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Single sample extraction and HPLC processing for quantification of NAD and NADH levels in *Saccharomyces cerevisiae*

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1 **Single sample extraction and HPLC processing for quantification of**
2 **NAD and NADH levels in *Saccharomyces cerevisiae***

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Abstract

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29 A robust redox extraction protocol for quantitative and reproducible metabolite isolation and
30 recovery has been developed for simultaneous measurement of nicotinamide adenine
31 dinucleotide (NAD) and its reduced form, NADH, from *Saccharomyces cerevisiae*. Following
32 culture in liquid media, approximately 10^8 yeast cells were harvested by centrifugation and then
33 lysed under non-oxidizing conditions by bead blasting in ice-cold, nitrogen-saturated 50-mM
34 ammonium acetate. To enable protein denaturation, ice cold nitrogen-saturated CH_3CN + 50-mM
35 ammonium acetate (3:1; v:v) was added to the cell lysates. After sample centrifugation to pellet
36 precipitated proteins, organic solvent removal was performed on supernatants by chloroform
37 extraction. The remaining aqueous phase was dried and resuspended in 50-mM ammonium
38 acetate. NAD and NADH were separated by HPLC and quantified using UV-VIS absorbance
39 detection. Applicability of this procedure for quantifying NAD and NADH levels was evaluated
40 by culturing yeast under normal (2% glucose) and calorie restricted (0.5% glucose) conditions.
41 NAD and NADH contents are similar to previously reported levels in yeast obtained using
42 enzymatic assays performed separately on acid (for NAD) and alkali (for NADH) extracts.
43 Results demonstrate that it is possible to perform a single preparation to reliably and robustly
44 quantitate both NAD and NADH contents in the same sample. Robustness of the protocol
45 suggests it will be 1) applicable to quantification of these metabolites in mammalian and
46 bacterial cell cultures; and 2) amenable to isotope labeling strategies to determine the relative
47 contribution of specific metabolic pathways to total NAD and NADH levels in cell cultures.
48

Introduction

Nicotinamide adenine dinucleotides are ubiquitous biological molecules that participate in many metabolic reactions. Recent studies indicate that these dinucleotides may play important roles in transcriptional regulation,^{1,2} calorie restriction mediated lifespan extension,³⁻⁵ and age-associated diseases.⁶⁻⁸ However, current methods for the quantitation of nicotinamide adenine dinucleotides can be complicated by the oxidation of reduced species. Most studies have relied on separate extractions for nicotinamide adenine dinucleotide (NAD) and NADH determinations: a basic extraction for the reduced species and a separate acidic extraction for the oxidized species.^{3,9-12} The extraction conditions are specific for the stabilization of either oxidized compounds, which are more stable in acid, or reduced compounds, which are more stable in base. Metabolites in the separate extracts are then commonly quantitated by enzymatic cycling assays^{3,9-12} that amplify a compound of interest through a series of cycling steps involving coupled reactions with final quantitation achieved fluorimetrically. While enzymatic cycling assays can quantitate the total amount of pyridine dinucleotides, they cannot be used to assess specific metabolic pathways using isotopically-labeled and unlabeled forms of a compound.

NAD and NADH measurements in yeast (*Saccharomyces cerevisiae*) indicate that levels of these metabolites may play important roles in lifespan extension.¹⁰ Yeast have frequently been used as a model organism to study calorie restriction (CR) where lifespan extension can be achieved by reducing the glucose concentration in the growth media from 2% to 0.5%.³⁻⁵ The benefit of CR appears to require Sir2, a sirtuin family protein that exhibits an NAD-dependent deacetylase activity and whose function, in part, includes regulation of chromatin silencing, genomic stability at the ribosomal DNA loci.⁵ NADH may regulate longevity by inhibiting the activity of Sir2.¹⁰

NAD is synthesized via two major mechanisms in yeast, the *de novo* and salvage pathways.^{1-3,13,14} In the *de novo* pathway, NAD is synthesized from tryptophan present within the cell. In the salvage pathway, NAD is generated from the recycling of degraded NAD products, such as nicotinamide, and through the incorporation of nicotinic acid from the extracellular environment. Both pathways play redundant yet essential roles in cell growth, but the salvage pathway is thought to play a more important role in life span regulation.^{4,13} To elucidate this role, it is important to determine the relative contributions of the salvage and *de novo* pathways to the total amount of cellular NAD and NADH. Culturing yeast in media containing isotopically-labeled nicotinic acid will enable approaches for quantitating levels of

81 metabolites derived from the salvage pathway.¹⁵ In conjunction with measurements of total
82 cellular NAD and NADH, isotopic measurements should afford estimates of NAD and NADH
83 contents from the *de novo* pathway. However, an extraction and isolation method that enables
84 quantitation of both labeled and unlabelled forms of NAD and NADH is required before such
85 quantitative assessments are feasible.

86 High performance liquid chromatography (HPLC) is well suited for separating and
87 quantitating metabolites and can be used to isolate isotopically-labeled molecules in biological
88 samples.¹⁶ A handful of single sample extraction protocols have been developed using HPLC
89 separation to quantitate nicotinamide adenine dinucleotides within the same sample.¹⁷⁻¹⁹ Some of
90 these techniques rely on acid extraction protocols and HPLC peak integration to measure NAD
91 directly and indirectly measure NADH by its acid degraded products.¹⁹ Another procedure
92 involves a rapid post-extraction labeling reaction with cyanide in basic solution that leads to the
93 reaction product NAD-CN for NAD.¹⁷ NADH and the derivitized NAD-CN isomers are then
94 quantified via fluorescence detection.

95 Here, we report the development of a single sample extraction and HPLC processing
96 procedure that enables the isolation and quantitation of total cellular NAD and NADH from
97 pools of yeast. The protocol avoids derivitization or the measurement of degradation products
98 from NAD or NADH making this approach amenable to isotope labeling strategies as the
99 potential for loss of an isotope label is minimized. Metabolite recovery and stability, as well as
100 reproducibility of the whole procedure are examined. Applicability of this procedure to quantify
101 NAD and NADH levels in yeast is evaluated by culturing yeast under normal (2% glucose) and
102 calorie restricted (0.5 % glucose) conditions.

103
104

Methods

104

105

106 **Yeast culture.**

107 *Saccharomyces cerevisiae* (BY4742) were cultured in 25-ml volumes of liquid synthetic
108 complete media at 30°C consisting of 6.7 g/L Bacto yeast nitrogen base without amino acids
109 (Becton-Dickinson, Franklin Lakes, NJ), 1.92 g/L yeast synthetic drop-out media supplement
110 without uracil (Sigma-Aldrich, St Louis, Mo), 0.08 g/L uracil (Sigma-Aldrich, St Louis, Mo) and
111 20 g/L glucose (anhydrous dextrose) (EMD Chemicals, Gibbstown, NJ) dissolved in distilled
112 H₂O. Media was filter sterilized (0.22-µm GP Express Plus Membrane, Millipore, Billerica, MA)
113 before use as previously described²⁰. For calorie restriction, 5 g/L instead of 20 g/L glucose was
114 used. Around 10⁴ yeast were used to inoculate each culture. 100-µl aliquots were periodically
115 taken from culture for growth and cell density measurement via hemacytometer. Cultures were
116 maintained until they contained ~ 7 x 10⁶ cells/ml corresponding to mid-log phase growth.

117

118 **Preparation of yeast pellets for metabolite extraction.**

119 Approximately 7 x 10⁷ yeast were harvested by aliquoting 10 ml of the culture into a 15 ml
120 Falcon tube (Becton-Dickinson, Franklin Lakes, NJ) followed by centrifugation for 3 minutes at
121 4,000 rpm at 4°C in a Sorvall RC 5C Plus (DuPont, Newtown CT). The supernatant was
122 discarded and the pellet resuspended in 1-ml PBS at 4°C, and transferred to a 2-ml polypropylene
123 bead blasting tube (Outpatient Services, Petaluma, CA). The walls of the Falcon tube were
124 washed with 1-ml ice-cold PBS and the rinsate added to the bead blasting tube. Samples were
125 centrifuged at 4,000 rpm for 3 minutes in a bench top mini-centrifuge (National Labnet,
126 Woodbridge NJ) at 4°C and the supernatant discarded.

127

128 **Single sample metabolite extraction for HPLC speciation.**

129 Using the work of Lazzarino et al.¹⁸ as a conceptual basis, an extraction protocol was developed
130 to recover water-soluble metabolites from yeast cells for fractionation by HPLC. 600 µl of 50-
131 mM ammonium acetate (Aldrich, Milwaukee, WI) saturated with N₂ gas was added to the
132 pelleted yeast in bead blasting tubes and the tubes filled to the meniscus with 212-300 µm
133 diameter acid-washed glass beads (Sigma-Aldrich, St Louis, Mo). Yeast were bead blasted at
134 maximum speed using a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK) for 30 seconds
135 followed by a 2-minute incubation on ice and another 30 seconds of bead blasting. The bead

136 blasting tube was then inverted and its base lanced with a red-hot 0.5 inch long 26-gauge needle
137 (Becton Dickinson & Co, Franklin Lakes NJ). An open-ended 1.5 ml-microfuge tube
138 (Eppendorf, Westbury NY) was placed over the base of bead blasting tube and the assembly
139 nested upright in a 50-ml Falcon tube (Becton-Dickinson, Franklin Lakes, NJ). The nested tubes
140 were centrifuged at 2000 rpm for 3 minutes in a Sorvall centrifuge to separate cell lysates from
141 glass beads. Cell lysates were then transferred to a cold 2 ml microfuge tube (Eppendorf,
142 Westbury NY) and kept on ice. The glass beads in the bead blasting tube were then washed twice
143 with 600 μ l of a N₂-saturated solution of acetonitrile (Sigma-Aldrich, St Louis, Mo) and 50-mM
144 ammonium acetate (3:1; v:v) vortexed and centrifuged for 3 minutes at 2000 rpm at 4°C. After
145 each wash, the rinsate was transferred to the microfuge tube containing the cell lysate.

146 Following washing, cell lysates were centrifuged at 10,000 rpm for 1 minute at 4°C. The
147 supernatant was transferred to a 10-ml glass test tube (Corning Inc., Corning NY) and stored on
148 ice. The remaining pellet was resuspended in 600 μ l of a N₂-saturated solution consisting of
149 acetonitrile and 50-mM ammonium acetate (3:1; v:v) and centrifuged at 10,000 rpm for 5
150 minutes at 4°C. The resulting supernatant was pooled with the previous supernatant and the
151 remaining pellet discarded. Lipids were extracted from the liquid volume using 10 ml of ice-cold
152 chloroform (VWR International, West Chester, PA) and 5 seconds vigorous agitation. Samples
153 were left to separate on ice for 30 minutes. The upper aqueous phase was collected in a 15-ml
154 Falcon tube and was quickly frozen at -80°C and lyophilized for 15 hours in a Christ Alpha 1-2
155 freeze dryer (Martin Christ GmbH, Germany) while the organic phase was discarded. To test
156 whether discarded protein pellets contained NAD or NADH, randomly selected pellets were
157 resuspended in 600 μ l of chloroform using vigorous agitation, dried under a stream of nitrogen,
158 resuspended in 600 μ l of acetonitrile, and stored in a 4°C refrigerator overnight. The lysate was
159 then vortexed vigorously for 30 seconds and centrifuged at 10,000 rpm for 5 minutes at 4°C.
160 The supernatant was frozen at -80°C and then lyophilized for at least 15 hours.

161 Lyophilized samples were resuspended in 250 μ l ice-cold 50-mM ammonium acetate and
162 vortexed vigorously. The suspension was filtered with a 0.45- μ m polyvinylidene fluoride
163 micro-spin filter tube (Alltech Associates Inc., Deerfield, IL) using a bench top mini-centrifuge
164 operating at 10,000 rpm for 1 minute and aliquots analyzed by HPLC.

165

166 **HPLC speciation of metabolites.**

167 HPLC measurements were performed using a Hydrosphere C18 column (5 micron, 150 x 4.6

168 mm I.D. (Waters, Milford MA)) with a direct connection UNIGUARD guard column (Thermo
169 Electron, Newington, NH) fitted with the appropriate PEEK tip and Hypersil Gold guard
170 cartridge (5 micron, 10 x 4.0 mm I.D., (Thermo Electron, Newington, NH)) on an Agilent 1100
171 HPLC (Hewlett-Packard Wilmington, DE). The C18 column and guard column were maintained
172 at room temperature (22°C). Up to 100- μ l aliquots of metabolites were injected and fractionated
173 by reversed-phase chromatography. A 1 ml/min gradient of mobile phase A (50-mM ammonium
174 acetate) and mobile phase B (100% acetonitrile) initially consisting of 100% mobile phase A,
175 with mobile phase B increasing to 5% over 30 minutes at a rate of 0.1% per minute was utilized.
176 The column was washed after each separation by increasing mobile phase B to 90% for 7
177 minutes. UV absorbance was monitored at 260 nm and 340 nm and pertinent peak areas
178 integrated using area under the curve algorithms. Quantitation of NAD was assessed using
179 absorbance at 260 nm. Since NADH absorbs at 340 nm whereas NAD does not, quantitation of
180 NADH was assessed using absorbance at both 260 and 340 nm.

181 Peak identification and quantitation of NAD and NADH were assessed using standard
182 solutions of NAD and NADH (Sigma-Aldrich, St Louis, MO) dissolved in 50-mM ammonium
183 acetate. Standard stock solutions were prepared fresh every week, while working standard
184 solutions were prepared daily by appropriate dilution of the stock solutions. Working standards
185 were always analyzed on the same day as samples of interest to generate standardization curves.

186 Metabolite contents were calculated from the measured absorbance, corrected for
187 recovery efficiency and processing volumes and, when relevant, converted to units of amol/cell
188 using the number of cells in the extraction and the molecular weight of the metabolite of interest.

189

190 **Mass spectrometry measurements.**

191 Fractions obtained via HPLC analysis of standards or metabolite extracts from yeast were dried
192 in a centrifugal vacuum evaporator (Jouan, Winchester, VA) at ambient temperature and stored
193 at -80°C prior to mass spectrometry (MS) analysis. Samples were resuspended in a 89.9:10:0.1
194 water/acetonitrile/formic acid (Sigma-Aldrich, St Louis, Mo) solution prior to direct infusion
195 into the MS system by a syringe pump without a column at a flow rate of 10 ml/min.
196 Electrospray ionization (ESI)-MS analyses were carried out using a Quattro Micro™ API
197 (Micromass UK, Manchester, UK) mass spectrometer. High-resolution MS (HR/MS) analyses
198 were done in ESI mode on a Micromass Fourier transform MS (Micromass UK Manchester,
199 UK). All ESI-MS spectra were obtained in positive ion mode with an electrospray capillary

200 potential of 3.0 kV and a cone potential of 21 V. Standard solutions of NAD and NADH,
201 resuspended in a 89.9:10:0.1 water/acetonitrile/formic acid mixture, were used for NAD and
202 NADH identification in the mass spectrometry measurements.

203

204 **Reproducibility, linearity and sensitivity provided by the chromatographic method.**

205 Reproducibility, linearity and sensitivity provided by the chromatographic method were studied
206 with solutions containing up to 1µg/µl NAD or NADH standards with HPLC injection volumes
207 varying between 5 and 100 µl. The quantities of NAD and NADH used in the linearity
208 experiments spanned the working range of metabolite contents obtained from processing pools of
209 yeast. Regression analysis was utilized to determine linear correlation coefficients of standard
210 curves for each standard. Limits of detection (LOD) were considered to be the quantities that
211 produced a peak whose area was three times that of the square root of the integrated background
212 underneath the peak. Reproducibility of the chromatographic method was evaluated using up to
213 100-µl injections of standard solutions on the same day (intra-day) and on various days (inter-
214 day) over a three-month period. Fresh standard solutions were prepared weekly for the inter-day
215 reproducibility measurements.

216

217 **Recovery and reproducibility of the extraction protocol.**

218 Recovery efficiency of the extraction protocol was determined with standard solutions
219 containing 2.5 µg of either NAD or NADH dissolved in 50-mM ammonium acetate. For standard
220 addition experiments yeast cultures were split into two samples prior to bead beating. One
221 sample was processed as previously described to determine yeast NAD and NADH contents.
222 Standard solutions containing 2.5 µg or 10 µg of either NAD or NADH (these amounts span the
223 range of NAD and NADH contents within yeast metabolite extracts) dissolved in 50-mM
224 ammonium acetate were added to bead beaten cell lysates from the other sample for subsequent
225 processing. Total quantities of NADH and NAD in spiked samples were corrected for the
226 contribution of yeast metabolites and recovery of NAD and NADH from the spikes assessed.

227

228 **Statistical analysis.**

229 Differences in NAD and NADH contents of yeast grown in 20 g/L or 5 g/L glucose were
230 assessed by unpaired two-tailed Student's t-tests. A significance level of less than 0.02 was
231 considered meaningful. A significance level between 0.02 and 0.10 was considered evidence of a

232 possible trend, while a significance level of greater than 0.10 was considered to indicate no
233 significant difference.

234

Results

Reproducibility, linearity and sensitivity provided by the chromatographic method.

Typical chromatograms of standard solutions containing NAD and NADH are shown in Figure 1. Intra-day variability observed in retention time of each authentic compound was typically less than 2 percent of the mean retention time. Inter-day variability observed in retention times of the authentic compounds was typically less than 60 seconds. Linearity for peak area quantitation at 260-nm absorbance of standard solutions of NAD and NADH is shown in Figure 2. Linearity for peak area quantitation at 340-nm absorbance of standard solutions of NADH (data not shown) was virtually identical to that observed for NADH in Figure 2. Limits of detection (LOD) at 260-nm absorbance were 10 ng for NAD and 25 ng for NADH, while the limit of detection at 340-nm absorbance was 20 ng for NADH. Linearity and LOD for the chromatographic method were insensitive to the range of injection volumes (5 to 100 μ l) utilized for each standard. Table 1 summarizes the intra-day and inter-day precision of the chromatographic method for the detection of NAD and NADH at 260-nm absorbance. The intra-day and inter-day precision of the chromatographic method for the detection of NADH at 340-nm absorbance was virtually identical to that shown for NADH in Table 1 (data not shown). Relative standard deviations (standard deviation expressed as a percentage of the corresponding mean value) for detection of the authentic compounds ranged from 1.6 to 2.3% for the intra-day reproducibility experiments and 3.3 to 4.0% for the inter-day reproducibility experiments.

Recovery and reproducibility of the metabolite extraction protocol.

Table 2 shows recoveries of NAD and NADH obtained with our extraction and speciation protocol for standard solutions containing 2.5- μ g of either NAD or NADH. Differences in measured NADH values via absorbance at 340-nm and 260-nm in the recovery experiments for standard solutions were typically less than 1%. Recovery of NAD was 95.9 +/- 4.9% (mean +/- standard deviation) while no detectable conversion or degradation of NAD to other metabolites was observed. Recovery of NADH was 83.4 +/- 6.6 % with 12.9 +/- 6.2 % of the NADH oxidized to NAD during sample processing.

Table 3 shows recoveries of NAD and NADH obtained from the standard addition experiments performed on yeast cultures grown in 20 g/L glucose after correction for the contribution from yeast to the NAD and NADH contents. Differences in measured NADH values

266 via absorbance at 340-nm and 260-nm in the standard addition experiments were typically less
267 than 1%. Recoveries from the standard addition experiments are consistent with the metabolite
268 recovery efficiency data shown in Table 2. Recovery of 2.5 μg NAD matrix spikes was 95.6 +/-
269 8.2%, while recovery of 10 μg NAD matrix spikes was 96.5 +/- 6.0 %. No detectable conversion
270 or degradation of NAD matrix spikes to other metabolites was observed. Recovery of 2.5 μg
271 NADH matrix spikes was 82.6 +/- 12.3%, while recovery of 10 μg NADH matrix spikes was
272 83.2 +/- 9.9%. 13.3 +/- 11.3% of the 2.5 μg NADH matrix spikes oxidized to NAD during
273 sample processing, while 12.0 +/- 8.0% of the 10 μg NADH matrix spikes oxidized to NAD.
274 Standard addition experiments performed on yeast cultures grown in 5 g/L glucose produced
275 similar matrix spike recoveries (data not shown) to those reported in Table 3.

276

277 **Measurement of NAD and NADH contents in yeast.**

278 Figure 3 shows a typical HPLC chromatogram of metabolites extracted from approximately 7×10^7
279 yeast grown in 20 g/L glucose. HPLC chromatograms of metabolites extracted from yeast
280 grown in 5 g/L glucose were qualitatively similar to that shown in Figure 3. Fractions of yeast
281 metabolite extracts with retention times corresponding to those of authentic NAD and NADH
282 standards were collected and their identity confirmed with mass spectrometry. Mass
283 spectrometry data from these metabolite fractions was highly consistent with mass spectrometry
284 data obtained from corresponding authentic standards of NAD and NADH. Briefly, mass
285 spectrometry of metabolite fractions with retention times corresponding to that of the authentic
286 NAD standard revealed a peak corresponding to a M/Z of 664.1 +/- 0.3 (expected 664.4 for
287 M+H) and a pattern of fragmentation ions that was similar to that of the authentic standard (data
288 not shown). Likewise, mass spectrometry of metabolite fractions with retention times
289 corresponding to that of the authentic NADH standard revealed a peak corresponding to a M/Z
290 of 666.1 +/- 0.3 (expected 666.4 for M) and a pattern of fragmentation ions of smaller M/Z that
291 was similar to that of the authentic standard (data not shown).

292 Metabolite extracts from yeast cultured in 20 g/L glucose typically contained 4 μg each
293 of NAD and NADH, while those cultured in 5 g/L glucose contained 5 μg of NAD and 2.5 μg
294 NADH. Differences in measured NADH values via absorbance at 340-nm and 260-nm for the
295 metabolite extracts were typically less than 1%. Figure 4 and the top row in Table 4 shows
296 contents (amol/cell) of NAD and NADH in yeast grown in synthetic complete media containing
297 20 g/L glucose or 5 g/L glucose. HPLC analysis of extracts from the remaining protein pellet

298 revealed no detectable NAD or NADH (data not shown). For growth in 20 g/L glucose (non-
299 calorie restriction), NAD is present in yeast cells at 97 +/- 13 amol/cell. This value increases
300 significantly ($P < 0.02$) to 122 +/- 19 amol/cell when cells are cultured in 5 g/L glucose (calorie
301 restriction. NADH values, however, significantly decrease ($P < 0.02$) from 99 +/- 13 amol/cell
302 under non-calorie restricted conditions to 63 +/- 10 amol/cell with calorie restriction. The sum of
303 NAD and NADH is not significantly different for both growth conditions at approximately 190
304 amol/cell. The ratio of NAD to NADH increases significantly ($p < 0.02$) from 1.0 for yeast
305 cultured in 20 g/L glucose to 1.9 for yeast cultured in 5 g/L glucose.

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Discussion

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310 Minimization of artifactual oxidation of reduced metabolite species is challenging to achieve for
311 any metabolite extraction protocol. Here, we have improved on previous approaches and
312 developed a method for measuring NAD and NADH in the same sample of yeast using a simple
313 extraction protocol. Reverse phase HPLC provided excellent speciation and resolution of NAD
314 and NADH from other metabolites within a 25-minute runtime. The dynamic range of the
315 chromatographic method was shown to be linear for the 38 ng to 18.3 μg quantities of the
316 authentic NAD standard studied and 68 ng to 13.9 μg quantities of the authentic NADH standard
317 studied. Meanwhile, metabolite extracts from $\sim 7 \times 10^7$ yeast cultured in 20 g/L glucose contained
318 approximately 4 μg each of NAD and NADH, while yeast cultured in 5 g/L glucose contained
319 approximately 5 μg of NAD and approximately 2.5 μg NADH. Consequently, the quantities of
320 authentic standards of NAD and NADH used in studying the linearity of the chromatographic
321 method correspond to NAD and NADH contents that one could expect to extract from $\sim 2 \times 10^6$
322 to $\sim 2 \times 10^8$ yeast. The sensitivity of the chromatographic method for NAD (10 ng) and NADH
323 (20 ng) detection compares favorably with that of other chromatographic methods¹⁷ and suggest
324 that NAD contents from as few as 2×10^5 yeast cells and NADH contents from as few as 5×10^5
325 yeast cells could be quantified. However, such cell numbers are at least as high those used for
326 enzymatic cycling measurements of NAD and NADH in yeast which are routinely performed on
327 10^5 .¹⁰ The chromatographic method produces acceptable intra-day and inter-day reproducibility
328 in retention time, speciation and subsequent quantitation of NAD and NADH. The higher inter-
329 day variation observed for quantitation of the authentic compounds plausibly arises as the intra-
330 day measurements were performed on the same set of standard solutions while fresh solutions
331 were prepared weekly for the inter-day measurements. The higher inter-day than intra-day
332 variation observed for retention time of the authentic compounds plausibly arises as day to day
333 variations in pump and column performance are likely greater than variation within a single day.

334 Measurements performed with authentic standards of NAD and NADH reveal that the
335 extraction protocol typically produces at least 95 % recovery of NAD and at least 80 % recovery
336 of NADH. No degradation products of the NAD standard were detectable while NADH
337 oxidation to NAD appears to be approximately 13%. However, great care must be exercised at
338 all points in the extraction protocol to prevent artifactual oxidation or degradation of NADH.
339 Precautions were taken at each step in the metabolite extraction protocol to minimize processing

340 time and sample temperature to minimize NADH oxidation while optimizing metabolite
341 recovery. We found that samples must be kept as cold as possible. Allowing the sample
342 temperature to supersede room temperature can cause oxidation of NADH to occur by as much
343 as 50%. Accordingly, yeast were bead blasted for two 30-second periods and left on ice for the
344 intervening period because greater consistency in NADH recovery was obtained over that from
345 bead blasting for one 60-second period. Frozen extracts may be kept in a -80°C freezer; however
346 standard solutions containing NADH frozen for more than 72 hours started to display signs of
347 NADH oxidation.

348 We also found that multiple washings of the glass beads in the bead blasting tubes
349 following bead beating, careful attention to vigorous vortexing and agitation during resuspension
350 or mixing activities, and conservative centrifugation times could improve metabolite recoveries
351 by as much as 20%. Interestingly, we found that lipid extraction using chloroform resulted in
352 lower recoveries of NADH if the organic and upper aqueous phases were left to separate on ice
353 for less than 15 minutes. NADH recoveries reached a stable plateau for separation times of 25
354 minutes or more. Because we observed that lyophilization of standard solutions of NADH in 50-
355 mM ammonium acetate can result in oxidation of NADH to NAD, the longer settling times
356 during lipid extraction possibly allow ammonium acetate in the metabolite extracts to partition
357 into the organic phase.

358 NAD and NADH levels in yeast determined in the current study are compared with those
359 obtained in prior studies via enzymatic assay in Table 4. Total NAD values for normal growth
360 conditions (i.e., 20 g/L glucose) have been reported between 79.8 and 210 amol/cell,^{3,9-12} which
361 is consistent with our findings of 97 +/- 13 amol/cell. There are few published reports of
362 measured NADH values in yeast presumably because of the difficulty in making measurements
363 on such readily oxidizable molecules. NADH values obtained from the current method are nearly
364 40% higher than those obtained through enzymatic cycling assays.^{3,10} Cells grown under calorie
365 restriction (i.e., 5 g/L glucose) have been reported to contain a similar range of NAD values as
366 cells grown without calorie restriction.^{3,10} We measured 122 +/- 19 amol/cell using the current
367 method, which falls well within the published range. NADH values obtained from yeast grown
368 under calorie restriction have been reported at 27 amol/cell.⁹ Using the current method for
369 metabolite extraction, we obtain 63 +/- 10 amol/cell of NADH from calorie-restricted cells.
370 However, similar to the study of Lin et al.,⁹ we found that under calorie restriction conditions,

371 NADH contents are significantly reduced and that the NAD/NADH ratio significantly increases
372 compared to yeast cultured in 20 g/L glucose.

373 Enzymatic cycling reactions for indirectly measuring NAD and NADH typically rely on a
374 heating step under either acidic or alkali conditions for cell lysis and preservation of either NAD
375 (acidic) or NADH (alkali)^{3,9-12} and there can be a large loss (more than half) of the metabolite of
376 interest during the harsh extractions conditions. Interestingly, during development of our assay,
377 we observed that the choice of buffer the yeast are maintained in prior to, and during bead
378 beating, had a profound influence on intracellular NAD and NADH contents. Compared to bead
379 beating in 50 mM ammonium acetate, bead beating yeast in an acetonitrile: 10-mM KH₂PO₄
380 buffer (3:1 v:v) resulted in reduction of cellular NADH levels by as much as 90%, while bead
381 beating yeast in a 50-mM KH₂PO₄ buffer resulted in reduction of cellular NADH levels by as
382 much as 20%. Stored NADH has been shown in various animal models to be rapidly utilized by
383 cells under stressful conditions, including oxidative stress²¹ and high osmolarity²², while NADH
384 has been shown to degrade more rapidly in KH₂PO₄ buffers than other buffers such as Pipes.²³ It
385 is plausible that the lower NADH levels observed in the enzymatic assays and by bead beating in
386 acetonitrile are a result of a cellular stress response to the buffer. These observations suggest that
387 it is crucial that exposure of intact cells to chemical or environmental stressors prior to, and
388 during, cell lysis be minimized.

389 The work of Lazzarino et al.¹⁸ served as a conceptual basis for the development of the
390 metabolite extraction protocols reported here. Their work was developed for recovering a broad
391 range of metabolites representative of both the redox and energy states of cells in animal tissue
392 and utilized acetonitrile and KH₂PO₄ containing buffers during both cell lysis and metabolite
393 extraction¹⁸. To enable robust, reliable measurement of NAD and NADH in yeast, it was
394 necessary to develop significantly different cell lysis and metabolite extraction methods.
395 Acetonitrile-containing buffers were avoided during cell lysis and KH₂PO₄-containing buffers
396 were avoided throughout the protocol. Owing to their robust cell wall bead-beating procedures
397 were used to rapidly and efficiently lyse yeast. We also found that multiple chloroform
398 extraction steps proposed by Lazzarino et al.¹⁸ did not increase the yields of NAD and NADH
399 recovered from yeast. Elimination of such steps reduced total sample processing time by at least
400 twelve hours. The extraction method reported here relies on rapid and efficient cell lysis and
401 relatively benign chemical conditions prior to lysis and during metabolite extraction. These

402 careful considerations likely minimize cellular use and degradation of NADH resulting in
403 recovery of higher amounts of NADH.

404 The protocol reported here measures the total amount (i.e., free + bound) of NAD and
405 NADH in yeast. Ratios of total NAD to total NADH in yeast seem to be within a reasonable
406 range for NAD regulation.²⁴ However, baseline ratios of the free pool of NAD:NADH have been
407 debated.²⁵⁻²⁹ The free pool NAD:NADH ratio has been estimated from concentrations of the
408 intracellular metabolites, pyruvate and lactate, for various tissues in different species animal
409 species to range from 0.1 to 10.²⁵⁻²⁹ During development of the assay reported here, we found that
410 yeast cell lysates that were passed through a 0.45- μ m glass fiber filter, immediately after bead
411 beating and prior to metabolite extraction, possessed NADH levels that were around one sixth of
412 those obtained from extractions of unfiltered lysates (data not shown). NAD levels, however,
413 were not altered resulting in NAD/NADH ratios of approximately 7. This observation of filtered
414 NAD and NADH levels is consistent with the hypothesis that the majority of cellular NADH is
415 likely membrane or protein bound, while the majority of NAD is not protein bound. In our
416 protocol, bound NADH is likely effectively released from proteins after bead beating by the use
417 of acetonitrile which is well-known for its ability to denature proteins.³⁰

418 Results of this study and that of others¹⁰ reveal that yeast NADH contents are
419 significantly reduced under calorie restricted conditions while NAD:NADH ratios increase. It
420 has been suggested that this shift may lead to an activation of Sir2, a sirtuin protein family
421 member.⁴ NADH functions as an inhibitor of Sir2,¹⁰ so lower levels of NADH found in yeast
422 cells grown under calorie restriction could result in less inhibition of Sir2 and therefore an
423 increased life span in yeast.¹⁰ By culturing yeast in ¹⁴C-labeled growth media, we are
424 investigating the relative contributions of the *de novo* and salvage pathways to total NAD and
425 NADH levels in yeast strains as a function of nutrient status.³¹ The protocol developed here
426 avoids derivitization or the measurement of degradation products from NAD or NADH so that
427 the potential for loss of the isotope label is minimized. Initial radiolabeling of yeast cells and
428 subsequent accelerator mass spectrometry (AMS) measurement for ¹⁴C quantitation indicate that:
429 1) yeast cells are amenable to such labeling and quantification,³¹ and 2) the single sample
430 extraction and HPLC speciation protocol developed here is amenable to AMS quantitation of
431 ¹⁴C in isolated fractions of the HPLC trace³¹ enabling us to quantify both total (by UV-Vis
432 absorption) and ¹⁴C labeled NAD and NADH (by AMS) from the same sample.

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Acknowledgements

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Tables

	NAD	NADH
Amount (μg)	0.125	0.125
Intra-day precision (% relative standard deviation) (n=3)	2.0	2.3
Inter-day precision (% relative standard deviation) (n=5)	3.9	4.0
Amount (μg)	0.625	0.375
Intra-day precision (% relative standard deviation) (n=4)	1.6	1.9
Inter-day precision (% relative standard deviation) (n=5)	4.0	3.6
Amount (μg)	5.44	3.33
Intra-day precision (% relative standard deviation) (n=3)	1.9	2.3
Inter-day precision (% relative standard deviation) (n=5)	3.4	3.3
Amount (μg)	15.26	13.87
Intra-day precision (% relative standard deviation) (n=4)	1.8	2.0
Inter-day precision (% relative standard deviation) (n=5)	3.5	3.5

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Table 1: Intra-day and inter-day reproducibility of the chromatographic method for the detection of NAD and NADH in standard solutions at 260-nm absorbance. (Relative standard deviation is the standard deviation expressed as a percentage of the corresponding mean value.)

451

Standard contents	% Recovered (SD)	% Conversion (SD)	% Total Recovered (SD)
NAD (2.5 µg)	95.9 (4.9)	None detected	95.9 (4.9)
NADH (2.5 µg)	83.4 (6.6)	12.9 (6.2)	96.3 (2.1)

452

453 **Table 2:** Percent recoveries obtained from the metabolite extraction protocol performed on
454 standard solutions containing NAD or NADH. 12.9% of NADH was converted to NAD during
455 sample processing. Values represent the mean and standard deviation of nine separate
456 experiments.

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Spike Contents	% Recovered (SD)	% Conversion (SD)	% Total Recovered
NAD (2.5 µg)	95.6 (8.2)	None detected	95.6 (8.2)
NAD (10 µg)	96.5 (6.0)	None detected	96.5 (6.0)
NADH (2.5 µg)	82.6 (12.3)	13.3 (11.3)	96.0 (9.2)
NADH (10 µg)	83.2 (9.9)	12.0 (8.0)	95.2 (1.9)

458

459 **Table 3:** Percent recoveries of authentic standard solutions of NAD or NADH spiked into 10 ml
460 aliquots of yeast cultures grown in 20 g/L glucose immediately after pelleting the yeast for
461 subsequent metabolite extraction. Each value represents the mean and standard deviation of at
462 least three separate experiments performed on different days.

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	Normal				Calorie restriction			
	NAD	NADH	NAD/NADH Ratio	NAD + NADH	NAD	NADH	NAD/NADH Ratio	NAD + NADH
Present study	97.30 (12.70)	99.30 (13.00)	1.00 (0.10)	196.60 (22.40)	121.88 (19.23)	63.44 (9.72)	1.94 (0.30)	185.33 (25.69)
Lin et al. ¹⁰ (2004) ^a	88.20 (4.20)	59.50 (9.10)	1.48	147.70	83.30 (5.60)	27.30 (7.70)	3.05	110.60
Anderson et al. ³ (2002) ^b	140	54	2.56	194	-	-	-	
Lin et al. ¹¹ (2001) ^b	140 - 210	-	-		140 – 210	-	-	
Ashrafi et al. ⁹ (2000) ^b	79.80	-	-		-	-	-	
Smith et al. ¹² (2000) ^b	149.80	-	-		-	-	-	

465

466 **Table 4:** Comparison of metabolite contents (amol/cell) of yeast grown under normal or calorie
467 restriction conditions determined in this study and those obtained in prior studies via enzymatic
468 assay. Data are presented as mean and standard deviation (SD) of replicates.

469 ^aReported values¹⁰ have been converted from mM/cell to amol/cell assuming a yeast cell volume
470 of 70 fL (10⁻¹⁵ L) and are expressed as the mean and standard deviation (SD) of 3 experiments
471 performed in duplicate.

472 ^bReported values³ were converted to mM/cell as reported¹⁰ and converted to amol/cell here
473 assuming a yeast cell volume of 70 fL.

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Figure Captions

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Figure 1: Typical HPLC chromatograms of 10- μ l aliquots of 50-mM ammonium acetate containing a) 2.50- μ g NAD and b) 2.34- μ g NADH (absorbance at 340 nm has been offset vertically by 10 mAu for clarity and an expanded view of the NADH peak is inset). Retention times (t_r) of the authentic compounds are: NAD t_r = 20.9 min., and NADH t_r = 24.4 min.

Figure 2: Standard curves for the chromatographic quantitation of authentic compounds of NAD and NADH. NAD contents range from 0.038 ng to 18.26 μ g. NADH contents range from 0.068 ng to 13.87 μ g. An expanded view of the standard curves near the origin is inset. Lines represent linear least squares fits to the data. Coefficients of correlation are 0.9975 for NAD and 0.9984 for NADH.

Figure 3: Typical HPLC chromatogram of a 75- μ l aliquot of yeast metabolite extracts from approximately 7×10^7 yeast cells grown in 20 g/L glucose. Retention times (t_r) of the examined compounds are: NAD t_r = 21.3 min., and NADH t_r = 24.9 min.

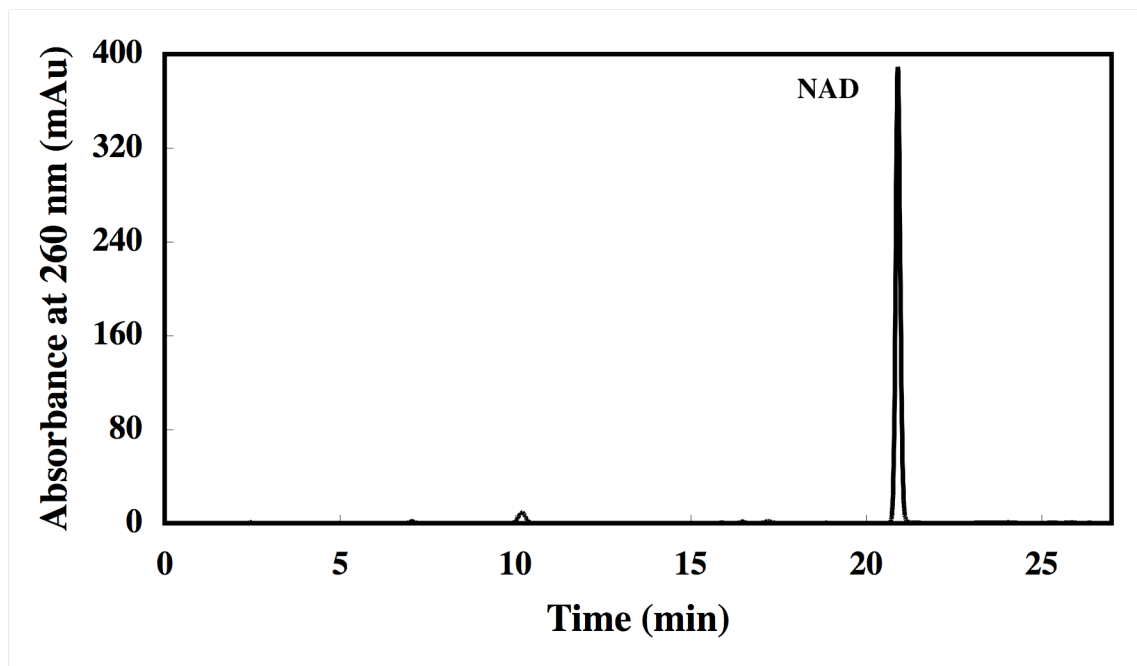
Figure 4: Metabolite contents (amol/cell) of yeast grown in synthetic complete media containing 20 g/L glucose (normal) or 5 g/L glucose (calorie restriction). Data are presented as mean and standard deviation (SD) of seven separate experiments for each culture condition. Student's t-test assessments were used to determine whether NAD, NADH and NAD+NADH are similar for the 20 and 5 g/L glucose growth conditions (* $p < 0.02$, ** $p > 0.10$).

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Figures

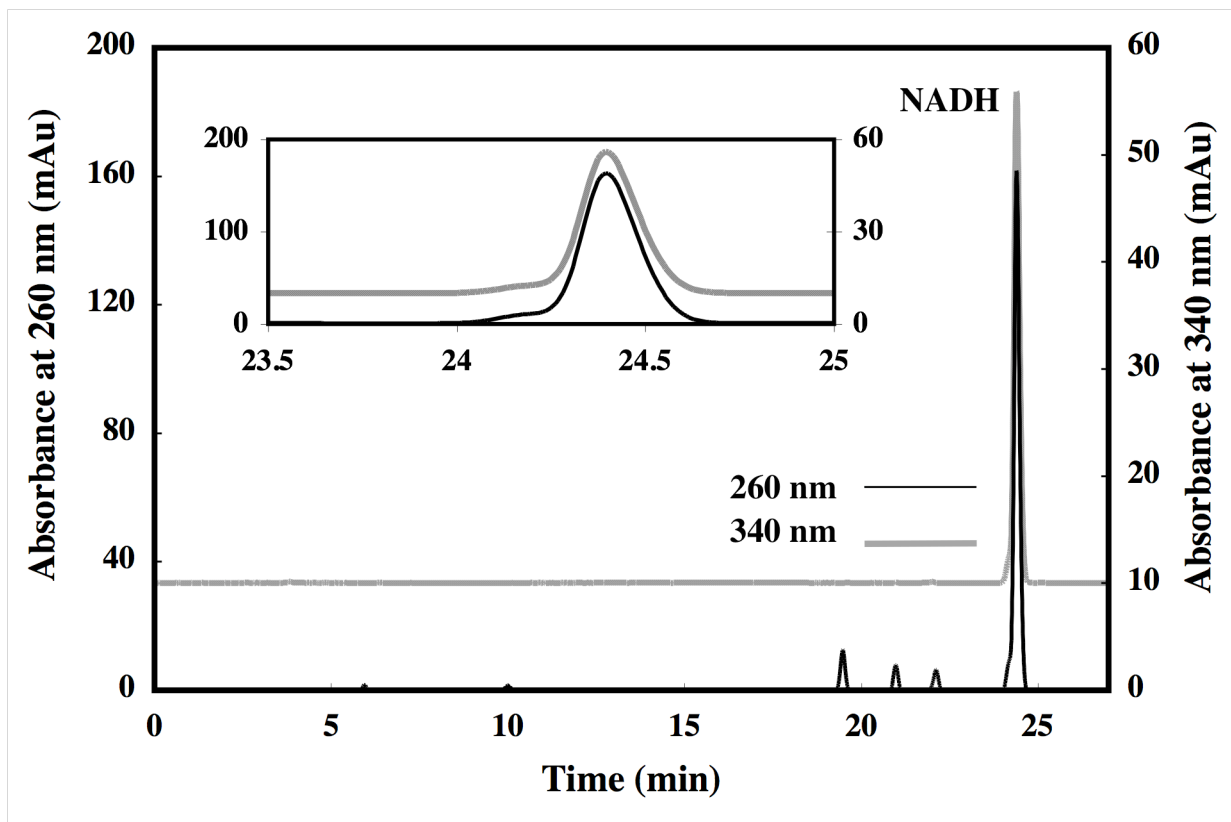
Figure 1:

A)



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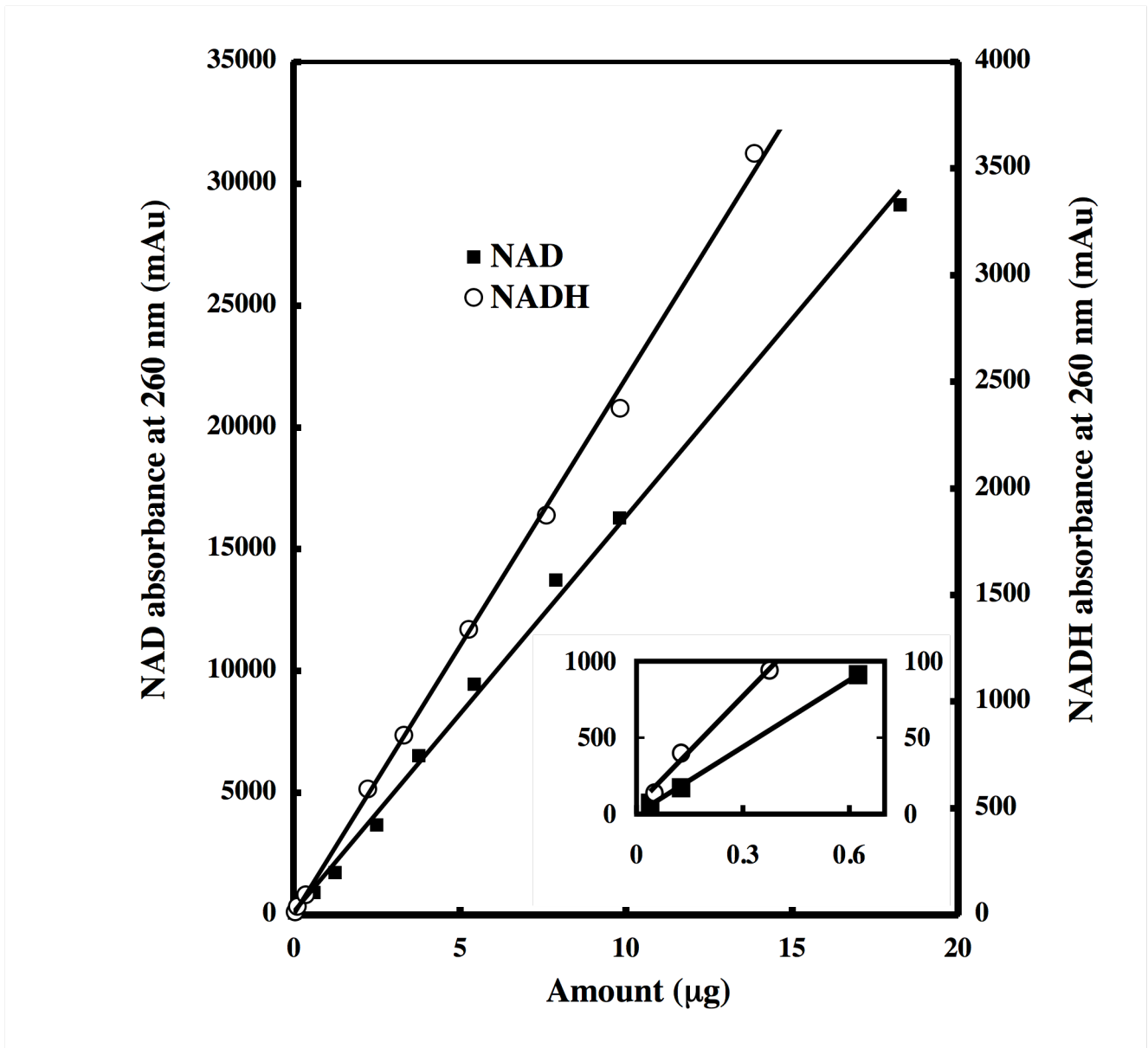
B)



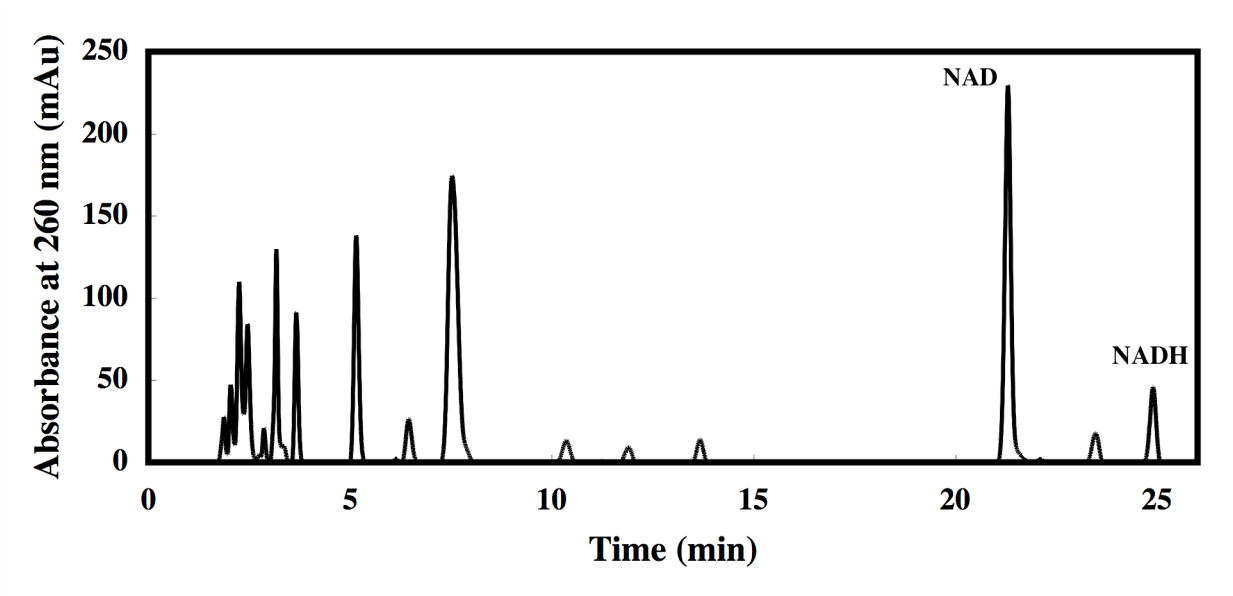
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Figure 2:

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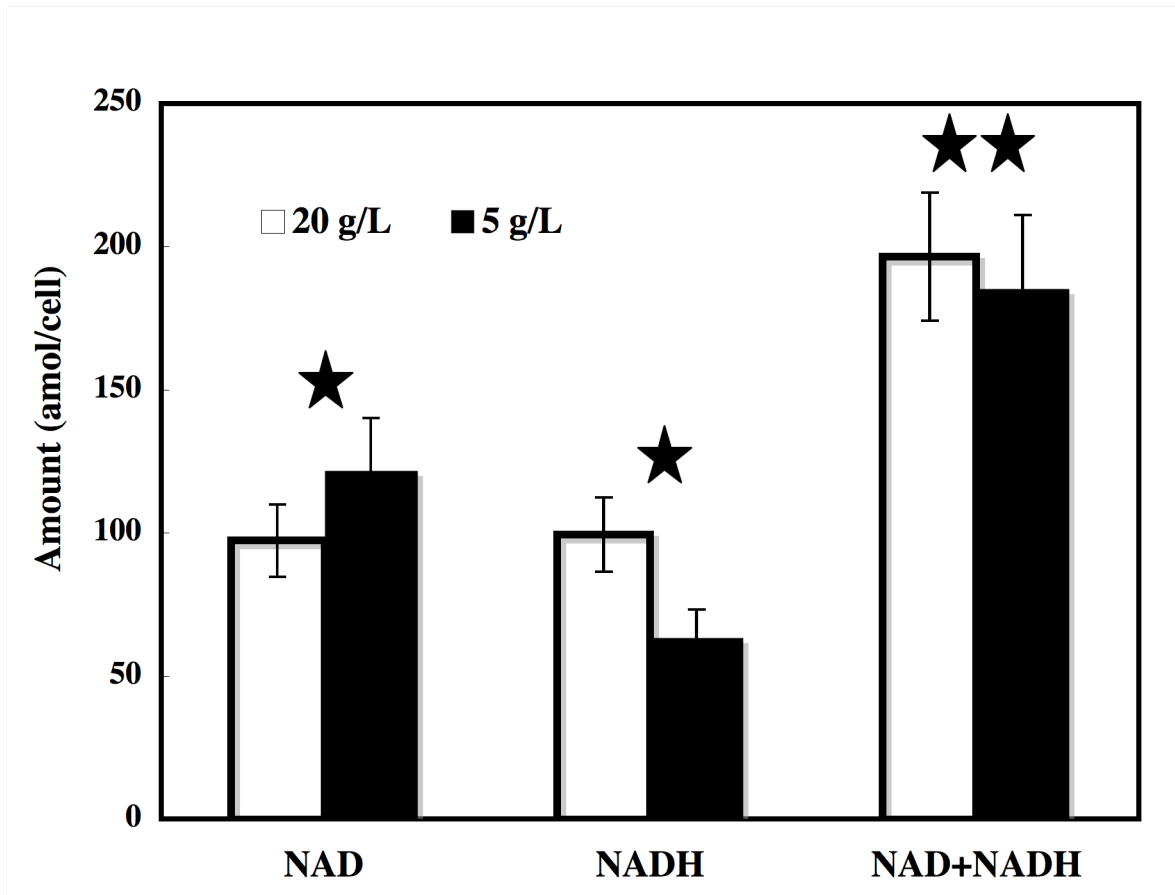


505 **Figure 3:**
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508 **Figure 4:**
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