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Stable isotope labeling by essential nutrients in cell culture (SILEC) for accurate measurement of nicotinamide adenine dinucleotide metabolism[†]

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Abstract

Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are conserved metabolic cofactors that mediate reduction-oxidation (redox) reactions throughout all domains of life. The diversity of synthetic routes and cellular processes involving the transfer of reducing equivalents to and from these cofactors makes the accurate quantitation and metabolic tracing of NAD(H) and NADP(H) of broad interest. However, current analytical techniques typically rely on standard curves that do not incorporate confounding effects of the sample matrix. We utilized the essential requirement of niacin and tryptophan for NAD synthesis in mammalian cells to devise a stable isotope labeling by essential nutrients in cell culture (SILEC) method for efficient labeling of intracellular NAD(H) and NADP(H) pools. Coupling this approach with detection by liquid chromatography-high resolution mass spectrometry (LC-HRMS), we demonstrate the utility of incorporating a [¹³C₃¹⁵N₁]-nicotinamide moiety into a library of NAD-derived metabolites for use as internal standards in matrixed samples. Using a two-label system incorporating [¹³C₃¹⁵N₁]-nicotinamide and [¹³C₁₁]-tryptophan, we quantify the relative contribution of salvage and *de novo* NAD synthesis, respectively, in *S. cerevisiae* and HepG2 human hepatocellular carcinoma cells under basal conditions. As a further proof-of-principle, we demonstrate an improvement in the linear range for quantification of NAD and apply this method to analysis of NAD(H) in mouse liver. This method demonstrates the generalizability of SILEC, and provides a simple method for generating a library of stable isotope labeled internal standards for quantifying and tracing the metabolism of cellular and tissue NAD(H) and NADP(H).

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Conflicts of interest

There are no conflicts to declare.

Introduction

The pyridine nucleotides nicotinamide adenine dinucleotide (NAD⁺ or NAD) and its phosphorylated derivative, NAD phosphate (NADP⁺ or NADP), are ubiquitous evolutionarily conserved metabolic cofactors. The oxidized metabolites undergo reversible cycles of reduction to NADH and NADPH, respectively, continuously shuttling the hydride ions that form the fundamental units of cellular energy currency.¹ In these roles, NAD and NADP help to maintain anti-oxidant defenses, as well as carbon flux through glycolysis, the pentose-phosphate pathway, β -oxidation, and fatty acid synthesis. NAD also forms the primary chemical link between the TCA cycle and mitochondrial electron transport chain.² Since eukaryotic cells also utilize NAD as a co-substrate in a wide variety of post-translational peptide modifications, including mono- and poly-ADP-ribosylation, deacylation, and the production of signaling molecules such as cyclic ADP-ribose and NaADP, the size of intracellular NAD(P) pools and the ratio of oxidized to reduced metabolites must be tightly regulated.³ Quantification of the amount of NAD(H) and NADP(H) thereby provides critical insight into the metabolic state of a cell or tissue.⁴

Established techniques for the measurement of NAD(H) and NADP(H) include variations of enzymatic cycling assays,⁵ as well as liquid chromatography-mass spectrometry (LC-MS) based approaches.^{4,6,7} LC-MS based assays of NAD(H) and NADP(H) are particularly attractive because of high sensitivity and specificity, even from complex samples.⁴ To best ensure accurate, precise, and reproducible measurement using LC-MS based assays, internal standards are used throughout the complete bioanalytical workflow to adjust for variations in extraction and analysis and to facilitate quantification by providing an internal normalization factor.⁸ Inter-conversion and protein-binding is of special concern in the case of NAD metabolites. Although these processes can be controlled to some extent by optimal sample handling,⁶ stable isotope-labeled analogs are an ideal means of accounting for this instability. A previous study by Ortmayr, *et al.* specifically noted the lack of available stable isotope labeled analogs as an impediment to conducting rigorous and reproducible NAD metabolite analysis.⁹ Furthermore, since LC-high resolution MS (LC-HRMS) also permits simultaneous metabolite quantitation and tracing with isotope-specific resolution, a diversity of stable isotope labels (*e.g.* ²H, ¹³C, and/or ¹⁵N) can be introduced by experimental design and distinguished by the HRMS.^{10,11}

The purpose of this study was to utilize tryptophan and/or niacin essentiality to generate a SILEC system with high labeling efficiency for NAD(H) and NADP(H). The system augments published techniques^{4,6} by using isotope encoded labeling (¹³C and/or ¹⁵N). For quantification, this SILEC approach allows more complete labeling and requires less rigorously controlled conditions than complete carbon labeling. For metabolic pathway tracing, this labeling allows determination of the preference for precursor biosynthetic substrates under different conditions. We applied this system to the quantitation of pyridine nucleotides across various biological matrices in order to assess performance of the assay with or without internal standardization. We demonstrate that a multi-label system can be used to improve the performance, specifically the linear range, of existing LC-MS based assays of NAD(H) and NADP(H).

Materials and methods

Chemicals

Optima LC-MS grade water, methanol, and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Waltham, MA). NADH, NADP, NADPH, diisopropylethylamine (DIPEA), niacin, tryptophan, 5-sulfosalicylic acid, and 1,1,1,3,3,3-hexafluoro 2-propanol (HFIP) were purchased from Sigma-Aldrich (St Louis, MO). NAD was from Roche. [$^{13}\text{C}_{11}$]-tryptophan was from Cambridge isotope laboratories (Tewksbury, MA) and [$^{13}\text{C}_3^{15}\text{N}_1$]-nicotinamide was from Isosciences (King of Prussia, PA). Defined yeast media was reconstituted from yeast drop-out mix from US Biological sciences (Salem, MA).

Cell culture

Niacin and tryptophan free RPMI1640 media from Corning Life Sciences (Tewksbury, MA), was reconstituted with natural isotopic abundance 2 mg L^{-1} niacin, 5 mg L^{-1} tryptophan, 2 mg L^{-1} [$^{13}\text{C}_{11}$]-tryptophan and/or 5 mg L^{-1} [$^{13}\text{C}_3^{15}\text{N}_1$]-nicotinamide as indicated for each experimental group. HepG2 human hepatocellular carcinoma cells were obtained from ATCC, and passaged at 80% confluence. Fetal bovine sera (FBS) that were dialyzed (dFBS) or charcoal-stripped (csFBS) were purchased from Golden West Biological (Temecula, CA). Reduced amounts of serum in the media increased doubling time in culture after the first passage. The protocol was therefore modified to passage cells in media containing 10% serum, then to perform an overnight incubation in 2% serum containing media before harvesting the cells for use as an internal standard. This “ultra-labeling” has been used successfully before in pantothenate SILEC.¹²

Trp5 mutant *S. cerevisiae* (Carolina Biological Supply, Burlington, NC) was cultured in standard defined medium as previously described¹³ but omitting tryptophan and niacin, which was then replaced with labeled analogs as above. Yeast culture was grown to the stationary phase, then harvested for extraction.

Analysis of NAD(H)/NADP(H) by LC-HRMS

HepG2 cells were harvested by lifting with a cell scraper, followed by centrifugation at $500g$ for 5 min at 4°C . Yeast cells were pelleted by centrifugation at $900g$ for 10 min at 4°C . Cell and yeast pellets were then extracted by adding 80: 20 (v/v) methanol: water at -80°C with 15 0.5 s pulses with a handheld sonicator (Fisher) and, centrifuged at $16\,000g$ for 10 min at 4°C to remove insoluble debris. The supernatant was diluted 200 times in 5% 5-sulfosalicylic acid and then $5 \mu\text{L}$ was injected for analysis by LC-HRMS with modifications as indicated from a previously described method.¹⁴ Animal experiments were conducted under an approved University of Pennsylvania Institutional Animal Care and Use Committee protocol, and in compliance with federal, state and local laws. Tissue samples were cut over dry ice to aliquots of 10–90 mg, weighed to on a balance with tolerance to 0.01 mg, spiked with $500 \mu\text{L}$ of SILEC cell extract, then extracted by -80°C 80: 20 (v/v) methanol: water, sonication to homogeneity with 60 0.5 s pulses with a handheld sonicator (Fisher), and centrifugation as above. Briefly, NAD(H)/NADP(H) was quantified by single ion monitoring (SIM) on an Ultimate 3000 UHPLC coupled to a Q Exactive HF (Thermo).

Statistical analysis

Data analysis was conducted in XCalibur 3.0 Quan Browser and Tracefinder 4.1 (Thermo) and statistical analysis was conducted in Excel 2016 (Microsoft) and Graph Pad Prism 7 (GraphPad Software). Isotopologue enrichment was calculated as previously published.¹⁵ Statistical comparisons for tissue levels of NAD(H) were conducted by Mann Whitney (non-parametric) tests.

Results and discussion

Labeling purity and distribution of tryptophan *versus* nicotinamide-derived NAD

Molecular pathways responsible for the biosynthesis of NAD and its derivatives can be interrogated at increasingly high resolution (biological and analytical) using modern techniques. Such approaches may exploit the fact that diverse species synthesize NAD from as many as six distinct nutrient precursors, while others are restricted to a more limited set of precursors.¹⁶ In most mammalian cells and tissues, a two-step salvage pathway regenerates NAD from nicotinamide (Nam), phosphoribosyl pyrophosphate (PRPP), and ATP to maintain local NAD homeostasis.¹⁷ However, if a sufficient supply of Nam or nicotinic acid (collectively termed niacin) are not provided in the diet, higher eukaryotes, including mammals, must synthesize the Nam moiety of NAD from the essential amino acid tryptophan (Fig. 1A).¹⁸ In contrast to prokaryotes and some other eukaryotes, this trophic requirement renders mammalian pyridine nucleotides amenable to an isotopic tracing strategy capable of labeling all metabolites derived from the essential precursors, called Stable Isotope Labeling with Essential nutrients in Cell culture (SILEC). SILEC, modified from the procedure originally developed by Ong and Mann for proteogenic essential amino acids and quantitative proteomics,^{12,19} allows the production of a library of stable isotope-labeled analogs for use as internal standards in mass spectrometry (MS) based assays.⁸ The primary advantage of SILEC over abiotic synthesis of MS standards is the *in vivo* generation of an entirely labeled cell system, allowing entire biochemical pathways to be interrogated, while preserving covalent modifications, metabolite ratios, and redox distribution of the labeled metabolites under different biological conditions. Since the essential nutrient is incorporated into every molecule in the pathway, SILEC is an efficient labeling strategy for entire biochemical pathways, as compared to developing a synthetic route for abiotic production of each individual molecule in the pathway. The importance of the source of NAD precursors extends to clinically relevant human biochemistry, where specific defects in NAD synthesis pathways are linked to birth defects.²⁰ The structure of NAD(H) includes a nicotinamide moiety joined to adenosine diphosphate by a ribose nucleotide, while NADP(H) includes an additional phosphate attached to C2 of the ribose ring, adjacent to the adenine moiety (Fig. 1B). The restricted number of precursors for the nicotinamide moiety of the NAD backbone potentially allows the introduction of both ¹³C and/or ¹⁵N labels at high efficiency. This has not previously been quantitatively examined in the literature.

Since the purity of isotopic labeling is often a limiting factor in sensitivity and precision of stable isotope dilution LC-MS based assays, we quantified incorporation of labels into NAD and NADP over multiple 2–3 day passages of HepG2 cells. Each passage allowed at least a doubling of cell number and time for basal turnover of the NAD pool. We found that

nicotinamide-derived [$^{13}\text{C}_3$ $^{15}\text{N}_1$] labels were efficiently incorporated into NAD/NADP within 1 passage (84–86% labeled), but only 1–2% of tryptophan-derived ^{13}C labeling were incorporated over the same period (Table 1). We therefore stopped passaging HepG2s in SILEC media containing $^{13}\text{C}_{11}$ -tryptophan and unlabeled nicotinamide due to futility. By the third generation, NAD/NADP labeling was over 99.5%, achieving an acceptable purity for analysis (Fig. S1[†]). Although the use of dialyzed *versus* charcoal-stripped FBS can affect the efficiency of acyl-CoA SILEC,¹² both dFBS and csFBS provided interference-free, high-efficiency labeling in this case. Previous results with pantothenate labeling indicate batch to batch variability of serum lots,^{13,21} however, we did not examine this phenomenon here since both lots tested were acceptable (>99.5% labeling by p3 in all cases).

Yeast culture allows a more rapid scaling of SILEC production due to easily defined media conditions and rapid cell division.¹³ We selected a strain of *S. cerevisiae* deficient in tryptophan synthetase (Trp5), the enzyme required to produce tryptophan from indole-3-glycerol-phosphate and L-serine, rendering this mutant auxotrophic for tryptophan. This system prevents the metabolism of unlabeled carbon sources (*e.g.* glucose, amino acids, and CO_2 -consuming reactions) into NAD metabolites. The yeast culture was conducted in non-nutrient limiting conditions with harvest at the entry into stationary growth phase as monitored by OD of the culture. In contrast to the HepG2 culture, the yeast culture derived 11.7% of the NAD *via* tryptophan, as indicated by $^{13}\text{C}_6$ isotopologue enrichment which is derived exclusively from tryptophan. This demonstrates the utility of an isotope-encoded two-label system in which NAD metabolites derived from tryptophan can be differentiated from those derived from niacin/nicotinamide. However, since the purpose of these experiments was to produce a uniformly labeled internal standard, we did not test conditions restricting niacin or tryptophan in either HepG2 or yeast culture but a nutrient-restriction may be useful for investigation of NAD synthesis.

Liquid chromatography high resolution mass spectrometry of NAD metabolites

We used a previously published ion pairing-LC-HRMS method for cellular and tissue metabolomics to quantitatively examine the labeling of NAD and the impact of labeling on analytical parameters.¹⁴ Chromatograms of NAD(H)/NADP(H) showed the expected co-elution of isotopically labeled analogs (Fig. 2). In contrast to ^2H labeling strategies,⁸ no significant shift in retention time was observed (4.2 vs. 4.3 min). As was pointed out by Trammell and Brenner, the LC separation prior to MS detection is critical, as there can be isotopic overlap in the detection of NAD metabolites when MS is used exclusively. At high resolution, NAD and NADH do not share significant overlap, since the M+2 isotopologue of NAD is sufficiently resolved from NADH. Unfortunately, the [$^{13}\text{C}_6$] labeling of NAD+/NADP+ overlaps with [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-NADH/NADPH (Fig. 2B), even at 60 000 resolving power, a commonly used resolving power for Orbitrap detectors, and above the resolving power of most time-of-flight (TOF) instruments as they are used.¹⁰ Therefore, analysts should take care when analyzing isotopic tracing experiments with poorly chromatographically resolved NAD metabolites. Mass windows used for quantitative and

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qualitative analysis are shown in Table 2, highlighting this overlap in the underlined m/z windows.

Absolute quantification of NAD

We next used an untargeted LC-HRMS method and a targeted LC-HRMS method to compare absolute quantitation with and without stable isotope dilution. Standard curves with NAD were prepared in water over the range of NAD expected from cell culture and tissue. Isotope dilution improved the linearity and increased the coefficient of determination (r^2), in both the untargeted and targeted methods (Fig. 3).

Finally, we investigated the utility of stable isotope dilution-LC-HRMS for quantification of NAD(H) in stored mouse liver samples. Since the NAD pool of mammalian liver is highly labile, responding to both circadian and dietary inputs,^{22,23} we compared tissues from mice orally treated with vehicle or 400 mg kg⁻¹ day⁻¹ of the NAD precursor, nicotinamide riboside (NR) (Fig. 4). We detected higher levels of NAD and NAD(H), with differences in the median amount (p -value) of NAD metabolites at 10.91 (0.0043) and 3.32 (0.0043) ng mg⁻¹ of tissue, respectively. Molar NAD/NADH ratio was higher in the liver from NR treated mice (3.09 vs. 2.31, p -value 0.013) is comparable to reported values, and especially close to the ratio of 2.63 reported previously.⁶ Quality controls at 10 and 20 ng on column of NAD and NAD(H) composed of samples prepared identically to the liver extracts but with pure standards in water ($n = 5$ each) yielded precision (coefficient of variation) of 5.06% and 4.01% and accuracy of 5.19% and 2.41% for NAD, and 10.43% and 10.35% and accuracy of -14.9% and 6.8% for NADH.

We did not report NADP(H) values because of the known instability of NADPH which would have potentially caused artifacts in this analysis of stored samples. Stability and interconversion of NAD metabolites in bioanalytical assays remains a notable challenge in their measurement at the extraction, storage, and analysis steps. Our sample preparation used a previously published general polar metabolite extraction using -80°C 80/20 methanol/water with dilution of the methanolic extract with 5% 5-sulfosalicylic acid. Lu, *et al.*, showed that the best extraction solvent that keeps the oxidation of NADH or NADPH to under 10%, is 4/4/2 (v/v/v) methanol/acetonitrile/water with 0.1% formic acid.⁶ 80% methanol on dry ice gave similar recovery, with less signal intensity using their LC-MS method. The same solvents yielded almost 100% recovery for NAD⁺/NADP⁺ from the cells and tissues. Ortmayr, *et al.*, conducted an extensive stability and analysis validation on NAD(H)/NADP(H) and reported that NADH and NADPH have a relatively short half-life at a pH lower than 6, and NADPH was only stable at pH 8.⁹ Therefore, for analysis of a large number of samples, buffering samples with 5 mM ammonium acetate at pH 8 may be desirable as the highly labile NADPH was stable for at least 24 hours under these conditions. LC-MS based analysis of NAD metabolites may also present some unique and unexpected challenges that analysts should be aware of. Although we did not test multiple stationary phases in this report, others have noted a large (~80%) loss of NADPH using the widely used Waters Amide HILIC stationary phase.⁹

Finally, our optimized protocol may serve as a valuable tool for conventional SILEC experiments in cultured cells (analogous to conventional SILAC) with complete labeling. In

this case, unlabeled or uniformly labeled cells may be subjected to different treatments and then mixed prior to extraction and analysis. As we have shown, this process is also amenable to spike in SILEC approaches, using the labeled lysate/extract as an internal standard.²⁴ This can provide the gold standard for measuring relative effects of a given treatment, eliminating variables associated with sample preparation and analysis as sources of error.

Conclusion

Application of SILEC to study NAD(H) and NADP(H) metabolism provides improved quantification of metabolites central to cellular processes that drive cancer, diabetes, microbiology, metabolic dysfunctions, and infection. Extending the linear range of assays to quantify NAD metabolism will reduce potential artifacts of measurement. The introduction of a variable labeling system with both ¹³C and ¹⁵N labeling also allows experimental resolution of different pathways of generating and salvaging NAD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Biosynthesis and stable isotope labeling of NAD(H)/NADP(H). (A) NAD biosynthetic pathway from tryptophan, niacin, or nicotinamide. (B) Stable isotope labeling using [$^{13}\text{C}_3$, $^{15}\text{N}_1$]-nicotinamide with the resulting incorporation of isotopes into NAD.

Fig. 2. Chromatograms of NAD analogs. (A) Co-elution of stable isotope analogs with their unlabeled analogs from second generation HepG2 cell extract analyzed by LC-HRMS. There was no appreciable pure $[^{13}\text{C}]$ labeling observed in HepG2s under conditions tested. (B) Interference in the channel for $[^{13}\text{C}_3]$ -NAD⁺/NADP⁺ (retention time 4.2 and 6.4 min, respectively) was observed from $[^{13}\text{C}_3^{15}\text{N}_1]$ -NADH/NADPH (retention time 6.2 and 6.6 min, respectively) even at a 5 ppm window with 60 000 resolving power.

Fig. 3.

Calibration curves for NAD by LC-HRMS. Calibration curve for quantification of NAD by an untargeted metabolomics method (A) without or (B) with isotope dilution (ID). A targeted NAD method (C) without or (D) with ID. Inserts of the lower points are provided to highlight the non-linearity observed without ID.

Fig. 4. Liver samples from NR versus vehicle treated mice. Liver samples from mice treated with vehicle or NR at 400 mg kg⁻¹ day⁻¹ were analyzed by SID-LC-HRMS for (A) NAD, (B) NADH content.

Table 1

Purity of isotopic labeling of NAD across different culture conditions. Tryptophan labeling alone was stopped after 1 generation due to futulity

Dialyzed serum (2%)				
Generation	¹³ C ₁₁ -tryptophan	¹³ C ₃ , ¹⁵ N ₁ -nicotinamide	NAD labeling %	NADP labeling %
1		+	84.6	85.5
1	+		0.6	1.6
1	+	+	82.4	83.2
2		+	98.7	>99.5%
2	+	+	97.9	>99.5%
3		+	>99.5%	>99.5%
3	+	+	>99.5%	>99.5%
Charcoal stripped serum (2%)				
Generation	¹³ C ₁₁ -tryptophan	¹³ C ₃ , ¹⁵ N ₁ -nicotinamide	NAD labeling %	NADP labeling %
1		+	84.8	85.3
1	+		1.3	2.3
1	+	+	85.8	85.2
2		+	98.4	98.4
2	+	+	97.8	>99.5%
3		+	>99.5%	>99.5%
3	+	+	>99.5%	>99.5%
Yeast culture				
Label	Unlabeled	¹³ C ₆ -NAD	¹³ C ₃ , ¹⁵ N ₁ -NAD	
(%)	1.3%	11.7%	88.2%	

Table 2

Mass windows for distinct NAD labeling using LC-HRMS. Potential isotopic overlap italicized and underlined for clarity

Analyte	Chemical formula	Neutral mass	Neg ion mass	Isolation window	Mass error at 5 ppm (Da) (+/-)	Mass range (<i>m/z</i> - <i>m/z</i>)
NAD ⁺	C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂	663.1091	662.1018	657-677.10	0.0017	662.1002 662.1035
NAD ⁺ ¹³ C ₃ ¹⁵ N ₁	[¹³ C ₃ C ₁₈ H ₂₇ [¹⁵ N ₁ N ₆ O ₁₄ P ₂	667.1162	666.1089	657-677.10	0.0017	666.1073 666.1106
NAD ⁺ ¹³ C ₆	[¹³ C ₆ C ₁₅ H ₂₇ N ₇ O ₁₄ P ₂	669.1293	668.1220	657-677.10	0.0017	<u>668.1203</u> <u>668.1236</u>
NADH	C ₂₁ H ₂₉ N ₇ O ₁₄ P ₂	665.1248	664.1175	661.10-671.10	0.0017	664.1158 664.1192
NADH ¹³ C ₃ ¹⁵ N ₁	[¹³ C ₃ C ₁₈ H ₂₉ [¹⁵ N ₁ N ₆ O ₁₄ P ₂	669.1319	668.1246	661.10-671.10	0.0017	<u>668.1229</u> <u>668.1263</u>
NADH ¹³ C ₆	[¹³ C ₆ C ₁₅ H ₂₉ N ₇ O ₁₄ P ₂	671.1449	670.1376	661.10-671.10	0.0017	670.1359 670.1393
NADP ⁺	C ₂₁ H ₂₈ N ₇ O ₁₇ P ₃	743.0755	742.0682	738.08-758.08	0.0019	742.0663 742.0700
NADP ⁺ ¹³ C ₃ ¹⁵ N ₁	[¹³ C ₃ C ₁₈ H ₂₈ [¹⁵ N ₁ N ₆ O ₁₇ P ₃	747.0826	746.0753	738.08-758.08	0.0019	746.0734 746.0771
NADP ⁺ ¹³ C ₆	[¹³ C ₆ C ₁₅ H ₂₈ N ₇ O ₁₇ P ₃	749.0956	748.0883	738.08-758.08	0.0019	<u>748.0864</u> <u>748.0902</u>
NADPH	C ₂₁ H ₃₀ N ₇ O ₁₇ P ₃	745.0911	744.0838	740.09-760.09	0.0019	744.0820 744.0857
NADPH ¹³ C ₃ ¹⁵ N ₁	[¹³ C ₃ C ₁₈ H ₃₀ [¹⁵ N ₁ N ₆ O ₁₇ P ₃	749.0982	748.0909	740.09-760.09	0.0019	<u>748.0890</u> <u>748.0928</u>
NADPH ¹³ C ₆	[¹³ C ₆ C ₁₅ H ₃₀ N ₇ O ₁₇ P ₃	751.1112	750.1040	740.09-760.09	0.0019	750.1021 750.1058