Supplementary Materials

A Method to Monitor the NAD$^+$ Metabolome—From Mechanistic to Clinical Applications

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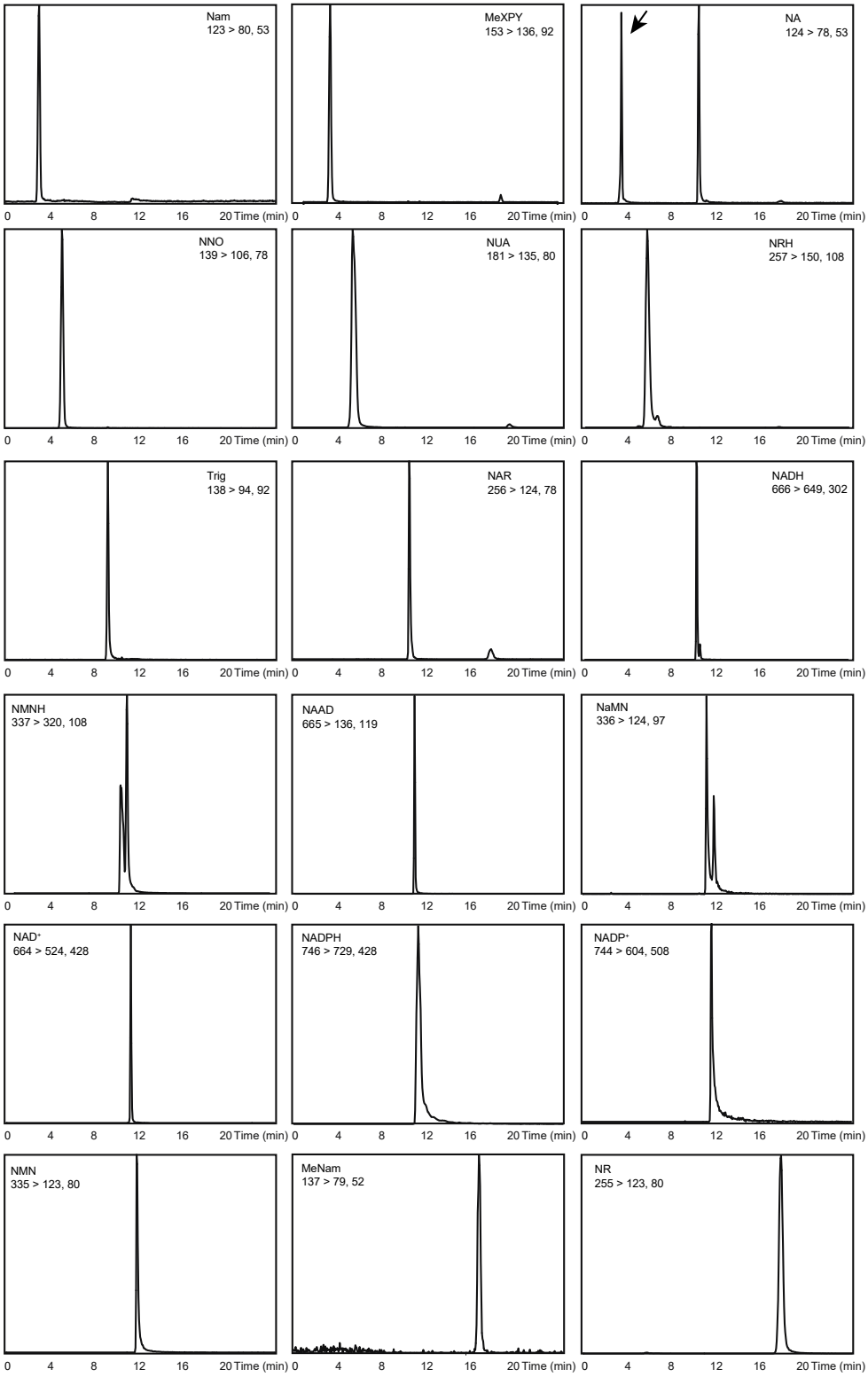

Figure S1. HILIC-ESI$^+$-MS/MS chromatograms of the 18 metabolites part of the NAD$^+$ metabolome (standard compounds) used in this study, obtained from the analysis of pools of standard compounds solutions, expressed in relative abundance (arbitrary units), and maximized per analyte. The arrow indicates the signal of NA (that has a chemical interference with NAR at a later retention time).

Table S1. Comparison of 3 sample preparation procedures in detecting the NAD+ metabolome of Hep G2 cells: biphasic extraction (BE), 80% cold methanol (80MeOH) and 40:40:20 with 0.1 M formic acid (40:40:20) extraction (n = 5, average ± SD). Values expressed in ratios of LC-MS signal intensity normalized to internal standard and total protein content (arbitrary units).

Extraction	Nam	NRH	Trig	NADH	NMN	NR	NMNH	NAD+	NADPH	NADP+
Average										
BE	0.25	0.01	0.01	6.85	0.07	0.14	0.00	11.02	2.66	0.67
80MeOH	0.15	0.02	0.01	6.17	0.06	0.10	0.01	10.37	1.40	0.63
40:40:20	0.10	0.01	0.00	4.64	0.05	0.00	0.01	9.44	2.11	0.59
Standard Deviation										
BE	0.04	0.00	0.00	0.91	0.01	0.03	0.00	0.68	0.19	0.09
80MeOH	0.02	0.00	0.01	0.65	0.01	0.01	0.01	0.91	0.23	0.12
40:40:20	0.02	0.01	0.00	0.11	0.00	0.00	0.01	0.83	0.22	0.04

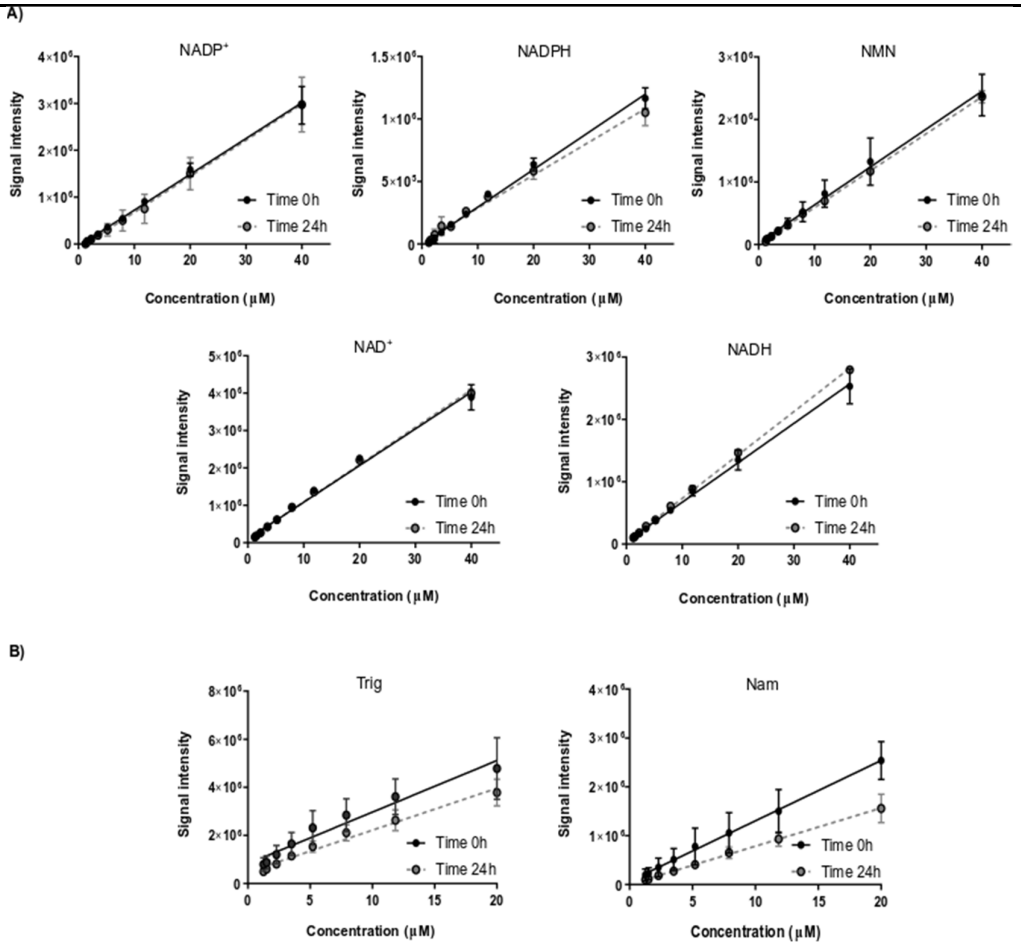

Figure S2. LC-MS metabolite signal intensity for NAD+ metabolites in standard solutions before and after 24 h at 4 °C (autosampler temperature) for a concentration range **A,** 1.25–40 µM (9 points) or **B,** 1.25–20 µM (8 points). Average ± standard deviation values (n = 3).

Table S2. NAD^+ metabolome of Hep G2 cells upon administration of Nam, NR or NRH (0.5 mM) and control (Ctrl), (*n* = 4, average ± SD). Values expressed in ratios of LC-MS signal intensity normalized to internal standard and total protein content (arbitrary units).

	Nam	NRH	NADH	NMN	NR	NMNH	NAD^+	NADPH	$NADP^+$
Average									
Ctrl	0.52	0.04	2.84	0.06	0.29	0.00	6.39	1.52	0.43
NA	0.38	0.02	3.32	0.06	0.21	0.00	6.51	1.46	0.50
Nam	5.58	0.01	3.14	0.06	0.21	0.01	7.24	1.47	0.48
NR	3.21	0.63	3.17	0.16	99.74	0.01	9.92	1.50	0.58
NRH	1.11	100.60	19.17	4.60	148.27	17.21	98.01	2.37	0.72
Standard Deviation									
Ctrl	0.19	0.01	0.49	0.01	0.10	0.01	1.22	0.26	0.09
NA	0.06	0.01	0.55	0.01	0.08	0.01	0.35	0.16	0.06
Nam	0.58	0.00	0.15	0.01	0.08	0.01	0.41	0.22	0.06
NR	0.97	0.15	0.75	0.04	24.44	0.02	0.87	0.20	0.05
NRH	0.13	11.10	1.24	0.16	25.77	1.06	2.25	0.33	0.03

Table S3. NAD^+ metabolome of murine plasma, urine, kidney, liver, pancreas, and muscle.

ID	Urine (*n* = 8)	Plasma (*n* = 7)	Kidney (*n* = 5)	Liver (*n* = 8)	Pancreas (*n* = 7)	Muscle (*n* = 8)
Nam	X	X	X	X	X	X
MeXPY	X	X	X	X	X	X
NA	-	-	-	-	-	-
NNO	X	X	-	-	-	-
NUA	-	-	-	-	-	-
NRH	X	X	X	X	X	X
Trig	X	X	X	X	X	X
NAR	-	-	-	-	-	-
NADH	-	-	X	X	X	X
NMNH	-	-	-	-	-	-
NAAD	-	-	-	-	-	-
NAMN	-	-	-	X	-	-
NAD^+	-	-	X	X	X	X
NADPH	-	-	X	X	X	X
$NADP^+$	-	-	X	X	X	X
NMN	X	-	X	X	X	X
MeNam	X	-	X	-	-	-
NR	X	X	X	X	X	-

ID: metabolite acronym; X = detected metabolite; - = not detected metabolite; n = number of biological replicates tested.

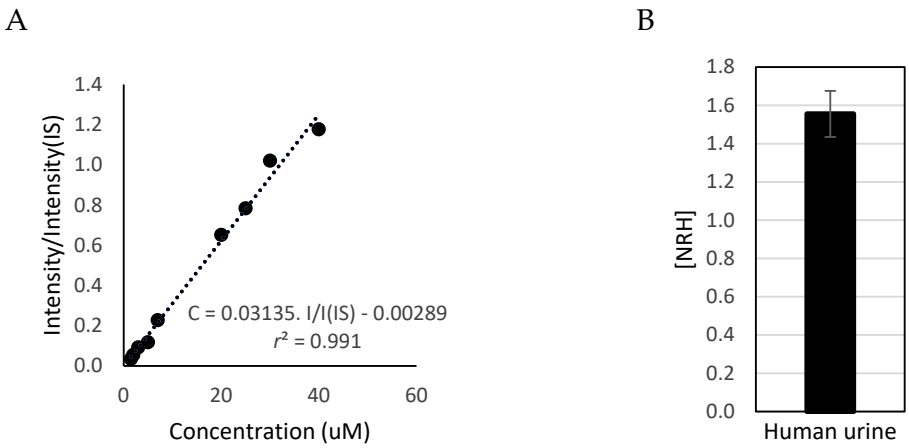

Figure S3. (**A**) Calibration curve of NRH; (**B**) quantification of NRH (μM) in 2 urine replicates (pool of at least 2 donors).

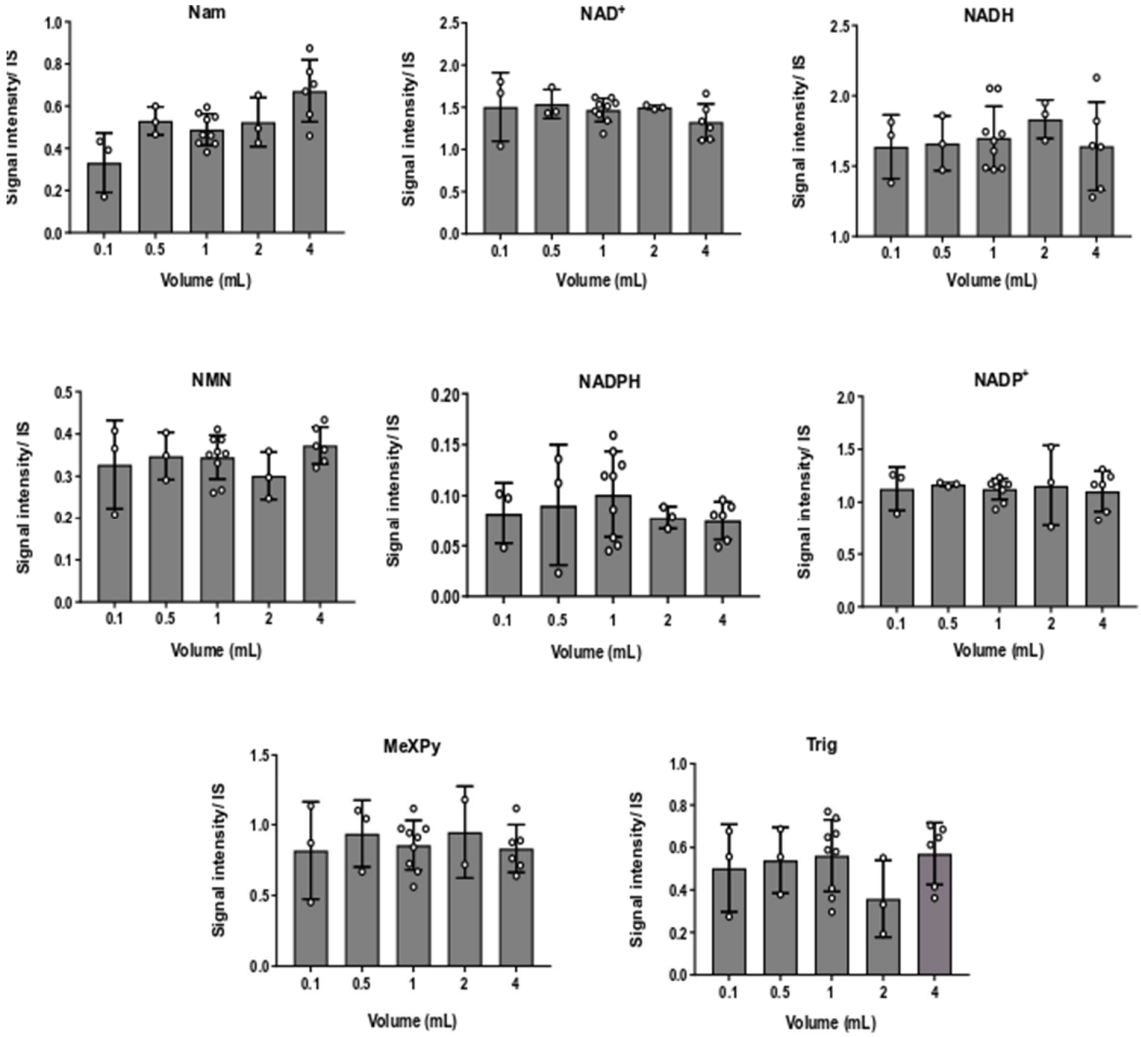

Figure S4. LC-MS metabolite signal intensity per internal standard for NAD^+ metabolites in human whole blood according to aliquot volume (0.1, 0.5, 1, 2, 4 mL) at blood draw. Average ±standard deviation values from 3 donors (except for MeXPY at 2 mL, 2 donors were analyzed; per aliquot and subject $n = 1$ for 0.1 mL, 0.5 mL, 2 mL, $n = 2$ for 4 mL, and $n = 3$ for 1 mL).

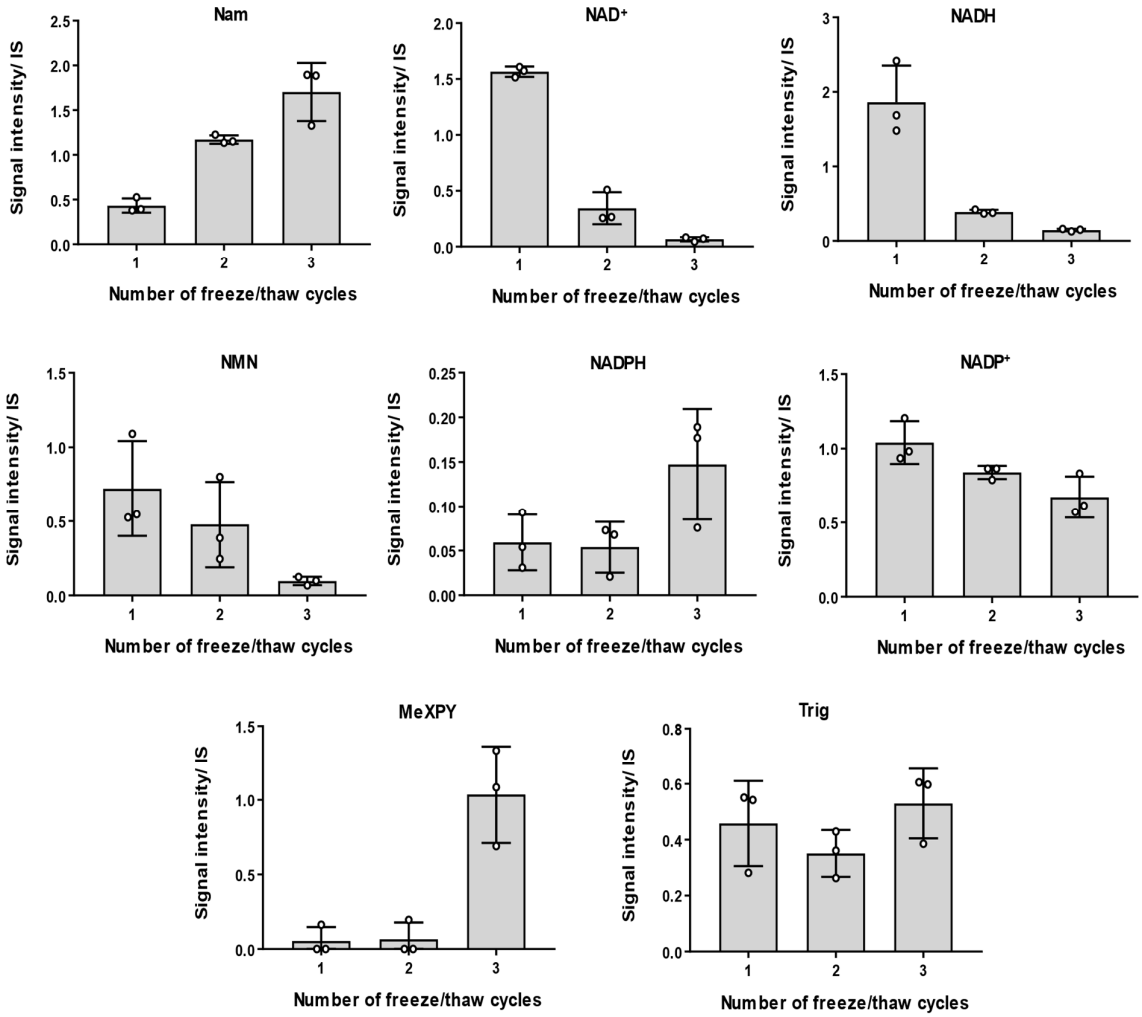

Figure S5. LC-MS metabolite signal intensity per internal standard for NAD^+ metabolites in human whole blood after 3 freeze-thaw cycles (1, 13, 21 days). Average ± standard deviation values from 3 donors ($n = 3$).

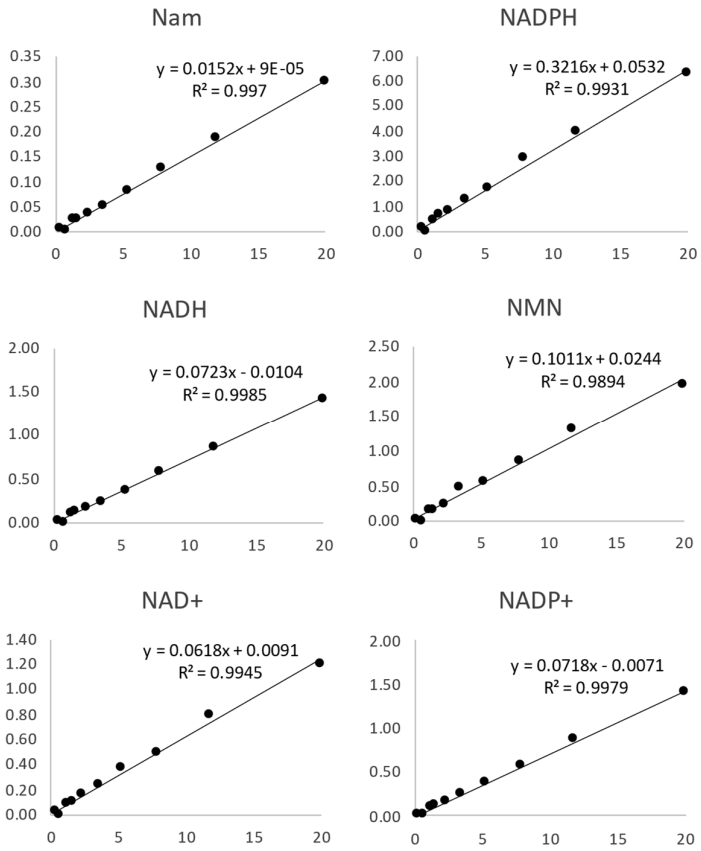

Figure S6. Calibration curves of NAD^+ metabolites relating LC-MS metabolite signal intensity per internal standard and metabolite concentrations (0.3-20 μM, 10 points). Average ± standard deviation values.

Table S4. NAD^+ metabolome quantification (expressed in μM) in human whole blood of 9 volunteers (average and standard deviation of 3 aliquots per donor).

Average	Nam	NADH	NAD+	NADPH	NMN	NADP+
D1	8.0	14.8	17.5	11.0	2.5	12.8
D2	7.8	14.7	17.7	16.9	2.2	15.8
D3	10.1	13.6	15.2	15.6	2.5	15.5
D4	8.8	15.8	18.1	15.3	2.5	19.3
D5	7.4	13.0	15.5	10.6	2.8	14.7
D6	9.8	13.4	15.6	11.1	2.0	14.2
D7	4.9	7.8	10.5	7.1	2.0	13.2
D8	7.9	13.2	15.6	8.8	3.5	19.0
D9	5.5	12.2	15.1	11.5	2.7	19.4
St Dev	**Nam**	**NADH**	**NAD+**	**NADPH**	**NMN**	**NADP+**
D1	0.3	1.4	2.0	0.3	0.4	1.2
D2	0.5	1.3	1.4	1.0	0.4	4.9
D3	1.4	1.2	1.6	2.5	0.6	5.7
D4	0.8	0.6	0.1	0.6	0.2	4.1
D5	0.7	1.3	1.1	1.2	0.1	4.3
D6	0.5	0.2	0.6	0.6	0.2	5.4
D7	0.3	1.0	1.4	0.8	0.0	2.7
D8	0.2	0.6	0.6	0.1	0.2	0.4
D9	0.4	1.0	0.4	0.4	0.3	5.9
Min	10.1	15.8	18.1	16.9	3.5	19.4
Max	4.9	7.8	10.5	7.1	2.0	12.8

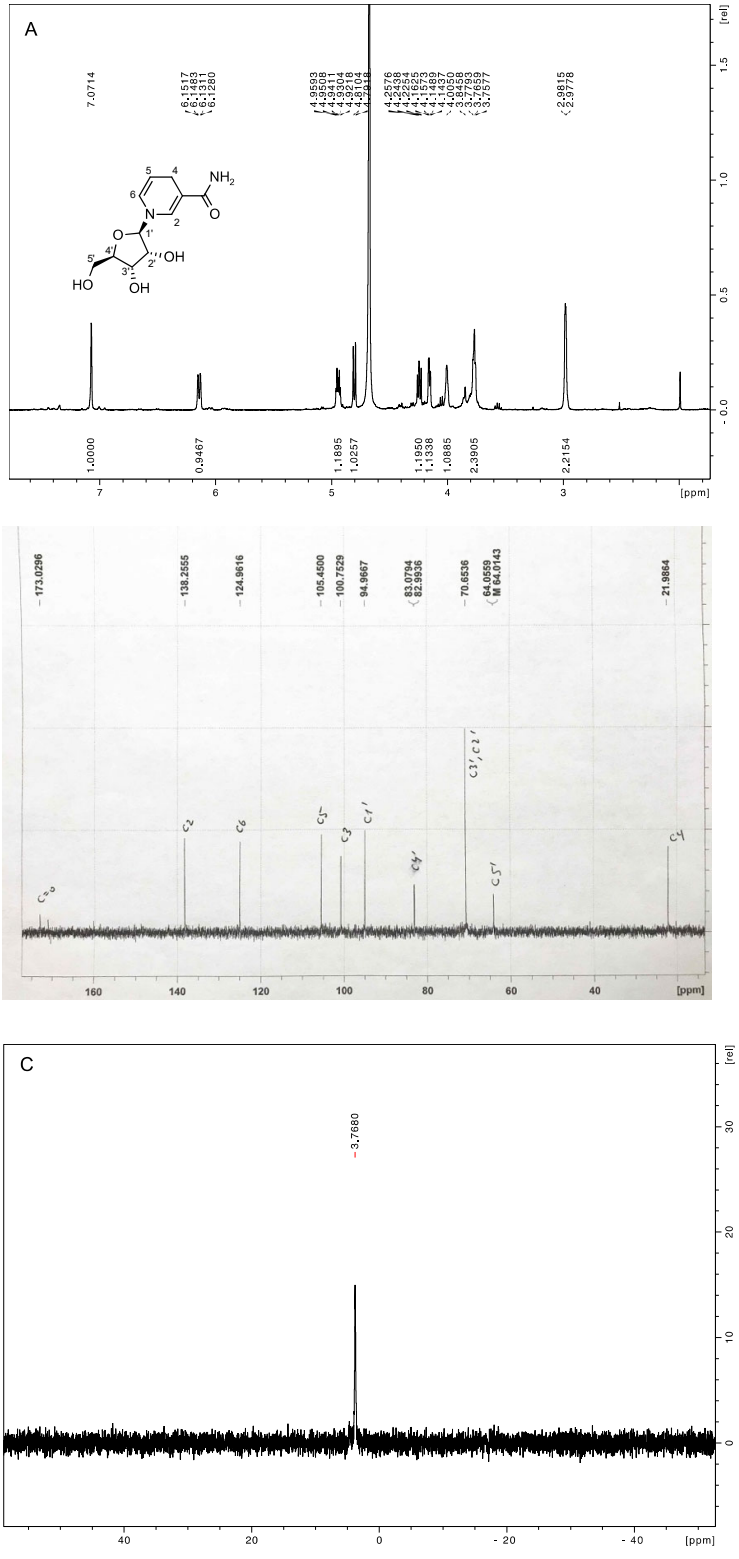

Figure S7. NMR spectra of NMNH in D_2O. **A**, ^{1}H NMR; **B**, ^{13}C NMR; and **C**, ^{31}P NMR.