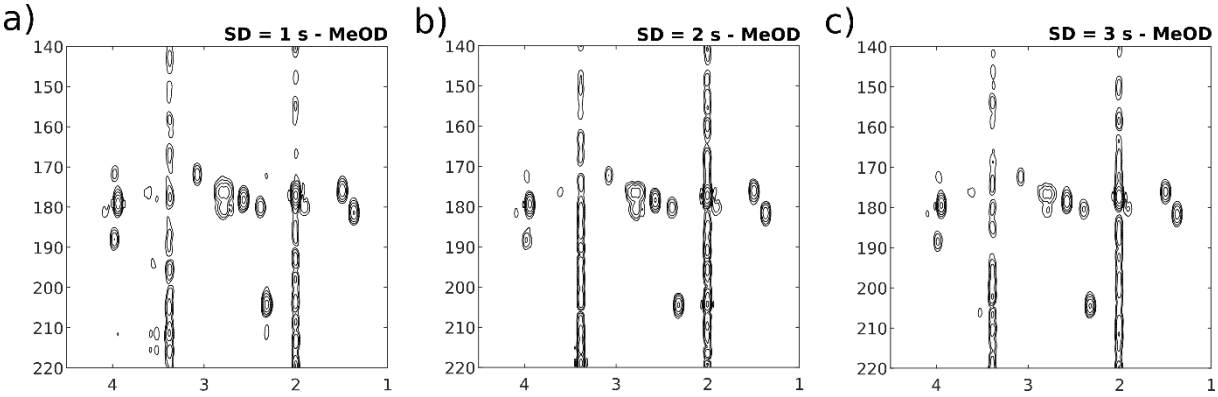

Fig. S6: UF long-range HETCOR spectra recorded after 21 minutes of hyperpolarization, 3.2 seconds of transfer and 1 to 3 seconds of stabilization delay (SD) respectively (from a to c). Each experiment has been recorded after a dissolution in MeOD solvent, on a 400 MHz equipped with a BBO prodigy probe at 293 K.

10. Comparison ^{1}H reference spectra in MeOD and in D_2O

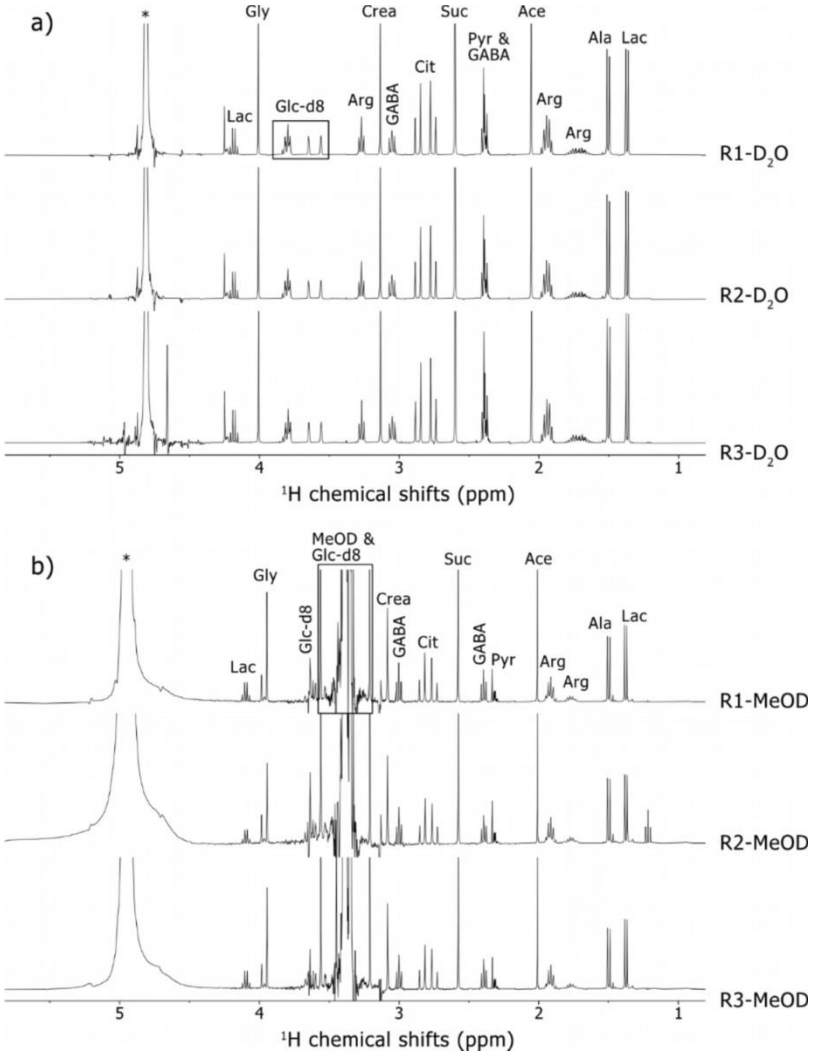

Fig. S7: ^{1}H reference spectra recorded at thermal equilibrium following d-DNP experiments (about 5 minutes after dissolution) on the post-dissolution NMR tube containing the 10-metabolites sample at 200 mM after dissolution. Individual metabolite concentration is about 3 mM in D_2O samples and 1.6 mM in MeOD ones, considering the dilution factors induced by the dissolution process and the metabolite solubility. a) Triplicates recorded after

*dissolution in D_2O and b) after dissolution in MeOD. Spectra were processed the same way, aligned and normalized by the same scale for comparison. * corresponds to the residual water signal. Glc-d8 stands for glyrcerol-d_8.*

11. Linear regression plots for metabolites in D_2O (not described in the main article)

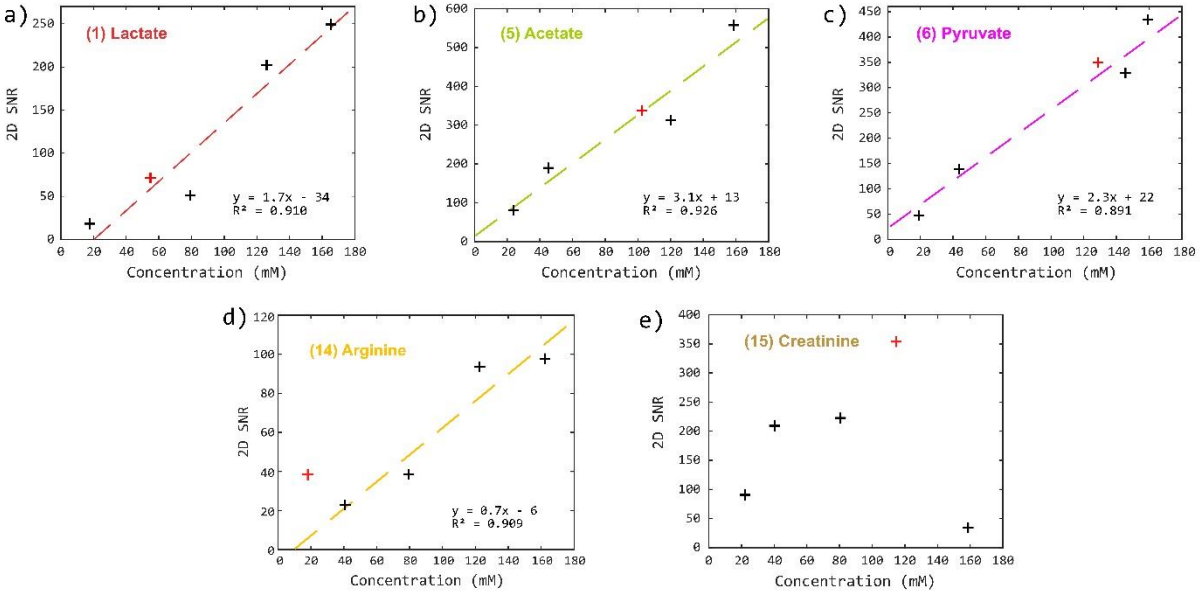

Fig. S8: Additional linear regressions for a) lactate (1), b) acetate (5), c) pyruvate (6), d) arginine (14) and creatinine (15) correlation signals, plotting 2D SNR of each 2D correlation with respect to their metabolite concentration in mM. Label correspondences are given in Table S2. No linear regression was performed for creatine (15) for the reason explained in the manuscript.

12. Linear regression plots for all metabolites in MeOD and the corresponding result Table.

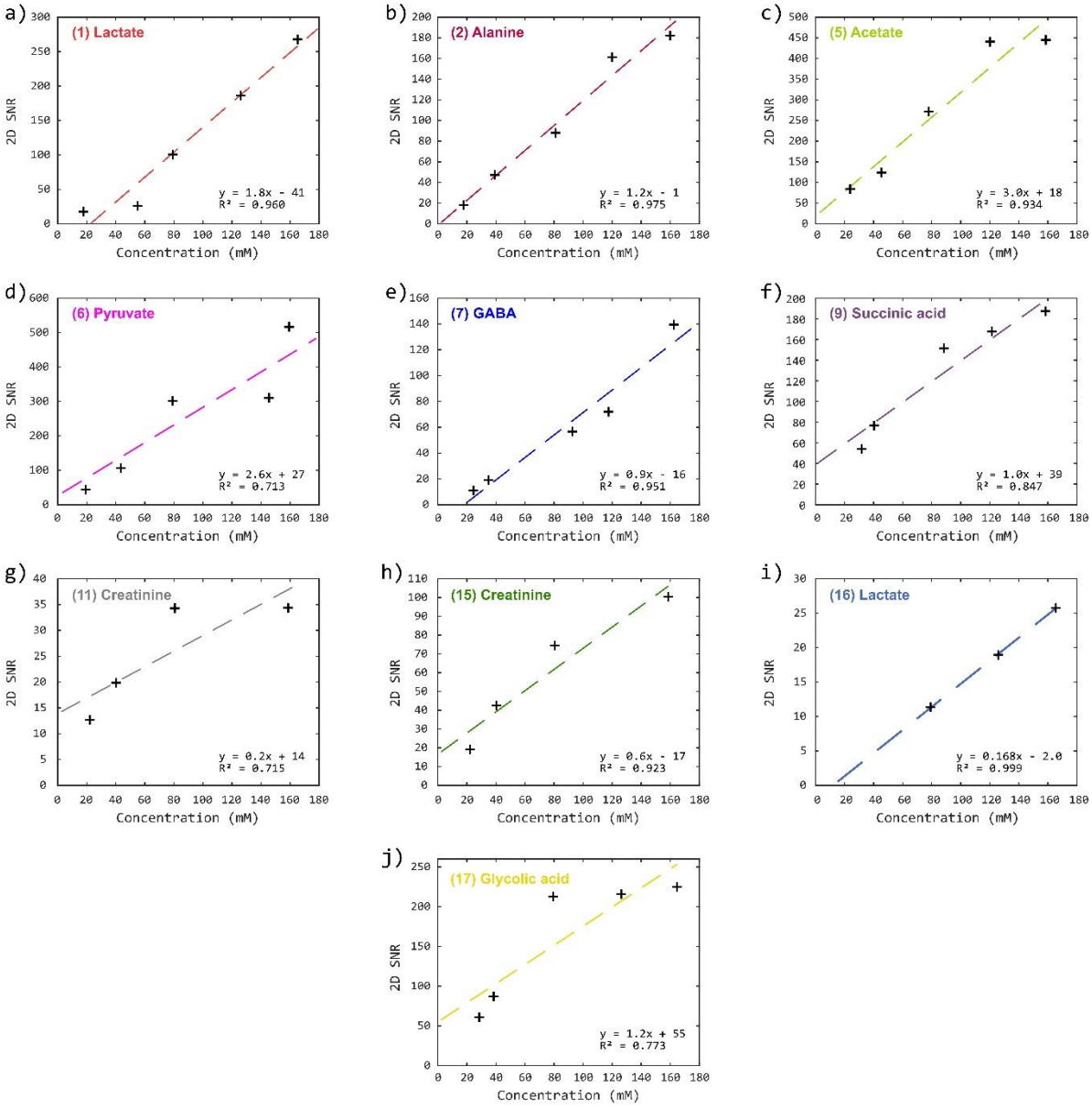

Fig. S9: Linear regressions for lactate (1 and 16), alanine (2), acetate (5), pyruvate (6), GABA (7), succinic acid (9), creatinine (11 and 15) and glycolic acid (17) correlation signals, plotting 2D SNR of each 2D correlation with respect to their metabolite concentration in mM. Label correspondences are given in ***the Table S2****. Creatinine (11) and (15) and lactate (19) 2D correlation SNR regressions were plotted with less than 5 points, as their 2D SNR were below the limit of detection (2D SNR < 3) for the lowest concentrated samples.*

Peak	Metabolite	Slope	y-intercept	R^2	LOQ_{calc} (input/ mM)
1	Lac ($^3J_{CH}$)	1.8 ± 0.2	-41 ± 22	0.960	28
2	Ala ($^3J_{CH}$)	1.2 ± 0.1	-2 ± 11	0.975	9.5
5	Ace ($^2J_{CH}$)	3 ± 1	18 ± 45	0.934	2.8
6	Pyr ($^2J_{CH}$)	3 ± 1	27 ± 99	0.902	6.5
7	GABA ($^2J_{CH}$)	0.9 ± 0.1	-16 ± 12	0.951	30
9	Suc ($^2J_{CH}$)	1.0 ± 0.2	39 ± 25	0.847	29
11	Crea ($^3J_{CH}$)	0.2 ± 0.1	14 ± 6	0.715	26
15	Crea ($^2J_{CH}$)	0.6 ± 0.1	17 ± 11	0.923	12
16	Lac ($^2J_{CH}$)	0.17 ± 0.03	$- 2.0 \pm 0.4$	0.999	72
17	Gly ($^2J_{CH}$)	1.2 ± 0.4	55 ± 38	0.773	37

Table S3: Linear regression equations. R^2 and calculated LOQ for 10 chemical sites, among 8 metabolites after dissolutions performed in MeOD solvent. LOQ is calculated by solving the linear regression equations with y = 10 (approximation of the SNR value that should correspond to the lower concentration that can be quantified with the method). It corresponds to the minimal input concentration that can be prepared in the DNP cup to have final signal 2D SNR of at least 10. ΔLOQ corresponds to the uncertainty of the LOQ calculation. determined by the linear fit.

13. Repeatability assessment at 25 mM, close to the mean LOQ determined in D_2O

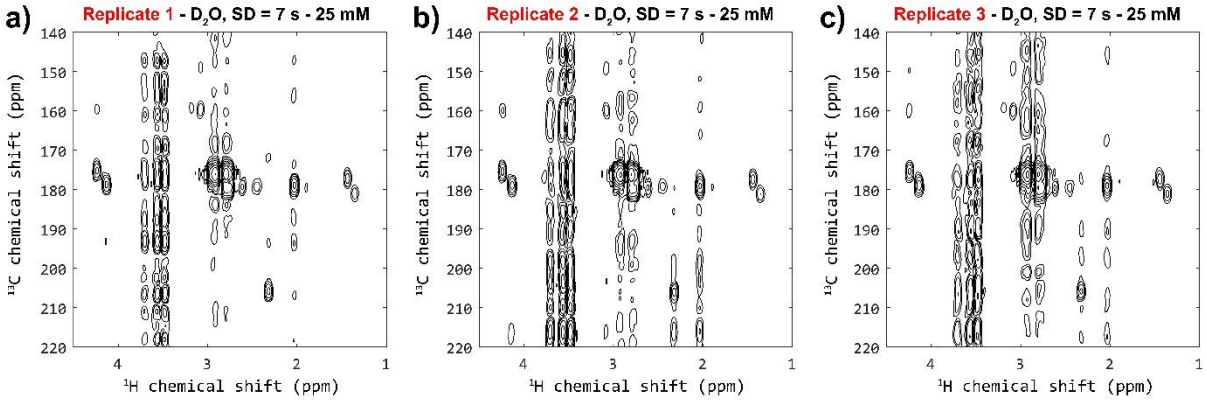

Fig. S10: UF long-range HETCOR spectra recorded after 20 min of hyperpolarization, 3.2 s of transfer and 7 seconds of stabilization in D_2O, on the same 10-metabolites mixture prepared at 25 mM each, and dissolved with 50 mM TEMPOL in the DNP juice. Liquid-state NMR detection was performed at 400 MHz, with a BBO prodigy probe and at 298 K.