EXPERIMENTAL ARTICLES

UDC 577.113.3:681.785.58 https://10.15407/biotech13.02.032

THE APPROACH FOR EXPRESS SPECTROMETRIC DETERMINATION OF THE REDUCED FORM OF NICOTINAMIDE ADENINE DINUCLEOTIDE (NADH) CONTENT

I. P. Krysiuk
I. R. Horak
S. G. Shandrenko

Palladin Institute of Biochemistry
of the National Academy of Sciences of Ukraine, Kyiv

E-mail: 4iryna.kr@gmail.com

Received 16.01.2020
Revised 23.03.2020
Accepted 30.04.2020

It is known that nicotinamide adenine dinucleotide (NADH/NAD^+) serves as a cofactor for many enzymes involved in the cell metabolism, redox control, signaling, biodegradation and other processes. Thereby determination of NADH/NAD^+ production is commonly used for the measurement of NADH/NAD^+-dependent enzymes activities. However, NADH may be oxidized spontaneously to NAD^+ form, so the aim of this study was to develop new approach for spectrometric determination of real NADH content in a sample.

There had been used optical absorbance intensities at wavelengths 234, 260, 290, 340, and 400 nm in order to calculate the percent of NADH in a sample.

An original formula for the calculation of NADH percent in a sample was figure out, and the example of its application was presented.

The proposed calculation method could be applied for quick and routine NADH content determination at any laboratory equipped with spectrometer.

Proposed method may be used for quick and routine determination of NADH content in any laboratory equipped with spectrometer.

Key words: NADH content determination, ultraviolet (UV) spectrometry.

Niacin (vitamin B_3) and its coenzyme forms NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate) play an important role in cellular metabolism, including glysolysis, Krebs cycle, fatty acids oxidation, etc [1–3]. As well, NAD^+/NADH have a critical impact on the cell signaling [2, 4], redox state [1], biodegradation and detoxification [5], stress response [6], DNA reparation [7], stemness and pluripotency [8, 9], differentiation, development, and transformation [10]. Novel studies demonstrate that NAD^+ supplementation may be used for treatment or correction of amyotrophic lateral sclerosis (ALS) [11], congenital malformations [12], and Friedreich's ataxia (FA) [13]. Also, Ye and colleagues demonstrated the essential role of NAD^+ salvage pathway and the enzyme nicotinamide phosphoribosyltransferase in the proliferation of colorectal cancer [14].

Nicotinamide adenine dinucleotide exists in two forms: an oxidized (NAD^+) and reduced (NADH) form. In cell metabolism, NAD^+/NADH are involved in redox reactions, carrying electrons and protons from one compound to another [1].

Biochemical studies use various enzymatic methods with the evaluation of produced NADH concentration [15–18]. However,

NADH may be spontaneously oxidized to NAD^+ during storage [19, 20], so there is a strong need to develop a quick procedure for measurement the real NADH content in NADH reagent for the proper estimation of the obtained results.

Both NAD^+ and NADH strongly absorb ultraviolet (UV) light because of the presence of adenine. For example, absorption peak of NAD^+ is at a wavelength of 259 nanometers (nm), with an extinction coefficient of 16,900 $M^{-1}cm^{-1}$. NADH also absorbs at higher wavelengths, with a second UV absorption peak at 339 nm with an extinction coefficient of 6,220 $M^{-1}cm^{-1}$. This difference in the ultraviolet absorption spectra between the oxidized and reduced forms of the coenzymes at higher wavelengths makes it simple to measure the conversion of one to another forms in enzyme assays by measuring the amount of UV absorption at 340 nm using a spectrophotometer [21, 22].

NAD^+ and NADH also differ in their fluorescence. NADH in solution has an emission peak at 445 nm (λ excitation — 340 nm) while the oxidized form of the coenzyme does not fluorescent [21, 22]. But sometimes such fluorescent estimation is not appropriate or not available for the determination of reduced/oxidized form ratio.

The aim of this study was to elaborate a method for the rapid evaluation of NADH content in NADH reagent by the UV spectroscopic measurement and calculation.

Materials and Methods

Chemicals and reagents. NADH and NAD^+ were obtained from AppliChem. Resazurin sodium was obtained from Sigma Chemical Company. Phenazine methosulfate and other reagents were obtained from Alfarus Company. All used reagents and chemicals were of analytical grade.

Equipment and sofrware. Spectrophotometer mQuant Microplate Reader and fluorimeter FL800 Microplate Reader (BioTek Instruments) were used. Data was plotted using Gen5™ Data Analysis Software.

Estimation of NADH samples fluorescence. Three 0.18 mM NADH samples in 50 mM phosphate buffer (pH 7.6) were prepared from NADH-reagents with different storage time. These NADH samples were going to be used as the NADH concentration standard solution for enzymatic dehydrogenase reactions. To these samples were added 0.1 mM PMS (phenazine methosulfate) and 0.1 mM Resazurin dye and in 10 min the fluorescence data (ex 545 nm; em 600 nm) were measured.

Data analysis. Fluorescence (ex 545 nm; em 600 nm) of NADH samples with PMS/resazurin dye was present as mean±sd. Percent of NADH in selected samples of NADH reagent was calculated in Microsoft Excel using developed formula "% NADH". Pearson correlation coefficients were determined using Microsoft Excel build-in procedure.

Results and Discussion

Development of the method. NADH evaluation may be conducted using spectrophotometric or fluorescent methods [21, 22]. BioTek's kit of reagents "Determination of NADH Concentrations" demonstrates linear concentration dependence on absorbance at 340 nm and fluorescence at 445 nm especially for low NADH concentration [22]. But for applying such methods, the standard of exact NADH concentration is needed for the correct calibration. Thereby, an express approach for the NADH content correct evaluation in NADH-reagents would be useful.

Spectral scan of the NADH solution is represented at Figure. It is supposed that five wavelength points reproduce such spectral feature. They are: 234 nm, 260 nm, 290 nm, 340 nm, and 400 nm. Three wavelength points (234 nm, 290 nm, 400 nm) describe the background of the spectra. Two other points (260 nm and 340 nm) are the maximum absorbance of NAD^+ and NADH, correspondingly.

To diminish the influence of background data, it was calculated "relative absorbance" (OD′):

$$OD'_\lambda = (OD_\lambda - OD_{234}) + \frac{OD_{234} - OD_{290}}{(290 - 234)} \cdot (\lambda - 234),$$

for wavelength range from 234 nm to 290 nm

$$OD'_\lambda = (OD_\lambda - OD_{290}) + \frac{OD_{290} - OD_{400}}{(400 - 290)} \cdot (\lambda - 290).$$

for wavelength range from 290 nm to 400 nm.

The sum of the relative absorbance points ("integral") for the two bands was calculated.

$$I_{NAD} = \sum_{\lambda=239}^{290} OD'_\lambda, \qquad I_{NADH} = \sum_{\lambda=290}^{400} OD'_\lambda.$$

Then, these two obtained integrals were normalized by the maximal relative absorbance for each range.

$$I'_{NAD} = I_{NAD} / OD'_{260}; \qquad I'_{NADH} = I_{NADH} / OD'_{340}.$$

The results of this calculation for the spectrum of the experimental solution of NADH (Figure) are following: absorbance points OD_{234} = 1,22; OD_{260} = 2,47; OD_{290} = 0,30; OD_{340} = 1,08; OD_{400} = 0,06; relative absorbance points OD'_{234} = 0,0; OD'_{260} = 1,67; OD'_{290} = 0,0; OD'_{340} = 0,89; OD'_{400} = 0,0; the value of integrals I_{NAD} = 41,49; I_{NADH} = 46,82; the value of normalized integrals I'_{NAD} = 24,78; I'_{NADH} = 52,78. It is necessary to take into account the coefficient of molar extinction: E_{NAD} = 16 900 $M^{-1}cm^{-1}$; E_{NADH} = 6 200 $M^{-1}cm^{-1}$.

It is proposed the formula for calculating the percentage of NADH in a solution containing NADH + NAD^+:

$$\%\,NADH = NADH/(NADH+NAD)\cdot 100 = 1/(1+NAD/NADH)\cdot 100,$$

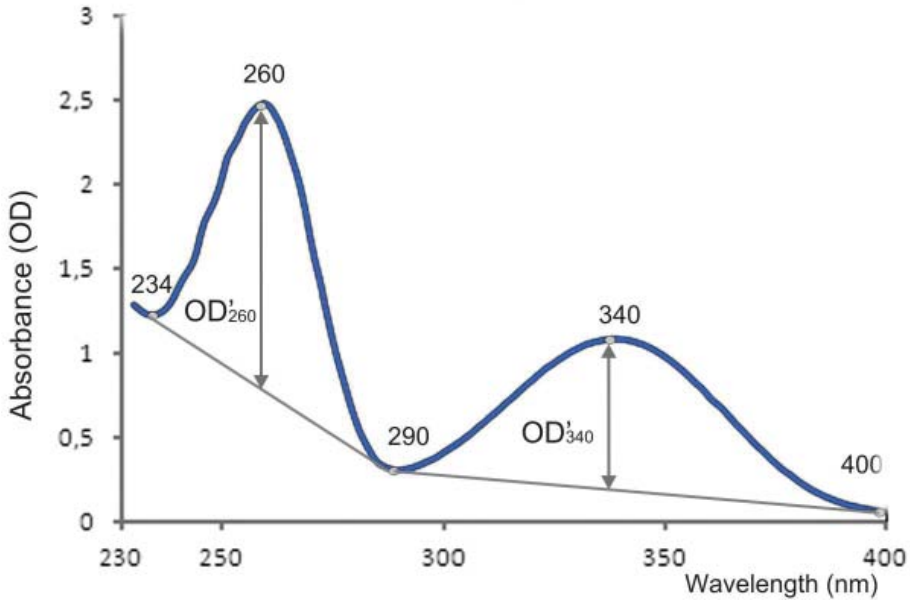
or:

$$\%\,NADH = 1/(1+(OD_{260} - OD_{234} + (OD_{234} - OD_{290})/(290 - 234) \times (260 - 234) \times 24.78/16900)/(OD_{340} - OD_{290} + (D_{290} - OD_{400})/(400 - 290) \times (340-290)\times 52.78/6200))\times 100.$$

This calculation is easy to carry out in Microsoft Excel. The formula in R1C1 reference style is:

$$\%\,NADH =1/(1 + (R[-3]C[-1]-R[-4]C[-1] + (R[-4]C[-1]-R[-2]C[-1])/(290-234)\times (260-234)\times 24.78/16900)/(R[-1]C[-1]-R[-2]C[-1] + (R[-2]C[-1]-RC[-1])/(400-290)\times (340-290)\times 52.78/6200))\times 100.$$

Application of the formula "%NADH". Three samples of 0,18 mM NADH solution were used in order to demonstrate possible application of developed "% NADH" formula. After addition of PMS/resazurin dye fluorescence (ex 545 nm; em 600 nm) was measured. Obtained fluorescence intensity from three NADH samples with PMS/resazurin were very different from one to another. The fluorescence intensity data (relative units) were for the 1-st sample — (630 ± 20); for the 2-nd sample — (400 ± 10); for the 3-rd sample — (260 ± 10). The non-coincidence of the results indicated the different concentration of NADH in these samples. During NADH reagent storage, its reduced form gradually becomes oxidized one. Therefore, in order to use NADH solution as a standard, it is necessary to control its current concentration. These NADH samples were then tested by the optical absorbance and fluorescence (ex 340 nm; em 440 nm) methods. The results are presented in the Table. Different fluorescence intensity of these samples confirms the varying of NADH concentration. Applying the formula "% NADH" to the optical absorbance data resulted in the evaluation of NADH content in these samples. The concentration of NADH varied from 39% to 19% in the samples. The correlation coefficient between the calculated concentration of NADH using the formula "% NADH" and the samples fluorescence intensity (ex 340 nm; em 440 nm)



Representative spectral scan of the NADH sample. Relative absorbances (OD') are shown graphically

Experimental data of NADH samples

l, nm	Samples		
	1	2	3
Optical absorbance			
234	0.97	0.85	0.92
260	1.96	2.1	2.47
290	0.24	0.315	0.4
340	0.,86	0.77	0.76
400	0.04	0.075	0.06
Calculation of NADN content (%)			
% NADH	38.5	26.7	18.9
Fluorescence (340/440 nm)			
Emission, rel. un.	230	201	146

was 0.95; and the fluorescence intensity of samples with PMS/resazurin (ex 545 nm; em 600 nm) was 0.98. Strong correlation between calculated NADH content and fluorescent data demonstrates precision of developed "%NADH" formula.

Conclusion

Taken together, this study developed an original formula for rapid and precise evaluation of NADH content in ($NADH+NAD^+$) reagent by the UV spectrometric measurement of the optical absorbance at the wavelengths 234 nm, 260 nm, 290 nm, 340 nm, and 400 nm. Proposed procedure is simple, quick, and require only routine laboratory equipment, such as spectrometer.

Financial support

This study was funded by budget themes "The Role of Amino Oxidases in the Development of Carbonyl-Oxidative Stress in Pathologies of Different Genesis", registration number 0115U003644 (2015–2017) and "Study of the features of extracellular matrix changes as an indicator of cancer progression and development of ways for its correction", registration number 0117U004344 (2017–2021).

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

REFERENCES

1. *Pollak N., Dölle C., Ziegler M.* The power to reduce: pyridine nucleotides — small molecules with a multitude of functions. *Biochem. J.* 2007, 402 (2), 205–218. https://doi.org/10.1042/BJ20061638
2. *Nikiforov A., Kulikova V., Ziegler M.* The human NAD metabolome: Functions, metabolism and compartmentalization. *Crit. Rev. Biochem. Mol. Biol.* 2015, 50 (4), 284–297. https://doi.org/10.3109/10409238.2015.1028612
3. *David L. Nelson, Michael M. Cox.* Lehninger Principles of Biochemistry. *New York: W. H. Freeman.* 2005, 1198 p.
4. *Berger F., Ramirez-Hernandez M. H., Ziegler M.* The new life of a centenarian: signalling functions of NAD(P). *Trends Biochem. Sci.* 2004, V. 29, P. 111–118. https://doi.org/10.1016/j.tibs.2004.01.007
5. *Sellés Vidal L., Kelly C. L., Mordaka P. M., Heap J. T.* Review of NAD(P)H-dependent oxidoreductases: Properties, engineering and application. *Biochim. Biophys. Acta Proteins Proteom.* 2018, 1866 (2), 327–347. https://doi.org/10.1016/j.bbapap.2017.11.005
6. *Grolla A. A., Miggiano R., Di Marino D., Bianchi M., Gori A., Orsomando G., Gaudino F., Galli U., Del Grosso E., Mazzola F., Angeletti C., Guarneri M., Torretta S., Calabrò M., Boumya S., Fan X., Colombo G., Travelli C., Rocchio F., Aronica E., Wohlschlegel J. A., Deaglio S., Rizzi M., Genazzani A. A.,*

Garavaglia S. A nicotinamide phosphoribosyltransferase-GAPDH interaction sustains the stress-induced NMN/NAD+ salvage pathway in the nucleus. *J. Biol. Chem*. 2020 [Epub ahead of print] pii: jbc.RA119.010571. https://doi.org/10.1074/jbc.RA119.010571

7. *Cohen M. S.* Interplay between compartmentalized NAD+ synthesis and consumption: a focus on the PARP family. *Genes. Dev*. 2020 [Epub ahead of print]. https://doi.org/10.1101/gad.335109.119
8. *Lees J. G., Gardner D. K., Harvey A. J.* Nicotinamide adenine dinucleotide induces a bivalent metabolism and maintains pluripotency in human embryonic stem cells. *Stem. Cells*. 2020 [Epub ahead of print]. https://doi.org/10.1002/stem.3152.
9. *Girotra M., Naveiras O., Vannini N.* Targeting mitochondria to stimulate hematopoiesis. *Aging (Albany NY)*. 2020, 12 (2), 1042–1043. https://doi.org/10.18632/aging.102807
10. *Fjeld C. C., Birdsong W. T., Goodman R. H.* Differential binding of NAD^+ and NADH allows the transcriptional corepressor carboxyl-terminal binding protein to serve as a metabolic sensor. *Proc. Natl. Acad. Sci. USA*. 2003, 100 (16), 9202–9207. https://doi.org/10.1073/pnas.1633591100
11. *Harlan B. A., Killoy K. M., Pehar M., Liu L., Auwerx J., Vargas M. R.* Evaluation of the NAD+ biosynthetic pathway in ALS patients and effect of modulating NAD+ levels in hSOD1-linked ALS mouse models. *Exp. Neurol*. 2020, V. 327, P. 113219. https://doi.org/10.1016/j.expneurol.2020.113219
12. *Cuny H., Rapadas M., Gereis J., Martin E., Kirk R. B., Shi H., Dunwoodie S. L.* NAD deficiency due to environmental factors or gene-environment interactions causes congenital malformations and miscarriage in mice. *Proc. Natl. Acad. Sci. USA*. 2020, pii: 201916588. https://doi.org/10.1073/pnas.1916588117
13. *Chiang S., Kalinowski D. S., Dharmasivam M., Braidy N., Richardson D. R., Huang M. L.* The Potential of the Novel NAD^+ Supplementing Agent, SNH6, as a Therapeutic Strategy for the Treatment of Friedreich's Ataxia. *Pharmacol. Res*. 2020, V. 4, P. 104680. https://doi.org/10.1016/j.phrs.2020.104680
14. *Ye C., Qi L., Li X., Wang J., Yu J., Zhou B., Guo C., Chen J., Zheng S.* Targeting the NAD+ salvage pathway suppresses APC mutation-driven colorectal cancer growth and Wnt/β-catenin signaling via increasing Axin level. *Cell. Commun. Signal*. 2020, 18 (1), 16. https://doi.org/10.1186/s12964-020-0513-5
15. *Kang J. H., Lee S. H., Hong D., Lee J. S., Ahn H. S., Ahn J. H., Seong T. W., Lee C. H., Jang H., Hong K. M., Lee C., Lee J. H., Kim S. Y.* Aldehyde dehydrogenase is used by cancer cells for energy metabolism. *Exp. Mol. Med*. 2016, 48 (11), e272. https://doi.org/10.1038/emm.2016.103
16. *Sharma N., Okere I. C., Brunengraber D. Z., McElfresh T. A., King K. L., Sterk J. P., Huang H., Chandler M. P., Stanley W. C.* Regulation of pyruvate dehydrogenase activity and citric acid cycle intermediates during high cardiac power generation. *J. Physiol*. 2005, 562 (Pt 2), 593–603. https://doi.org/10.1113/jphysiol.2004.075713
17. *Fontaine J. X., Tercé-Laforgue T., Armengaud P., Clément G., Renou J. P., Pelletier S., Catterou M., Azzopardi M., Gibon Y., Lea P. J., Hirel B., Dubois F.* Characterization of a NADH-dependent glutamate dehydrogenase mutant of Arabidopsis demonstrates the key role of this enzyme in root carbon and nitrogen metabolism. *Plant Cell*. 2012, 24 (10), 4044–4065. https://doi.org/10.1105/tpc.112.103689
18. *Irimia A., Madern D., Zaccaï G., Vellieux F. M.* Methanoarchaeal sulfolactate dehydrogenase: prototype of a new family of NADH-dependent enzymes. *EMBO J*. 2004, 23 (6), 1234–1244. https://doi.org/10.1038/sj.emboj.7600147
19. *Hentall P. L., Flowers N., Bugg T. D.* Enhanced acid stability of a reduced nicotinamide adenine dinucleotide (NADH) analogue. *Chem. Commun. (Camb)*. 2001, V. 20, P. 2098–2099. https://doi.org/10.1039/b107634p
20. *Fukazawa K., Ishihara K.* Enhanced stability of NADH/dehydrogenase mixture system by water-soluble phospholipid polymers. *Biomaterials and Biomechanics in Bioengineering*. 2016, 3 (1), 37–46. https://doi.org/10.12989/bme.2016.3.1.037
21. *Ince C., Coremans J. M. C. C., Bruining H. A. In vivo* NADH Fluorescence. In: Erdmann W., Bruley D. F. (eds). Oxygen Transport to Tissue XIV. 1992. *Advances in Experimental Medicine and Biology*, V. 317. *Springer, Boston, MA.* https://doi.org/10.1007/978-1-4615-3428-0_30
22. *Paul Held.* Determination of NADH Concentrations with the Synergy™ 2 Multi-Detection Microplate Reader using Fluorescence or Absorbance. *BioTek, Application Note*. 2011.

СПОСІБ ШВИДКОГО СПЕКТРОМЕТРИЧНОГО ВИЗНАЧЕННЯ ВМІСТУ ВІДНОВЛЕНОГО НІКОТИНАМІД-АДЕНІНДИНУКЛЕОТИДУ (НАДН)

І. П. Крисюк
І. Р. Горак
С. Г. Шандренко

Інститут біохімії ім. О. В. Палладіна
НАН України, Київ

E-mail: 4iryna.kr@gmail.com

Нікотинаміддинуклеотид (НАДН/$НАД^+$) функціонує як коензим для багатьох ензимів, що беруть участь у клітинному метаболізмі, контролі окисно-відновної рівноваги, сигналюванні, біотрансформації та інших процесах. Тому визначення рівня продукування НАДН/$НАД^+$ зазвичай використовують для вимірювання активності НАДН/$НАД^+$-залежних ензимів. Проте НАДН може спонтанно окиснюватись до $НАД^+$, тому метою цієї роботи було розроблення нового методу визначення реального вмісту НАДН у препараті.

Для обчислення відсоткового вмісту НАДН у препараті визначали інтенсивність поглинання зразків за довжин хвиль 234 нм, 260 нм, 290 нм, 340 нм, 400 нм.

Було отримано оригінальну формулу для обчислення відсоткової концентрації НАДН у зразках і наведено приклад її застосування.

Запропонований метод може бути використаний для швидкого рутинного визначення вмісту НАДН у будь-якій лабораторії, обладнаній спектрометром.

Ключові слова: визначення концентрації НАДН, ультрафіолетова (УФ) спектрометрія.

СПОСОБ БЫСТРОГО СПЕКТРОМЕТРИЧЕСКОГО ОПРЕДЕЛЕНИЯ СОДЕРЖАНИЯ ВОССТАНОВЛЕННОГО НИКОТИНАМИД-АДЕНИНДИНУКЛЕОТИДА (НАДН)

И. П. Крысюк
И. Р. Горак
С. Г. Шандренко

Институт биохимии им. А. В. Палладина
НАН Украины, Киев

E-mail: 4iryna.kr@gmail.com

Никотинаміддинуклеотид (НАДН/$НАД^+$) служит кофактором для многих энзимов, участвующих в клеточном метаболизме, контроле окислительно-восстановительного равновесия, передаче сигналов, биодеградации и других процессах. Поэтому определение уровня продуцирования НАДН/$НАД^+$ обычно используется для измерения активности НАДН/$НАД^+$-зависимых энзимов. Однако НАДН может спонтанно окисляться до $НАД^+$, поэтому целью этой работы была разработка нового метода определения реального содержания НАДН в препарате.

Для вычисления процентного содержания НАДН в препарате определяли интенсивность поглощения образцов при длинах волн 234 нм, 260 нм, 290 нм, 340 нм и 400 нм.

Была получена оригинальная формула для вычисления процентной концентрации НАДН в образцах и представлен пример ее применения.

Предложенный метод может быть использован для быстрого рутинного определения содержания НАДН в любой лаборатории, оборудованной спектрометром.

Ключевые слова: определение концентрации НАДН, ультрафиолетовая (УФ) спектрометрия.