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LLNL-JRNL-400434

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January 11, 2008

Seperation Science

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

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Keywords: yeast, HPLC, NAD, NADH, isotope label

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Abstract

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

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 flourimetrically. 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

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

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

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

Methods

Yeast culture.

Saccharomyces cerevisiae (BY4742) were cultured in 25-ml volumes of liquid synthetic complete media at 30°C consisting of 6.7 g/L Bacto yeast nitrogen base without amino acids (Becton-Dickinson, Franklin Lakes, NJ), 1.92 g/L yeast synthetic drop-out media supplement without uracil (Sigma-Aldrich, St Louis, Mo), 0.08 g/L uracil (Sigma-Aldrich, St Louis, Mo) and 20 g/L glucose (anhydrous dextrose) (EMD Chemicals, Gibbstown, NJ) dissolved in distilled H_2O. Media was filter sterilized (0.22-µm GP Express Plus Membrane, Millipore, Billerica, MA) before use as previously described[20]. For calorie restriction, 5 g/L instead of 20 g/L glucose was used. Around 10^4 yeast were used to inoculate each culture. 100-µl aliquots were periodically taken from culture for growth and cell density measurement via hemacytometer. Cultures were maintained until they contained ~ 7 x 10^6 cells/ml corresponding to mid-log phase growth.

Preparation of yeast pellets for metabolite extraction.

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

Single sample metabolite extraction for HPLC speciation.

Using the work of Lazzarino et al.[18] as a conceptual basis, an extraction protocol was developed to recover water-soluble metabolites from yeast cells for fractionation by HPLC. 600 µl of 50-mM ammonium acetate (Aldrich, Milwaukee, WI) saturated with N_2 gas was added to the pelleted yeast in bead blasting tubes and the tubes filled to the meniscus with 212-300 µm diameter acid-washed glass beads (Sigma-Aldrich, St Louis, Mo). Yeast were bead blasted at maximum speed using a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK) for 30 seconds followed by a 2-minute incubation on ice and another 30 seconds of bead blasting. The bead

blasting tube was then inverted and its base lanced with a red-hot 0.5 inch long 26-gauge needle (Becton Dickinson & Co, Franklin Lakes NJ). An open-ended 1.5 ml-microfuge tube (Eppendorf, Westbury NY) was placed over the base of bead blasting tube and the assembly nested upright in a 50-ml Falcon tube (Becton-Dickinson, Franklin Lakes, NJ). The nested tubes were centrifuged at 2000 rpm for 3 minutes in a Sorvall centrifuge to separate cell lysates from glass beads. Cell lysates were then transferred to a cold 2 ml microfuge tube (Eppendorf, Westbury NY) and kept on ice. The glass beads in the bead blasting tube were then washed twice with 600 µl of a N_2-saturated solution of acetonitrile (Sigma-Aldrich, St Louis, Mo) and 50-mM ammonium acetate (3:1; v:v) vortexed and centrifuged for 3 minutes at 2000 rpm at 4°C. After each wash, the rinsate was transferred to the microfuge tube containing the cell lysate.

Following washing, cell lysates were centrifuged at 10,000 rpm for 1 minute at 4°C. The supernatant was transferred to a 10-ml glass test tube (Corning Inc., Corning NY) and stored on ice. The remaining pellet was resuspended in 600 µl of a N_2-saturated solution consisting of acetonitrile and 50-mM ammonium acetate (3:1; v:v) and centrifuged at 10,000 rpm for 5 minutes at 4°C. The resulting supernatant was pooled with the previous supernatant and the remaining pellet discarded. Lipids were extracted from the liquid volume using 10 ml of ice-cold chloroform (VWR International, West Chester, PA) and 5 seconds vigorous agitation. Samples were left to separate on ice for 30 minutes. The upper aqueous phase was collected in a 15-ml Falcon tube and was quickly frozen at -80°C and lyophilized for 15 hours in a Christ Alpha 1-2 freeze dryer (Martin Christ GmbH, Germany) while the organic phase was discarded. To test whether discarded protein pellets contained NAD or NADH, randomly selected pellets were resuspended in 600 µl of chloroform using vigorous agitation, dried under a stream of nitrogen, resuspended in 600 µl of acetonitrile, and stored in a 4°C refrigerator overnight. The lysate was then vortexed vigourously for 30 seconds and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was frozen at -80°C and then lyophilized for at least 15 hours.

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

HPLC speciation of metabolites.

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

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

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

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

Mass spectrometry measurements.

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

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

Reproducibility, linearity and sensitivity provided by the chromatographic method.

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

Recovery and reproducibility of the extraction protocol.

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

Statistical analysis.

Differences in NAD and NADH contents of yeast grown in 20 g/L or 5 g/L glucose were assessed by unpaired two-tailed Student's t-tests. A significance level of less than 0.02 was 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 µl) 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-µg 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

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

Measurement of NAD and NADH contents in yeast.

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

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

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

Discussion

Minimization of artifactual oxidation of reduced metabolite species is challenging to achieve for any metabolite extraction protocol. Here, we have improved on previous approaches and developed a method for measuring NAD and NADH in the same sample of yeast using a simple extraction protocol. Reverse phase HPLC provided excellent speciation and resolution of NAD and NADH from other metabolites within a 25-minute runtime. The dynamic range of the chromatographic method was shown to be linear for the 38 ng to 18.3 μg quantities of the authentic NAD standard studied and 68 ng to 13.9 μg quantities of the authentic NADH standard studied. Meanwhile, metabolite extracts from ~ 7x 10^7 yeast cultured in 20 g/L glucose contained approximately 4 μg each of NAD and NADH, while yeast cultured in 5 g/L glucose contained approximately 5 μg of NAD and approximately 2.5 μg NADH. Consequently, the quantities of authentic standards of NAD and NADH used in studying the linearity of the chromatographic method correspond to NAD and NADH contents that one could expect to extract from ~ 2 x 10^6 to ~ 2 x10^8 yeast. The sensitivity of the chromatographic method for NAD (10 ng) and NADH (20 ng) detection compares favorably with that of other chromatographic methods[17] and suggest that NAD contents from as few as 2 x 10^5 yeast cells and NADH contents from as few as 5 x 10^5 yeast cells could be quantified. However, such cell numbers are at least as high those used for enzymatic cycling measurements of NAD and NADH in yeast which are routinely performed on 10^5.[10] The chromatographic method produces acceptable intra-day and inter-day reproducibility in retention time, speciation and subsequent quantitation of NAD and NADH. The higher inter-day variation observed for quantitation of the authentic compounds plausibly arises as the intra-day measurements were performed on the same set of standard solutions while fresh solutions were prepared weekly for the inter-day measurements. The higher inter-day than intra-day variation observed for retention time of the authentic compounds plausibly arises as day to day variations in pump and column performance are likely greater than variation within a single day.

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

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

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

NAD and NADH levels in yeast determined in the current study are compared with those obtained in prior studies via enzymatic assay in Table 4. Total NAD values for normal growth conditions (i.e., 20 g/L glucose) have been reported between 79.8 and 210 amol/cell,[3,9-12] which is consistent with our findings of 97 +/- 13 amol/cell. There are few published reports of measured NADH values in yeast presumably because of the difficulty in making measurements on such readily oxidizable molecules. NADH values obtained from the current method are nearly 40% higher than those obtained through enzymatic cycling assays.[3,10] Cells grown under calorie restriction (i.e., 5 g/L glucose) have been reported to contain a similar range of NAD values as cells grown without calorie restriction.[3,10] We measured 122 +/- 19 amol/cell using the current method, which falls well within the published range. NADH values obtained from yeast grown under calorie restriction have been reported at 27 amol/cell.[9] Using the current method for metabolite extraction, we obtain 63 +/- 10 amol/cell of NADH from calorie-restricted cells. 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 KH_2PO_4
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 KH_2PO_4 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 stress[21] and high osmolarity[22], while NADH
384 has been shown to degrade more rapidly in KH_2PO_4 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 KH_2PO_4 containing buffers during both cell lysis and metabolite
393 extraction[18]. 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 KH_2PO_4-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

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

The protocol reported here measures the total amount (i.e., free + bound) of NAD and NADH in yeast. Ratios of total NAD to total NADH in yeast seem to be within a reasonable range for NAD regulation.[24] However, baseline ratios of the free pool of NAD:NADH have been debated.[25-29] The free pool NAD:NADH ratio has been estimated from concentrations of the intracellular metabolites, pyruvate and lactate, for various tissues in different species animal species to range from 0.1 to 10.[25-29] During development of the assay reported here, we found that yeast cell lysates that were passed through a 0.45-μm glass fiber filter, immediately after bead beating and prior to metabolite extraction, possessed NADH levels that were around one sixth of those obtained from extractions of unfiltered lysates (data not shown). NAD levels, however, were not altered resulting in NAD/NADH ratios of approximately 7. This observation of filtered NAD and NADH levels is consistent with the hypothesis that the majority of cellular NADH is likely membrane or protein bound, while the majority of NAD is not protein bound. In our protocol, bound NADH is likely effectively released from proteins after bead beating by the use of acetonitrile which is well-known for its ability to denature proteins.[30]

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

Acknowledgements

The authors would like to thank Dr. Magnus Palmblad for performing some of the initial sample extraction and HPLC speciation development and Dr. Sang Soo Hah for performing the ESI mass spectrometry measurements. This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344 and was supported by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program (P41 RR13461).

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

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.)

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)

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

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)

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

	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	-	-		-	-	-	

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

[a]Reported values[10] have been converted from mM/cell to amol/cell assuming a yeast cell volume of 70 fL (10^{-15} L) and are expressed as the mean and standard deviation (SD) of 3 experiments performed in duplicate.

[b]Reported values[3] were converted to mM/cell as reported[10] and converted to amol/cell here assuming a yeast cell volume of 70 fL.

Figure Captions

Figure 1: Typical HPLC chromatograms of 10-µl aliquots of 50-mM ammonium acetate containing a) 2.50-µg NAD and b) 2.34-µg 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 µg. NADH contents range from 0.068 ng to 13.87 µg. 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.

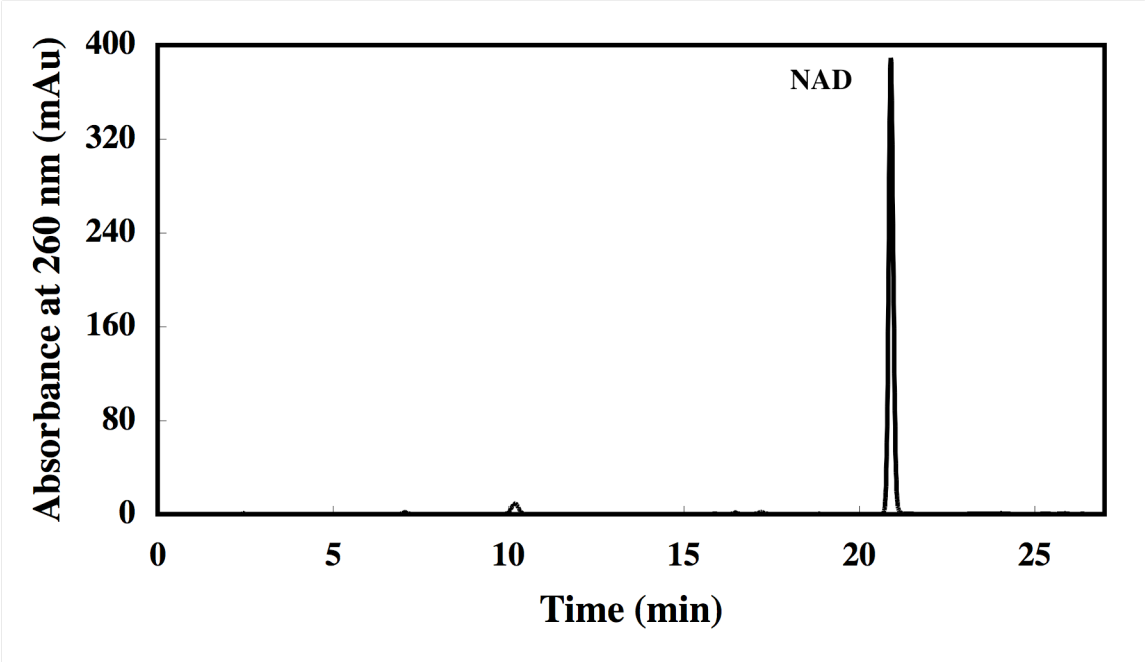
Figure 3: Typical HPLC chromatogram of a 75-ul aliquot of yeast metabolite extracts from approximately 7 x 10^7 yeast cells grown in 20 g/L glucose. Retention times (t_r) of the examined compounds are: NAD t_r = 21.3 min., and NADH t_r = 24.9 min.

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 (* $p < 0.02$, ** $p > 0.10$).

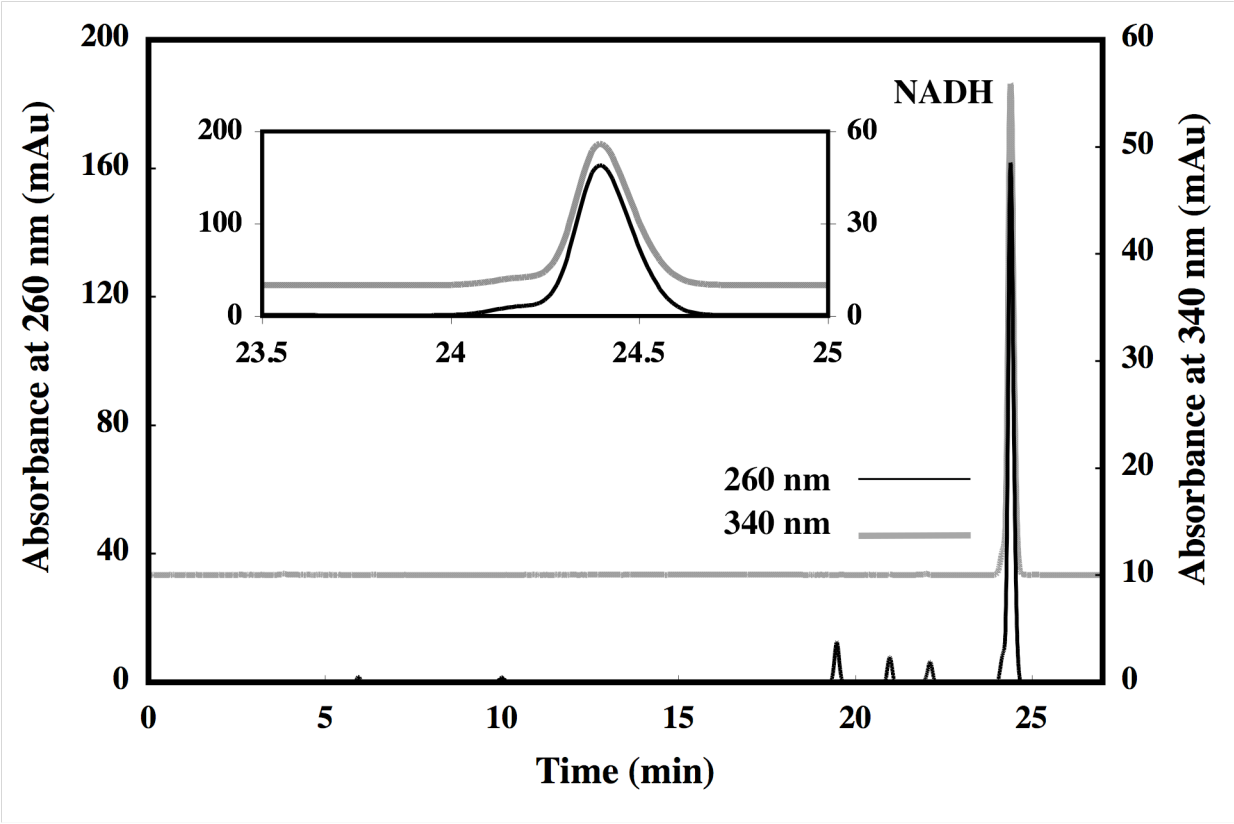
Figures

Figure 1:

A)



B)



504 **Figure 2:**

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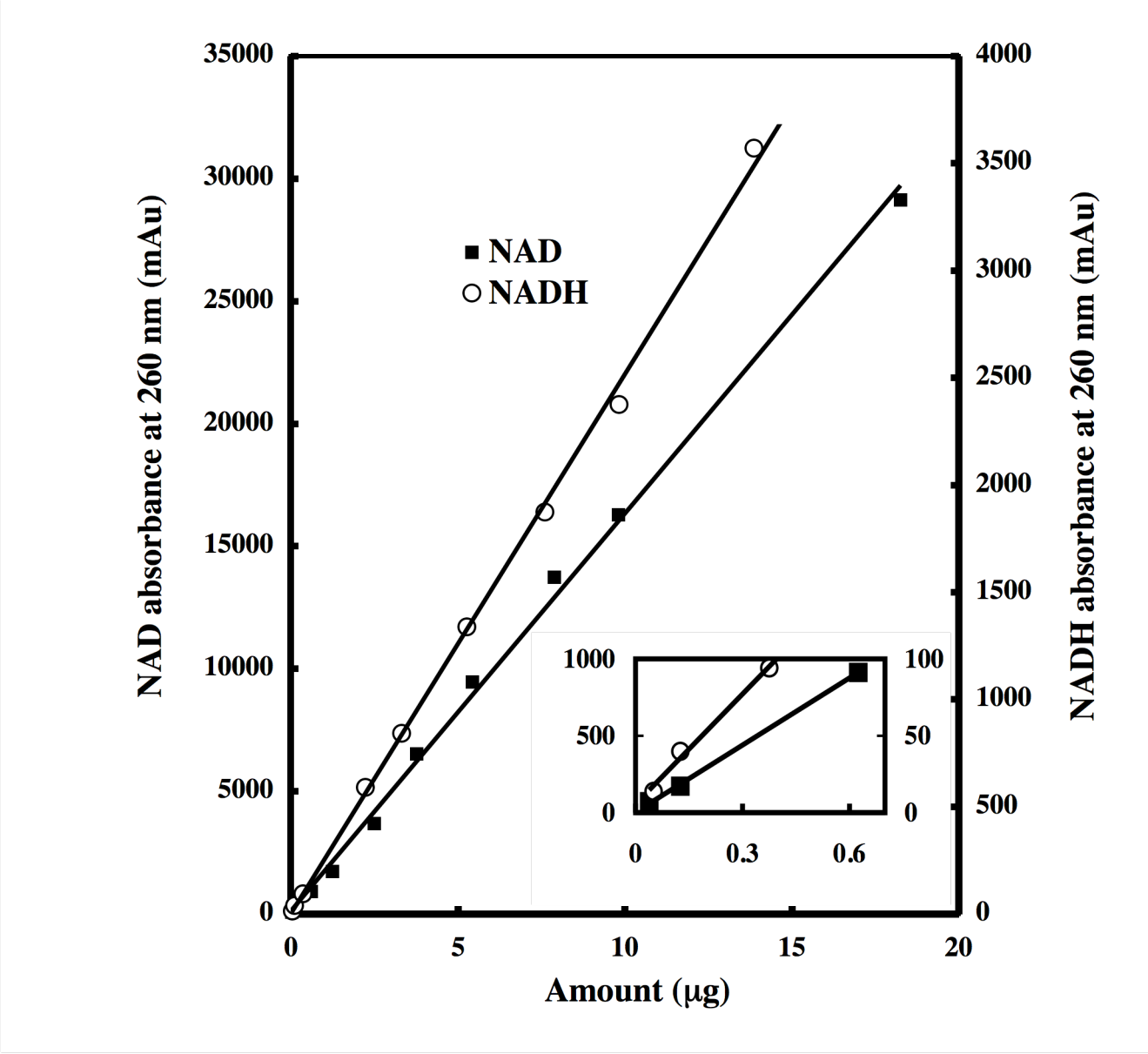

505 **Figure 3:**
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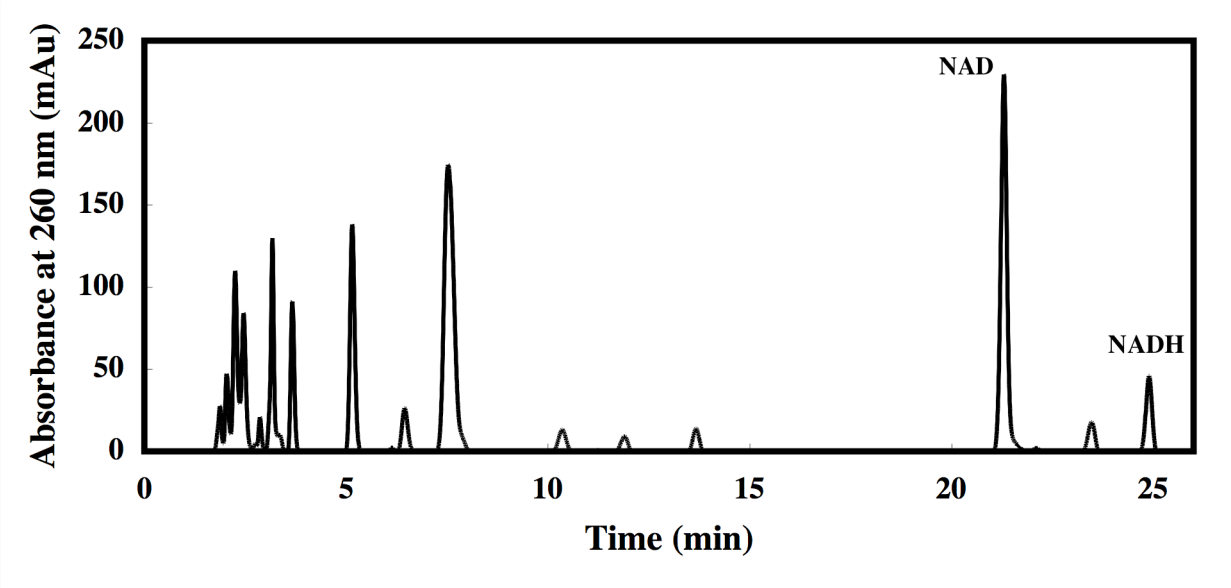

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508 **Figure 4:**
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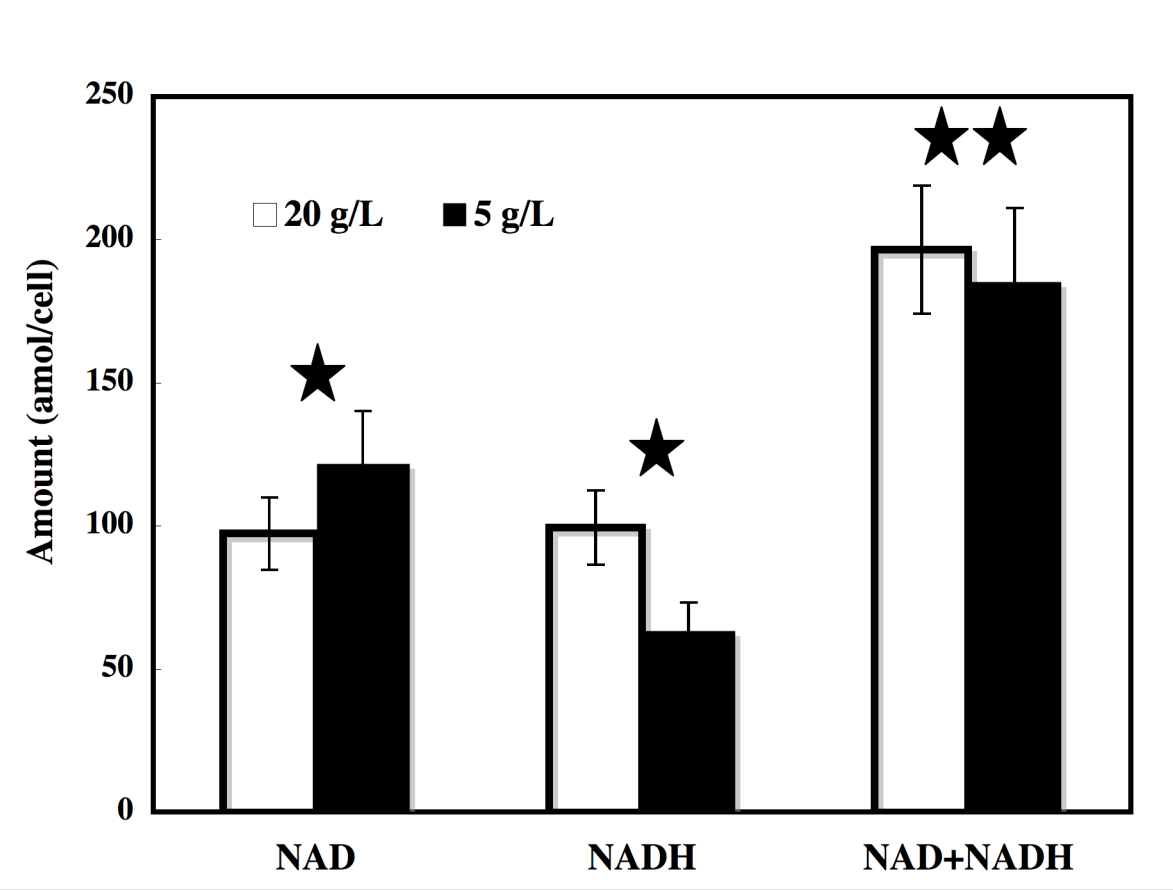

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