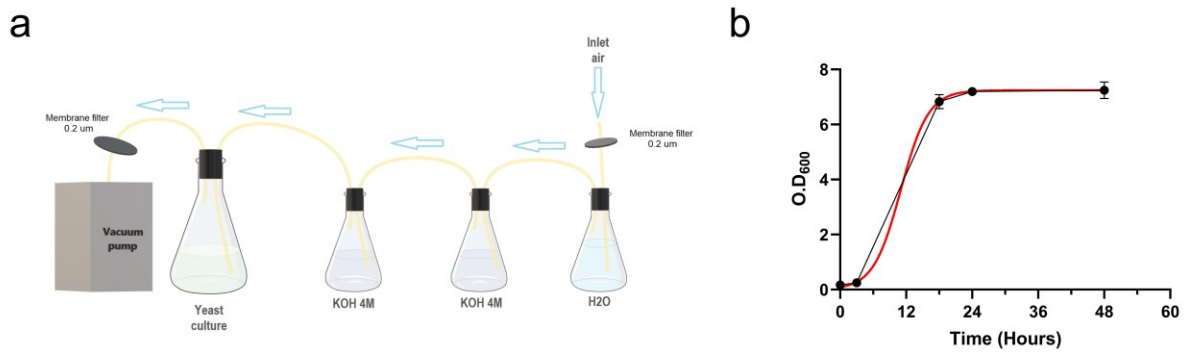
Appendix 2: Supplementary files for chapters 3 and 4

Supplementary table 5: Minimal media composition for Pichia pastoris yeast culture.

Trace Salt solution composition	
Component	**Concentration (g/L) or volume**
$CuSO_4$*5 H_2O	6
NaI	0.08
$MnSO_4$* H_2O	3
Na_2MoO_4*2 H_2O	0.2
H_3BO_3	0.02
$CoCl_2$*6 H_2O	0.05
$ZnCl_2$	20
H_2SO_4 (97% w/w)	5 mL
$FeSO_4$*7 H_2O	5
ddH_2O	q.s. for 1L
Yeast culture media composition	
Component	**Concentration (g/L) or volume**
$MgSO_4$*7 H_2O	0.5
KCl	0.9
$CaCL_2$*2 H_2O	0.022
85% (v/v) H_3PO_4	10.98 mL
Trace Salt Solution	4.6 mL
Biotin (0.2 g/L solution)	2 mL
($U^{13}C$) Glucose	10
25% v/v ammonia	Use to adjust pH to 5
ddH_2O	q.s. for 1L

Supplementary figure 13: *Pichia pastoris* yeast culture in minimal media with CO_2 quenching. a: Schematic diagram of the yeast culture system including the CO_2 adsorbing solutions. b: Yeast growth curve in minimal media at 28°C with agitation (250 rpm) and CO_2 quenching.



Supplementary table 6: NAD-related metabolites concentrations in external calibrators prepared by serial dilution of the NAD standard mix (NSM).

	Metabolites concentrations (µM)										
Compounds	**NSM 1**	**NSM 2**	**NSM 3**	**NSM 4**	**NSM 5**	**NSM 6**	**NSM 7**	**NSM 8**	**NSM 9**	**NSM 10**	**NSM 11**
All NAD^+-related metabolites excluding NUA, NR, and cADPR	0.0195	0.0391	0.0781	0.1563	0.3125	1.5625	3.125	6.25	12.5	25	50
NUA and NR	0.0020	0.0039	0.0078	0.0156	0.0313	0.1563	0.3125	0.625	1.25	2.5	5
cADPR	0.0012	0.0023	0.0047	0.0094	0.0188	0.0938	0.1875	0.375	0.75	1.5	3

Supplementary table 7: HPLC and MS/MS parameters for quantitative NAD metabolomics analysis

HPLC parameters
Flow rate: 0.25 mL/min.
Column temperature: 30 °C.
The chromatographic solvent gradients were applied as detailed below:
For positive Ionization mode:
A: 10 mM Ammonium formate in water + 0.1% formic acid
B: 10 mM ammonium formate in 90% Acetonitrile + 0.1% formic acid
Gradient:

Time	% of B
0.00	98
1.00	98
1.50	90
5.00	80
8.00	60
10.70	5
12.00	5
12.70	98
16:30	98

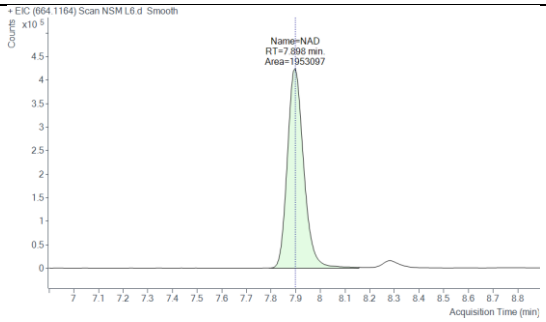
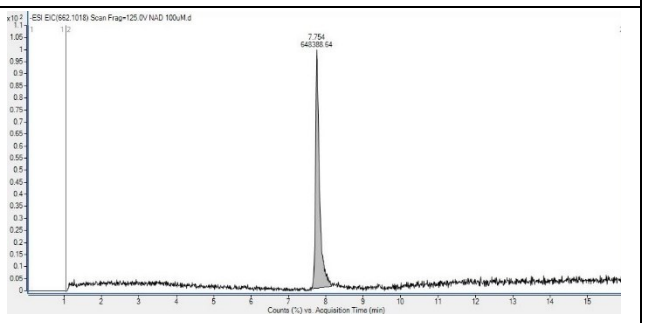
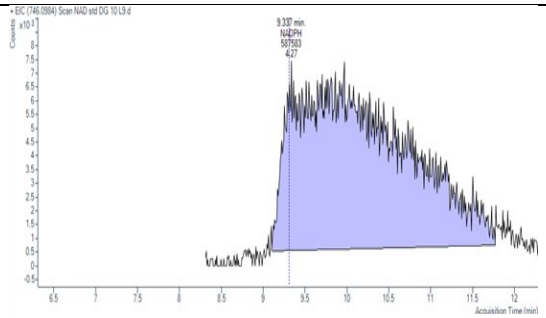
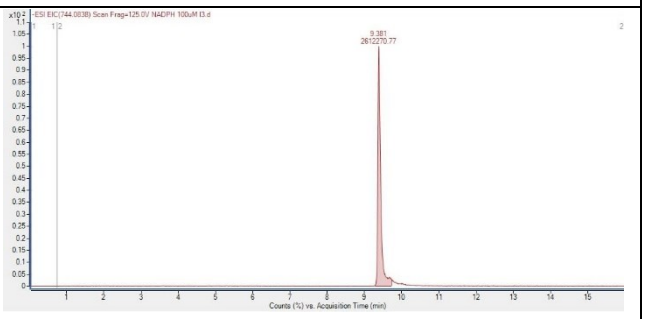
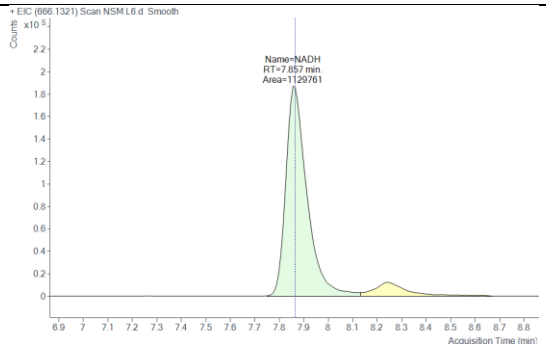
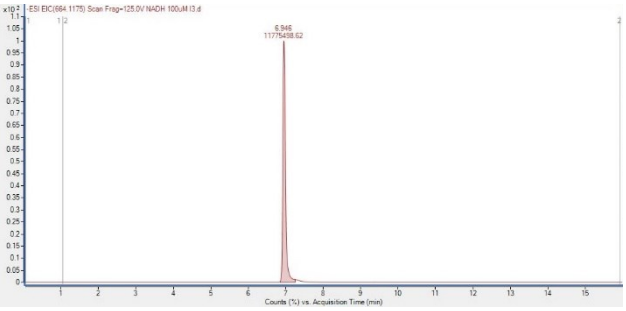
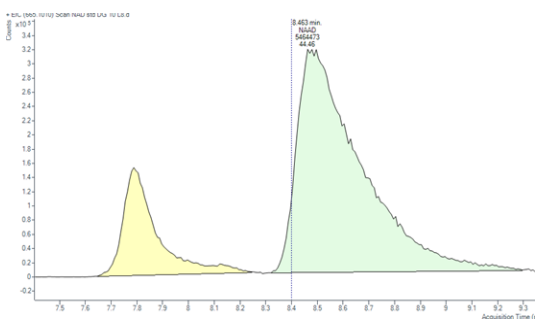
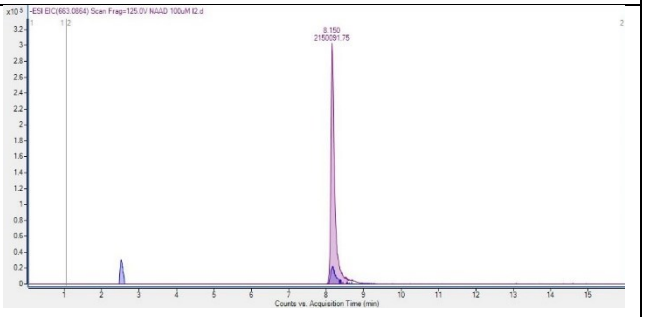
For negative ionization mode:
A: 10 mM ammonium acetate, pH 9.0 in water + 0.1% deactivator additive
B: 10 mM ammonium acetate, pH 9.0 in 85% Acetonitrile + 0.1% deactivator additive
Gradient:

Time	% of B
0.00	96
1.30	96
8.00	65
10.00	65
16:00	96

Q-TOF MS conditions:

Ionization mode	ESI (Electro-spray ionization) in negative or positive mode (Dual Agilent Jet Stream ESI)
Gas temperature	200 °C
Drying gas flow	10 L/min
Nebulizer	40 psi
Fragmentor voltage	125 V
Capillary voltage	1700 V
Collision energy	0 V
Collision cell gas pressure	22.0 psi
Acquisition parameters	Data acquired at 2 GHz extended dynamic range.

Supplementary figure 14: Examples of NAD metabolites external standards extracted ion chromatograms (EICs) in positive and negative ionization mode. EICs for (a) NAD^+, (b) NADPH, (c) NADH, and (d) NAAD in Positive (left) and Negative (right) ionization modes.

	Positive ionization mode	Negative ionization mode
a. NAD^+		
b. NADPH		
c. NADH		
d. NAAD		 (Unstable, decomposition to NA)

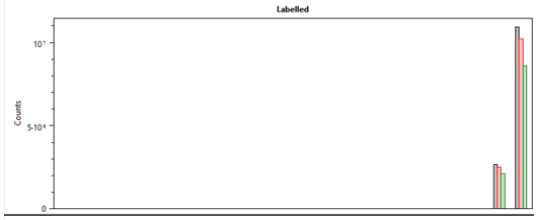
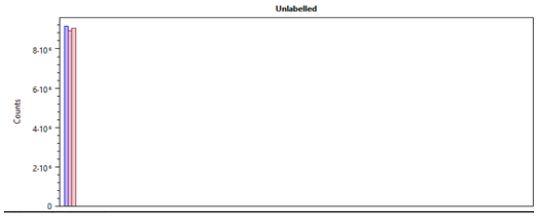
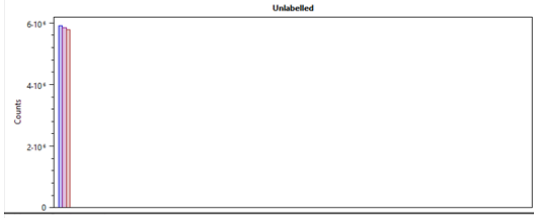
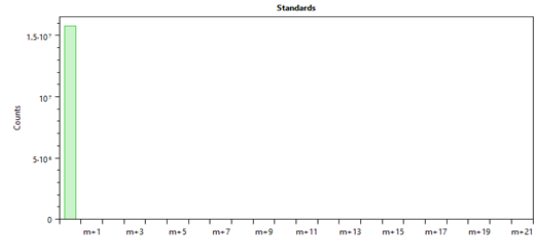
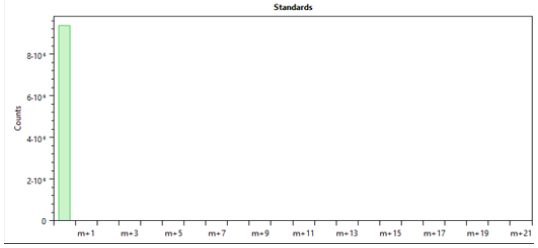
Supplementary table 8: NAD^+-related metabolites retention time (RT) repeatability in the NSM calibration levels and QC samples run on the HILIC-QTOF system for 2 consecutive days. QC samples were prepared by mixing mouse liver extracts. All the detected metabolites retention times were repeatable (RSD≤20%) and within the usual +/- 1 minute retention time window for HILIC columns. S.D.: standard deviation. R.S.D.: relative standard deviation. N.D : not detected.

	NAD standard mix calibrants						**QC samples**					
	Day 1 (n=11)			Day 2 (n=11)			Day 1 (n=9)			Day 2 (n=10)		
Metabolite	Mean RT (Min)	S.D.	**Intra-day RSD (%)**	Mean RT (Min)	S.D.	**Intra-day RSD (%)**	Mean RT (Min)	S.D.	**Intra-day RSD (%)**	Mean RT (Min)	S.D.	**Intra-day RSD (%)**
Melatonin	1.09	0.01	**0.8%**	1.10	0.01	**1.1%**	N.D			N.D		
3-HAA	1.20	0.04	**3.6%**	1.22	0.04	**3.0%**	N.D			N.D		
NAM	1.48	0.01	**0.4%**	1.47	0.00	**0.3%**	1.46	0.00	**0.3%**	1.46	0.00	**0.3%**
Me2PY	1.66	0.02	**1.1%**	1.66	0.01	**0.7%**	1.64	0.01	**0.4%**	1.64	0.01	**0.3%**
NA	2.80	0.07	**2.6%**	2.80	0.07	**2.6%**	2.84	0.07	**2.6%**	2.81	0.06	**2.3%**
Serotonin	3.48	0.01	**0.2%**	3.49	0.01	**0.2%**	3.45	0.02	**0.7%**	3.40	0.15	**4.3%**
L-Kyn	3.85	0.01	**0.2%**	3.86	0.01	**0.2%**	N.D			N.D		
L-Tryp	3.98	0.01	**0.3%**	4.00	0.00	**0.1%**	3.98	0.04	**1.1%**	3.99	0.01	**0.1%**
MeNAM	4.14	0.03	**0.7%**	4.15	0.03	**0.8%**	4.10	0.00	**0.0%**	4.12	0.00	**0.1%**
NUA	4.01	0.07	**1.8%**	4.00	0.01	**0.3%**	N.D			N.D		
3-HK	4.20	0.01	**0.1%**	4.20	0.01	**0.2%**	N.D			N.D		
5-HTP	4.64	0.01	**0.3%**	4.65	0.01	**0.2%**	N.D			N.D		
NR	4.87	0.01	**0.2%**	4.88	0.01	**0.2%**	4.85	0.00	**0.1%**	4.87	0.00	**0.1%**
NMN	7.18	0.40	**5.5%**	7.34	0.02	**0.3%**	7.54	0.07	**0.9%**	7.48	0.07	**1.0%**
NADH	7.89	0.01	**0.1%**	7.89	0.02	**0.3%**	7.90	0.01	**0.1%**	7.91	0.00	**0.0%**
NAD	7.95	0.01	**0.1%**	7.97	0.02	**0.2%**	7.95	0.01	**0.1%**	7.95	0.00	**0.0%**
cADPR	7.96	0.02	**0.2%**	7.98	0.02	**0.2%**	7.95	0.00	**0.1%**	7.95	0.00	**0.1%**
ADPR	8.01	0.01	**0.2%**	8.04	0.02	**0.2%**	8.01	0.01	**0.1%**	8.03	0.01	**0.1%**
NAMN	8.07	0.12	**1.5%**	8.06	0.01	**0.2%**	N.D			N.D		
NAAD	8.70	0.02	**0.3%**	8.73	0.02	**0.2%**	N.D			N.D		
NADP	9.44	0.03	**0.4%**	9.46	0.03	**0.3%**	9.56	0.12	**1.3%**	9.54	0.04	**0.4%**

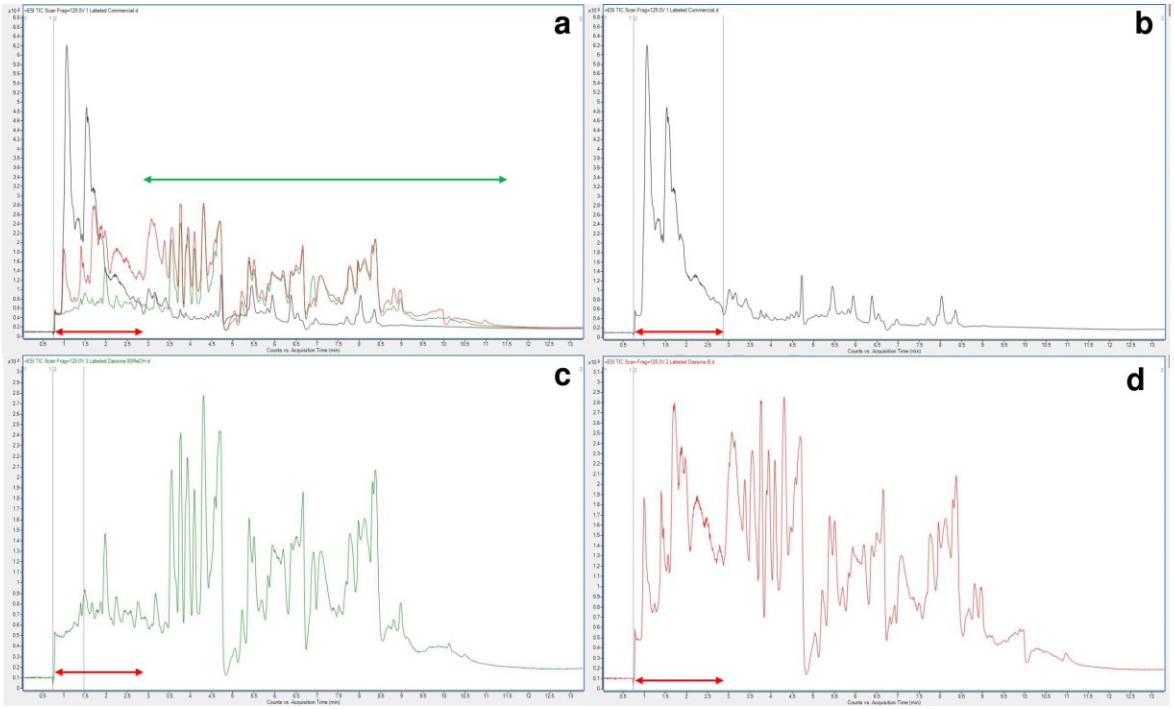
Supplementary table 9: NAD^+-related metabolites concentrations repeatability in the QC samples run on the HILIC-QTOF system for 2 consecutive days. To prepare the QC samples, mouse liver extracts were mixed and diluted 50 times before being injected in the LC/MS system. These QC samples were only used to confirm the stability of the mass spectrometer signal. All the measured metabolites relative standard deviations (RSD) were within guidelines requirements for biological samples (≤20%). Only metabolites that were within the linear dynamic range were used for the RSD determination. S.D.: standard deviation. R.S.D.: Relative standard deviation.

	QC samples					
	Day 1 (n=9)			**Day 2 (n=10)**		
Metabolite	**Mean concentration (µM)**	**S.D.**	**Intra-day RSD (%)**	**Mean concentration (µM)**	**S.D.**	**Intra-day RSD (%)**
ADPR	92.08	0.86	**0.9%**	94.09	1.63	**1.7%**
cADPR	105.70	1.63	**1.5%**	106.40	1.89	**1.8%**
L-Tryp	0.55	0.02	**3.2%**	0.56	0.03	**5.3%**
Me2PY	0.24	0.02	**6.6%**	0.26	0.01	**4.7%**
MeNAM	0.65	0.02	**2.4%**	0.67	0.01	**1.4%**
NAAD	194.14	2.86	**1.5%**	201.41	2.66	**1.3%**
NAD	1547.78	25.21	**1.6%**	1551.17	12.64	**0.8%**
NADH	144.45	3.75	**2.6%**	144.02	3.58	**2.5%**
NAM	30.71	5.72	**18.6%**	38.33	6.82	**17.8%**
NR	0.30	0.01	**3.7%**	0.37	0.02	**4.6%**

Supplementary figure 15: Isotopic enrichment in ^{13}C-labeled yeast extract versus unlabeled yeast extract and external standards calibration level 7 (NSM7). Figures below show the metabolites for which a small fraction of isotopologues with one unlabeled carbon atom were observed (M+20). No unlabeled peaks were observed in the ^{13}C-labeled yeast. For comparison, the corresponding unlabeled metabolites observed in the unlabeled yeast extract and the external standards (NSM7) are shown below.

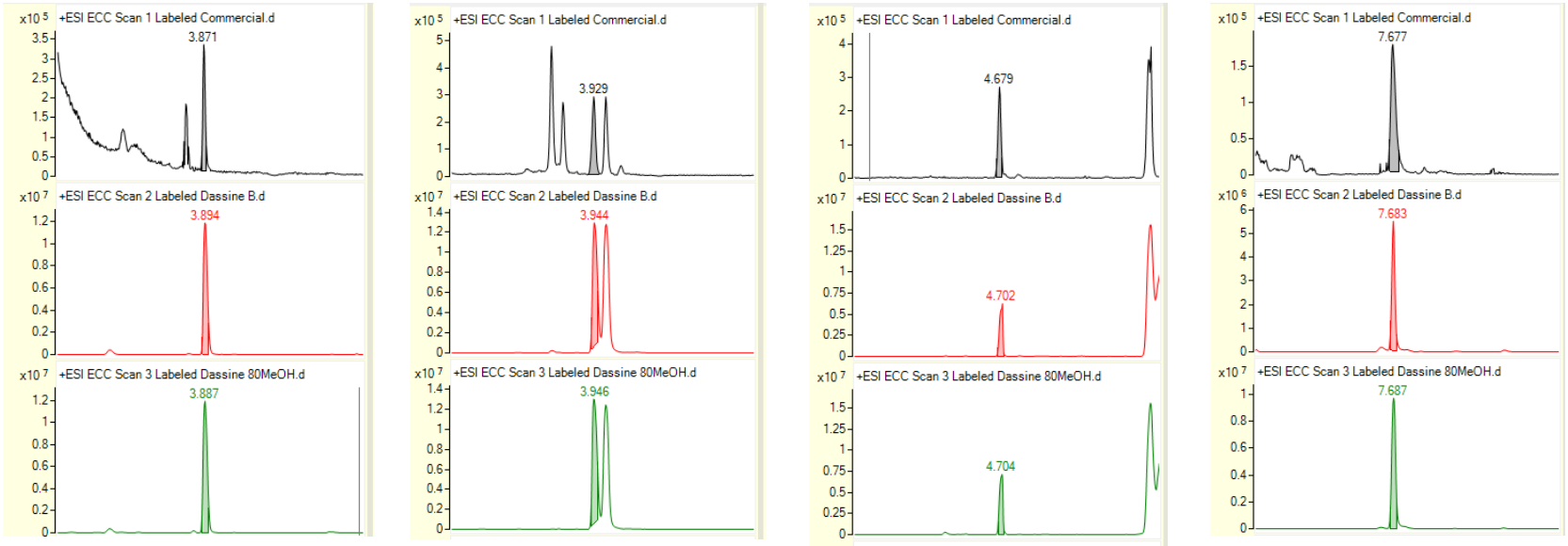
	NAD$^+$	**NADH**
^{13}C-labelled yeast extract		
Unlabelled yeast extract		
External standard (NSM7)		

Supplementary figure 16: Comparison of commercial (Black) and in-house generated ^{13}C-labeled yeast extracts (boiling ethanol extraction: red, cold methanol/dichloromethane extraction: green). a: Overlaid total ion chromatograms (TICs) showing the retention time range of interest for NAD^+-related metabolites (Green arrow) and the most contaminated area (red arrow) in the commercial yeast extract and boiling ethanol in-house extract which contain mass spectrum peaks and patterns suggesting the presence of lipid species and contaminants such as polyethylene Glycol (PEG) or PEG-Like compounds. b: Commercial yeast extract TIC, c: TIC of the cold methanol/dichloromethane extracted in-house yeast extract showing the absence of contaminants in the 1 to 3 minutes retention time window (red arrow), d: TIC of the boiling ethanol extracted in-house yeast extract.



Supplementary figure 17: Examples of extracted compound chromatograms (ECCs) of Tryptophan, leucine, NR and NAD^+ from the commercial ^{13}C-labeled yeast extract (Top row, black) and the in-house generated ^{13}C-labeled yeast extracts obtained through the hot ethanol (Middle row, red) or the cold methanol (Bottom row, green) methods.

Tryptophan **Leucine** **NR** **NAD^+**



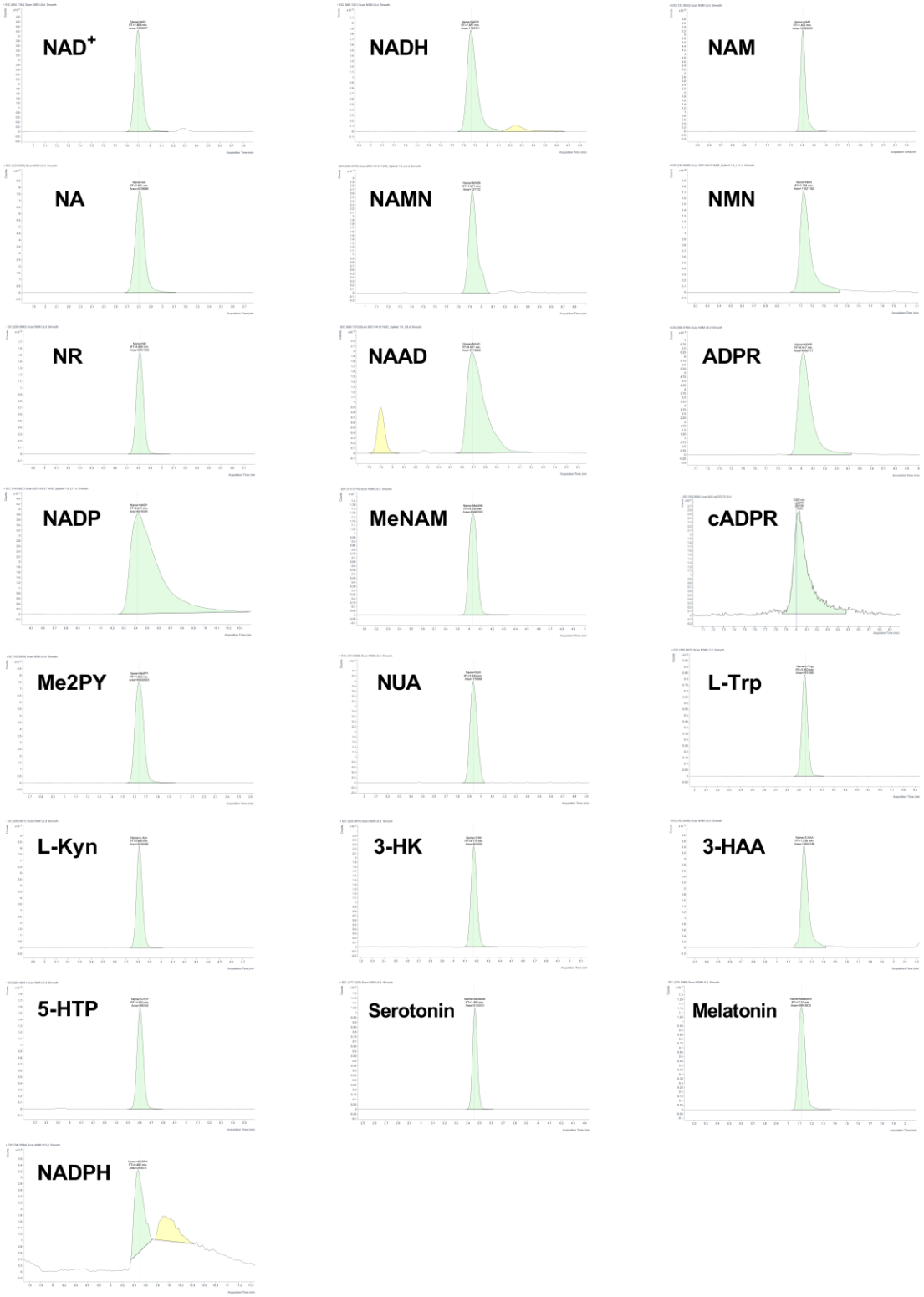
Supplementary table 10: Unlabeled and labeled metabolites peak areas in liver samples spiked with various amounts of ITSD (In-house generated ^{13}C-labeled yeast extract). The 5% spiking displayed low signal and bad signal-to-noise ratio for the majority of the metabolites. On the other hand, the 100% spiking resulted in increased ion suppression due to the addition of the yeast matrix. Both the 20% and 40% spiking ratios were suitable for most of the metabolites but the 20% was preferred as it reduced matrix effects.

Compound name	Unlabeled metabolites peak area				^{13}C-Labeled metabolites peak area				Unlabeled/labeled metabolites peak area ratios			
ITSD spiking	100%	40%	20%	5%	100%	40%	20%	5%	100%	40%	20%	5%
3-HK	2.46 x10^5	1.25 x10^5	4.88 x10^4	4.05 x10^3	1.78 x10^7	4.93 x10^6	4.88 x10^5	2.59 x10^4	0.01	0.03	0.10	0.16
cADPR	6.20 x10^5	6.68 x10^5	1.12 x10^6	1.84 x10^5	3.33 x10^5	3.10 x10^5	1.85 x10^4	2.39 x10^3	1.9	2.2	60.3	76.9
L-Tryp	2.68 x10^7	3.56 x10^7	3.47 x10^7	5.40 x10^6	1.05 x10^7	7.34 x10^6	2.28 x10^6	1.98 x10^5	2.6	4.9	15.2	27.2
NAD	1.32 x10^7	1.38 x10^7	2.27 x10^7	4.41 x10^6	7.38 x10^6	6.65 x10^6	3.71 x10^5	7.46 x10^4	1.8	2.1	61.4	59.1
NADH	2.71 x10^6	2.41 x10^6	5.87 x10^6	9.58 x10^5	1.13 x10^6	8.60 x10^5	1.08 x10^5	2.08 x10^4	2.4	2.8	54.6	46.0
NR	2.76 x10^6	3.20 x10^6	3.94 x10^6	1.28 x10^6	2.42 x10^7	1.93 x10^7	2.20 x10^6	5.40 x10^5	0.11	0.17	1.79	2.38

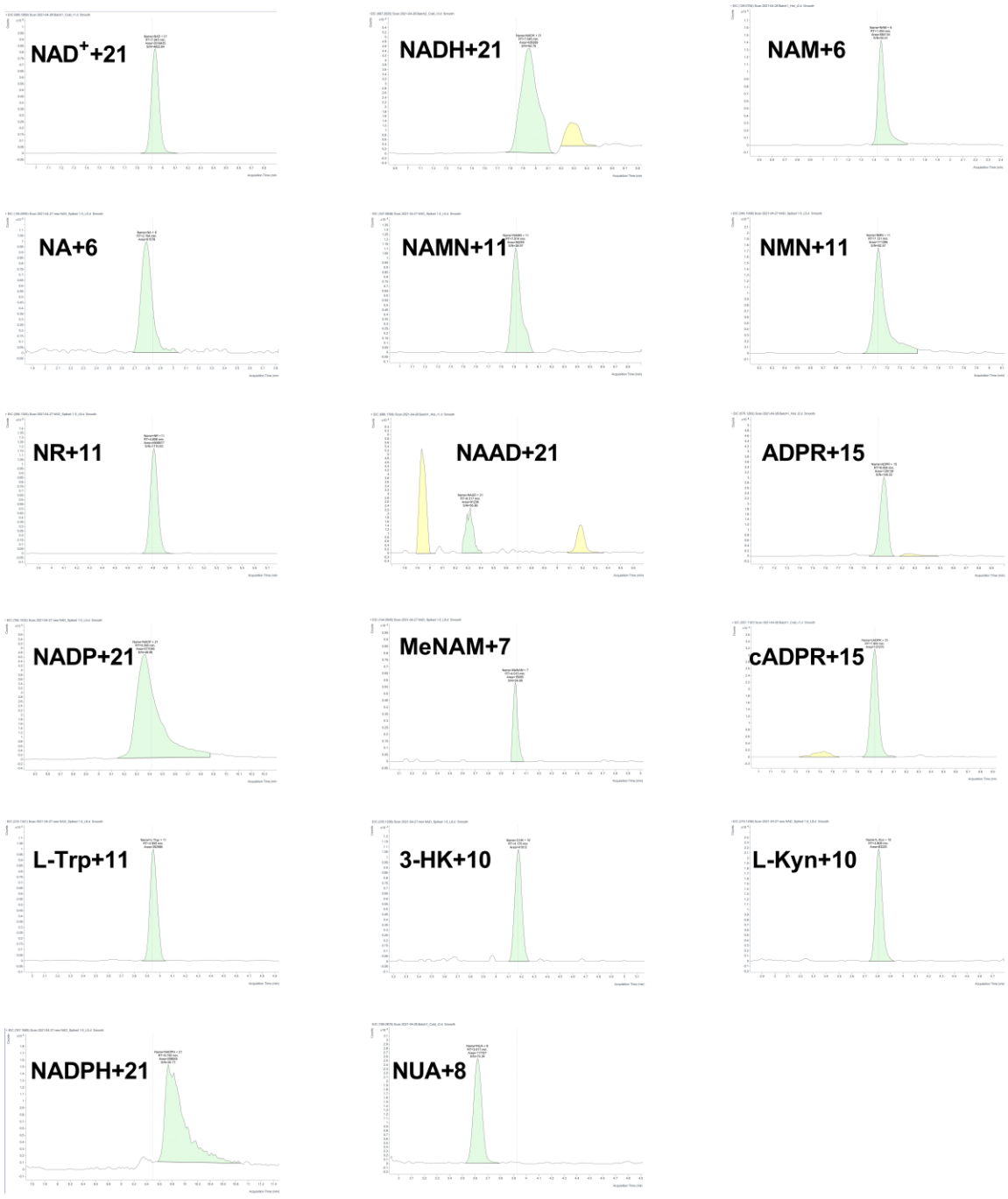
Supplementary table 11: Effect of ISTD spiking before or after tissue extraction on the measured metabolites concentrations. A pool of 3 liver extracts was spiked with 2 different ratios of ITSD: 100% and 20%, before and after the extraction with the cold MeOH/DCM method. For the ISTD spiking before the extraction, powdered liver samples were spiked with 20% or 100% of each ISTD aliquot before proceeding to the metabolite extraction as described in the methods section. For the ISTD spiking after the extraction, dry liver extracts were reconstituted with 20% (16 µl of ISTD reconstituted with 80 µl of 75% acetonitrile) and 34 µl of 75% acetonitrile for the 20% spiking or 100% (50 µl of ISTD reconstituted with 50 µl of 75% acetonitrile) to obtain a final volume of 50 µl. As previously determined, the spiking with 20% of the ITSD was the optimal ratio for internal normalization and showed a metabolite recovery between 82-121% with a mean of 98%. The spiking with 100% of ITSD affected the signal by causing ion suppression which explains the discrepancies in the calculated recovery ratio.

	Metabolite concentrations (µM) in liver extracts spiked with 100% ITSD		**Metabolite concentrations (µM) in liver extracts spiked with 20% ITSD**		**Signal recovery % (After/before extraction)**	
Compounds	**Before extraction**	**After extraction**	**Before extraction**	**After extraction**	**100%**	**20%**
3-HAA	0.000	0.028	0.068	0.066	/	97
5-HTP	0.423	1.755	2.705	2.228	414	82
ADPR	5.306	0.265	41.203	48.743	5	118
cADPR	1.850	7.267	15.569	13.093	393	84
L-Kyn	0.131	0.110	0.197	0.161	83	82
L-Tryp	12.73	36.89	41.53	47.73	290	115
Me2PY	0.273	0.243	0.347	0.347	89	100
MeNAM	0.338	0.369	0.700	0.673	109	96
NAD	33.11	105.95	199.24	170.37	320	86
NADH	13.13	41.21	80.36	89.36	314	111
NAM	10.18	6.92	11.72	11.22	68	96
NAMN	0.036	0.049	0.099	0.094	136	95
NR	1.135	2.186	2.572	3.133	193	122
Serotonin	0.717	1.123	0.806	0.693	157	86

Supplementary figure 18: Representative extracted ion chromatograms (EICs) of unlabeled (^{12}C) NAD-related metabolites from the NAD standard mix (NSM) measured by HILIC-MS (Q-TOF) in positive ESI mode.



Supplementary figure 19: Representative extracted ion chromatograms (EICs) of labeled (^{13}C) NAD-related metabolites from the in-house generated yeast extract internal standard measured by HILIC-MS (Q-TOF) in positive ESI mode.



Supplementary table 12: NAD^+-related metabolites concentrations in pre- and post-mortem liver samples (nmol/g tissue) in controls and treatment groups (2-hours timepoint).

	Controls			**2 Hours Post-NR supplementation**		
	Pre-mortem average conc.	Post-mortem average conc.	**Paired T test (p value)**	Pre-mortem average conc.	Post-mortem average conc.	**Paired T test (p value)**
NAD^+	364.56	694.64	**0.060**	1646.91	3309.34	**0.209**
NADH	91.33	169.74	**0.136**	280.79	651.82	**0.175**
NAD^+/ NADH	4.25	4.68	**0.477**	5.95	5.08	**0.179**
NAM	949.12	907.71	**0.874**	2699.68	2957.78	**0.677**
MeNAM	0.73	2.61	**0.131**	25.13	39.64	**0.430**
ADPR	88.87	100.34	**0.759**	74.12	54.13	**0.207**
cADPR	19.82	17.68	**0.551**	97.39	83.59	**0.560**
NA	1.40	1.07	**0.326**	3.38	3.93	**0.547**
NAAD	3.00	0.26	**0.376**	75.97	121.60	**0.551**
NADP	20.65	10.97	**0.260**	20.43	8.36	**0.003****
NAMN	0.85	4.35	**0.723**	0.50	2.89	**0.689**
NMN	0.90	0.50	**0.201**	3.90	1.24	**0.035***
NR	0.34	0.42	**0.203**	1.02	1.31	**0.262**
L-Trp	93.13	49.70	**0.140**	59.43	69.83	**0.827**
3-HK	0.18	0.06	**0.258**	1.04	0.06	**0.176**
L-Kyn	0.29	0.15	**0.053**	0.71	0.58	**0.221**
Serotonin	2.78	0.95	**0.040***	7.18	1.41	**0.086**
Me2PY	1.69	3.63	**0.031***	21.40	27.42	**0.408**
NUA	< LOD	< LOD	/	0.08	0.09	**0.864**

Supplementary table 13: NAD^+-related metabolites concentrations in pre- and post-mortem muscle samples (nmol/g tissue) in controls and treatment groups (2-hours timepoint).

	Controls			**2 Hours Post-NR supplementation**		
	Pre-mortem average conc.	Post-mortem average conc.	**Paired T test (p value)**	Pre-mortem average conc.	Post-mortem average conc.	**Paired T test (p value)**
NAD^+	1322.98	3940.35	**0.229**	6653.49	3565.06	**0.631**
NADH	128.44	331.65	**0.178**	464.88	232.90	**0.543**
NAD^+/ NADH	7.15	8.67	**0.608**	11.68	8.10	**0.519**
NAM	587.91	374.57	**0.043***	583.04	831.70	**0.383**
MeNAM	1.36	0.84	**0.077**	6.29	5.17	**0.674**
ADPR	68.16	155.20	**0.160**	100.61	69.33	**0.757**
cADPR	75.32	213.59	**0.253**	343.55	183.23	**0.617**
NA	32.85	16.49	**0.043***	3.98	16.78	**0.273**
NADP	36.51	24.66	**0.205**	24.81	16.76	**0.393**
NR	0.20	0.40	**0.262**	1.13	1.92	**0.729**
L-Trp	22.86	27.79	**0.426**	31.66	18.05	**0.162**
Serotonin	7.14	2.67	**0.247**	3.04	7.80	**0.198**
Me2PY	1.39	0.87	**0.255**	10.41	10.16	**0.944**

Supplementary figure 20: Example of individual subjects NAD^+-related metabolites concentrations in mouse liver showing the variable results obtained from the different subjects within the NR-supplemented groups.