核磁共振波谱法同时定量测定保健品中烟酰胺腺嘌呤二核苷酸及其前体化合物

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摘 要: 目的 建立可同时测定保健品中烟酰胺腺嘌呤二核苷酸(nicotinamide adenine dinucleotide, NAD^+)及其前体化合物含量的核磁共振波谱法。**方法** 采用核磁共振波谱技术, 优化检测条件后, 开发了标准曲线法和定量核磁共振法(quantitative nuclear magnetic resonance, qNMR)两种定量检测方法, 用于测定保健品中两种形式的 NAD 及其 4 种前体化合物。**结果** 确定了各目标化合物的特征峰。对于标准曲线法, 6 种目标化合物的线性关系良好($r \geqslant 0.9999$), 除烟酰胺(nicotinamide, NAM)的定量限为 0.5 mmol/L 外, 其他 5 种目标化合物的定量限均为 0.2 mmol/L。考察了两种具有代表性的 NAD^+前体化合物的日间和日内重现性, 烟酰胺单核苷酸(nicotinamide mononucleotide, NMN)和 NAM 的日间重现性分别为 0.21%和 0.37%, 日内重现性分别为 1.03%和 1.44%。用标准曲线法和 qNMR 两种方法分别对收集的 10 个NAD^+补充剂保健品进行检测, 误差合理(<5%)。**结论** 建立的核磁共振方法具有良好的可靠性和灵敏度, 可用于保健品中 NAD^+及其前体化合物的生产控制和市场监管。

关键词: 烟酰胺腺嘌呤二核苷酸; 烟酰胺单核苷酸; 烟酰胺核糖; 核磁共振; 定量检测

Simultaneously quantitative determination of nicotinamide adenine dinucleotide and its precursors in food supplements using nuclear magnetic resonance

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ABSTRACT: Objective To develop and establish nuclear magnetic resonance-based method which could simultaneously determine the content of nicotinamide adenine dinucleotide (NAD^+) and its precursors in food supplement products. **Methods** Based on nuclear magnetic resonance technique, after the selection of optimal acquisition conditions, 2 kinds of quantitative methods, calibration curve and quantitative nuclear magnetic resonance (qNMR), were established to determine two forms of NAD and its 4 kinds of precursors in food supplements. **Results** The characteristic peaks of each target were selected. The linearity of calibration curve method of 6 kinds of investigated compounds was good ($r \geqslant 0.9999$).

基金项目: 国家市场监管总局科技项目(2021MK166)

Fund: Supported by the Science and Technology Project of the State Administration for Market Regulation, China (2021MK166)

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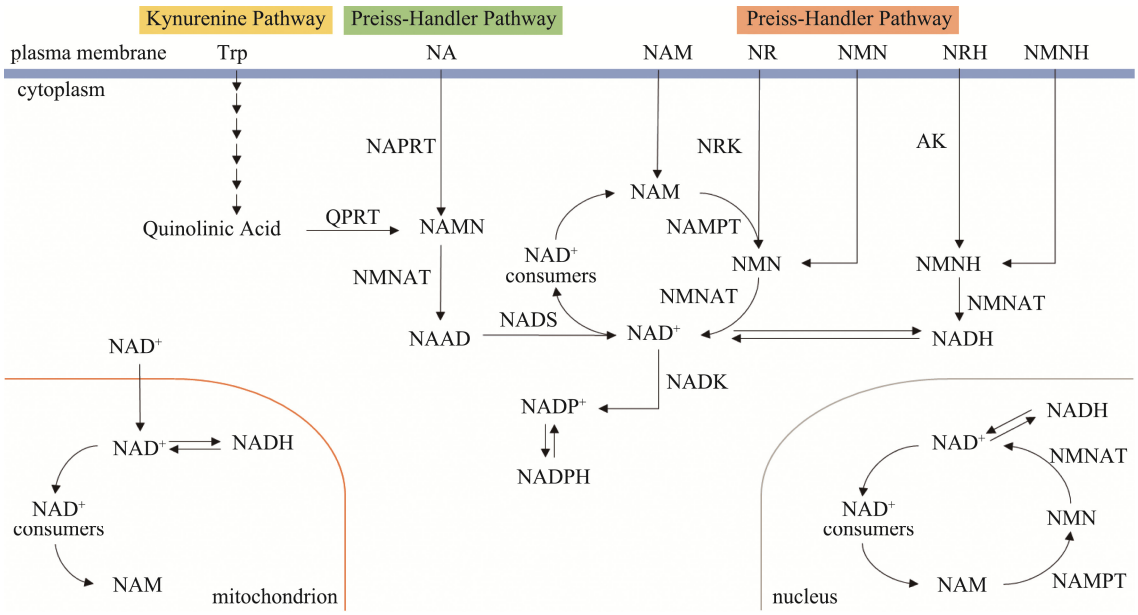
The limits of quantitation were 0.5 mmol/L for nicotinamide (NAM) or 0.2 mmol/L for 5 kinds of other target compounds. The inter-day and intra-day reproducibility of two representative NAD^+ precursors were also studied. The inter-day reproducibility was 0.21% for nicotinamide mononucleotide (NMN) and 0.37% for NAM respectively, and the intra-day reproducibility were 1.03% and 1.44%, respectively. Ten commercial samples of NAD^+ supplementary products collected were tested by both calibration curve and qNMR methods with reasonable error (less than 5%). **Conclusion** The proposed nuclear magnetic resonance methods have good reliability and sensitivity and could be applied in production control and administrative regulation for food supplements of NAD^+ and its precursors.

KEY WORDS: nicotinamide adenine dinucleotide; nicotinamide mononucleotide; nicotinamide riboside; nuclear magnetic resonance; quantitative determination

0 Introduction

Nicotinamide adenine dinucleotide (NAD^+) is either an important coenzyme involved in the redox reactions-based energy metabolism or an essential cofactor of non-redox NAD^+ dependent enzymes reactions of eukaryotes[1]. In the past two decades, several studies carried out in rodents or humans have proved that diseases status in nervous system, liver, kidney, intestine, hematopoietic system, muscle, and cardiovascular system, were associated with altered NAD^+ homeostasis and diminished NAD^+ content, and contrarily NAD^+ repletion in various ways showed health benefits and therapeutic effects in certain circumstance[2]. Since such unhealthy and diseases status may result in impaired NAD^+ biosynthesis and subsequently cause deficit of NAD^+, therefore using NAD^+ precursors as well as different forms of NAD^+ to restore NAD^+ pools could be a therapist rebalancing NAD^+ homeostasis for human[3]. NAD^+ precursors including nicotinic acid (NA), nicotinamide (NAM), nicotinamide riboside (NR), dihydronicotinamide riboside (NRH), nicotinamide mononucleotide (NMN), and dihydronicotinamide mononucleotide (NMNH) can be transported into eukaryotic cells and in turn metabolized into NAD^+. Within the cell, NAD^+ can be reversibly converted to NADH, the reduced form of NAD^+; NADH can be irreversibly converted to nicotinamide adenine dinucleotide phosphate ($NADP^+$) the phosphorylated form of NAD^+; furthermore, $NADP^+$ can also be reversibly converted to its reduction state, NADPH. Anabolism and catabolism regarding to the NAD^+ precursors and the 4 kinds of forms of NAD in cytosol, nucleus, and mitochondria establish the subcellular equilibrium of NAD^+ and its homeostasis[4]. Figure 1 shows metabolic pathways of NAD^+ biogenesis in eukaryotic cell.



Annotate: Trp: tryptophan; QPRT: quinolinate phosphoribosyltransferase; NA: nicotinic acid; NAPRT: nicotinic acid phosphoribosyltransferase; NAMN: nicotinamide mononucleotide; NMNAT: nicotinamide mononucleotide adenylyltransferases; NAAD: nicotinic acid adenine dinucleotide; NADS: NAD^+ synthetase; NADK: NAD^+ kinase; NAM: nicotinamide; NAMPT: nicotinamide phosphoribosyltransferase; NR: nicotinamide riboside; NRK: nicotinamide riboside kinases; NMN: nicotinamide mononucleotide; NRH: dihydronicotinamide riboside; AK: adenosine kinase; NMNH: dihydronicotinamide mononucleotide; NAD^+: nicotinamide adenine dinucleotide.

Fig.1　Biosynthesis pathways of NAD^+ in eukaryotic cell

To date, many studies using NAD^+ precursors as a therapy to boost NAD^+ levels have reported both clinical benefits and risks in various murine and primate models as well as in human, especially NMN and NR which are the most likely candidates for NAD^+ therapy[5], or so-called NAD^+ boosting strategy. Numbers of preclinical and clinical trials of NR and NMN have been conducted and some of those are really promising, such as the clinical trials of oral NR supplementations as the potential neuroprotective therapies for Alzheimer and Parkinson diseases[6–7], and a clinical trial of oral NMN supplementation to increase muscle insulin sensitivity to prediabetic women[8]. Plenty of clinical studies continually provide clinical evidence for validating the therapeutic promises of NAD^+ boosters in humans[9], especially for the ageing and age-related diseases[10–11].

Although it's reported in the limited number of clinical studies with different intervention periods various from 2 days to 12 weeks that NMN or NR oral intakes from 125 to 2000 mg/day in healthy middle age and older adult are safe[12–14] and can efficiently elevate NAD^+ level[15–19], the risks of NMN and NR supplementations like tumorigenesis and toxic metabolites accumulation are still existed[5]. Moreover, nutrient timing[20], synergistic supplementation with other compounds[21] and concurrent with exercise[22] are new challenges in the studies of NAD^+ boosting strategy.

Nevertheless, the commercial products of NAD^+ precursor as food supplements, especially NMN and NR, are popular and easily purchased in many countries and regions via online store or local market. The market volume of NMN and NR as anti-ageing arena is blooming[23], though this growing tendency might confront certain obstacles from regulations[24–25]. Due to the different efficacy of NAD^+ biosynthesis *in vivo*[26] and the different cost for different precursors, the NMN or NR product with high efficacy and high cost has the adulteration risk by NA or NAM with low efficacy and low cost. Besides, those food supplement products that claimed NMN or NR may have the risk of actual content over or under the label claim, even no target content can be detected[27]. Consequently, quantification of NAD^+ and its precursors in supplement products with NMN or NR as major compounds in the formulation is not only important for the consumer protection and market regulation, but also significant in providing precise functional ingredient level for precision medicine and nutrition intervention.

Quantification methods for trace analysis of NAD^+ and its metabolites in biological samples like blood and tissues are well established during the preclinical and clinical studies of NAD^+, and the most widely used ones are enzymatic cycling assay and liquid chromatography-mass spectrometry (LC-MS)[12–13,15,20,22,28]. The isomers in bio-system are beta configurations; alpha configurations mainly from chemical synthesis is not included during in this method development process[29]. Whereas, the matrix interference and target concentration magnitude in food supplement products are greatly different from the biological samples. NAD^+ precursors in the supplement products are considered as macronutrients with the level of mg/100 g, directly using those quantitative methods for biological samples without modifications might result in the huge bias of the measurement from dilution. It's also worth to pointing out that for the approach with high sensitivity such as LC-MS the uncertainty from different devices and instruments, inter-day and intra-day variance, and personal differentiation may negatively influence the accuracy and repeatability of measurement. As a powerful, non-destructive, and reproducible tool to analyze the molecular structures of chemicals, nuclear magnetic resonance (NMR) spectroscopy is widely applied in food analysis with simple sample preparation and relatively short analysis time[30–32]. It could be used for qualitative and quantitative analysis simultaneously. qNMR is a branch of NMR which is rapidly developed in the past two decades, especially in pharmaceutical, natural products and food supplement fields, which has been proven as a valuable orthogonal quantification method and alternative choice of chromatographic techniques[33–34]. Though high sensitivity methods developed for example chromatography-mass spectrometry[12–13], enzyme-mediated[35] or probe strategy[36], which limits of quantitation (LODs) could be as low as nmol/L or even fmol/L level, NMR is still a powerful tool in macrocompositions scenario. In this study, two NMR-based methods of simultaneous quantification for NAD^+ and its precursors in food supplement products are established and validated, which can be applied in NAD^+ supplement manufacture for quality control or in administrative regulation for market inspection.

1 Materials and methods

1.1 Chemicals and samples

Commercial food supplement samples (JD platform or local market).

Deuterium oxide (D_2O, 99.9%), 2,2,3,3-D_4 sodium-3-trimethylsilylpropionate (TSP-d_4, 98% D) (Cambridge Isotope Laboratories Inc., Andover, MA, USA); maleic acid (MLA), tert-butanol (*t*-BuOH), NMN, NAD, NR, NA, NAM, NADH (>97%, Shanghai Macklin Biochemical Co., Ltd., Shanghai, China).

1.2 Instruments and equipment

Agilent DD2 600 MHz Nuclear Magnetic Resonance with OneNMR 5 mm probe and 7510 automatic injector (Agilent Technologies., CA, USA); Mettler Toledo XS104 balance (±0.1 mg, Mettler-Toledo International Ltd., Shanghai, China); adjustable pipettes (Eppendorf, Hamburg, Germany); AS ONE TRIO TM-1N tube mixer (AS ONE Corporation, Japan); KQ-500E Ultrasonic cleaner (KunShan Ultrasonic Instrument Co. Ltd., JiangSu, China); KDC-40 centrifuge (Anhui Zhongke Zhongjia Scientific Instrument Co., AnHui, China); 5 mm NMR test tubes (Norell, Inc., Morganton, NC, USA); 0.22 μm nylon membrane (Tianjin Jinteng Biotechnology Co., Ltd., Tianjin, China).

1.3 Methods

1.3.1 Internal standard solution

A certain weight of TSP-d_4, MLA and *t*-BuOH was dissolved in 10 mL deuterated water to obtain 4 mmol/L TSP-d_4 deuterated water solution, 20 mmol/L MLA deuterated water solution and 5 mmol/L *t*-BuOH deuterated water solution,

respectively, then all solutions were stored at room temperature before using.

1.3.2 Stock solutions and working solutions of investigated compounds

Appropriate amount of NA, NAM, NR, NMN, NAD^+, and NADH were dissolved in 4 mmol/L TSP-d_4 deuterated water solution to make 100 mmol/L NA, NAM, NR, NMN, NAD^+ and 50 mmol/L NADH as corresponding stock solutions, then all stock solutions were stored in dark at 4℃.

The working solutions were prepared by serial dilutions of the corresponding stock solutions or high concentration working solutions with 4 mmol/L TSP-d_4 deuterated water solution. The concentrations of working solutions were 20.0, 10.0, 2.0, 1.0, 0.5 and 0.2 mmol/L for each analyte correspondingly.

1.3.3 Sample preparation of commercial food supplements

Twenty tablets or capsules (removing the cover) of each purchased food supplement sample were first pulverized and milled into fine powder. Afterwards, for calibration curve method, about 10 mg of sample powder in 1000 μL of 4 mmol/L TSP-d_4 deuterated water were vortexed, ultrasonicated for 10 min, centrifuged for 5 min, and filtered through 0.22 μm nylon membrane, 550 μL of clear solution were taken for NMR acquisition. Similar preparation procedure was taken for qNMR method, except the sample solutions were prepared by dissolving about 50 mg sample powder and 10 mg TSP-d_4 in 1000 μL deuterated water.

1.3.4 NMR parameters

Proton pulse sequence was selected for sample signal acquisition. The test temperature was set 25℃. Deuterated water was used to lock field. The spectral width was 16 ppm; the observed pulse width and degree were 9.6 μs and 90°, respectively. After the systematic optimization of each investigated compound, the duration delay time was 60 s, and the number of acquisition was 8. The phases of all NMR spectra were adjusted and corrected automatically by Vnmrj 4.2. The baseline and integral of signal peaks were carried out by MestReNova 11.0.

2 Results and analysis

2.1 Optimization of NMR acquisition

The optimization of NMR acquisition was conducted on each investigated compound. Only NMN was selected as the representative compound to show the optimization process. By comparing the ratio of peak areas of NMN (9.47 ppm) over TSP-d_4 (0 ppm, chemical shift internal standard), the optimal parameters of delay time and number of acquisition were chosen. Since all investigated compounds have quite similar chemical structures in molecular level, it's not surprising that the optimal parameters for NMR acquisition were the same for different compounds of NAD^+ and precursors.

2.1.1 Delay time

The delay time should be set long enough to allow the nucleus to fully return to the ground state after the last pulse excitation. Generally, it should be equal to or greater than 5 times of the longitudinal relaxation time (t_1). Short delay time might imbalance the ratio of peak areas of the target compound. It affects the integration result and causes inaccurate quantification result. The delay time was set to 30, 40, 50, 60 and 70 s. Based on the quantitative comparison of the peak aera ratios of NMN/TSP-d_4 as shown in figure 2, the delay time was selected as 60 s. All peak areas of all investigated analytes were accurately proportional to the corresponding hydrogen atoms.

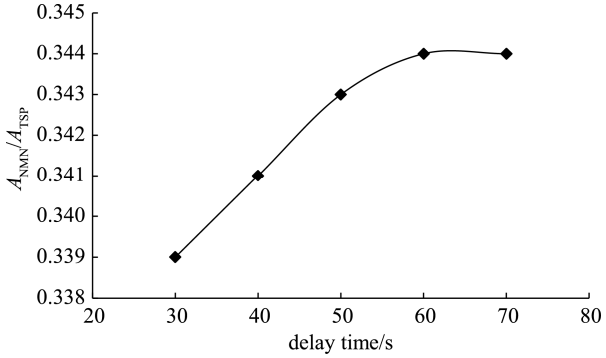


Fig.2 Optimization of delay time with NMN

2.1.2 Number of acquisition

To have good signal/noise ratio as well as to facilitate discrimination and integration, the number of acquisition also needs to be optimized for the characteristic peaks of the investigated compounds, theoretically the larger the better. However, greater number of acquisitions would lead to longer acquisition time and total quantification time of NMR method. As the number of acquisitions set at 2, 4, 8, 16 and 32, the quantitative comparison of the results from different number of acquisitions was shown in figure 3. It's clearly indicated that the ratios of the characteristic peak areas of NMN and TSP-d_4 reach a maximum with the number of acquisitions at 8 and keep almost constant as the number of acquisitions further increasing. Hence, the number of acquisitions was chosen as 8.

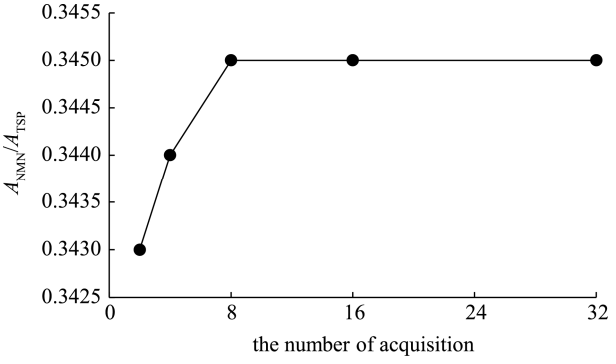


Fig.3 Optimization of number of acquisition with NMN

2.2 Selection of internal standard

The criteria for the selection of internal standard, such as the stability, simple and unique signal, no overlap with analytes *etc*.. TSP-d_4, MLA and *t*-BuOH were investigated as

internal standard of NMR methods. All of them could meet the above requirements. However, the addition of MLA might change the pH of the testing system and cause the signal shifting of the characteristic peaks of analytes. The melting point of *t*-BuOH is 23–26℃; at room temperature, it is mixed as liquid and solid states, which causes the difficulty of weighing accurately. TSP-d_4 is widely used as internal standard of chemical shift in aqueous solution. Its thermal and chemical stability has been regarded as consensus. Its singlet peak is defined as 0 ppm and far from the signal peaks from analytes. Hence, TSP-d_4 is chosen as internal standard in this study. It can be used both as a quantitative internal standard and as a chemical shift internal standard, which is "one stone two bird" strategy.

2.3 NMR spectra of analytes and characteristic peaks for quantification

A few milligrams of NA, NAM, NR, NMN, NAD^+ and NADH standards were dissolved with TSP-d_4 deuterated water individually. Their ^{1}H NMR spectra were acquired separately and the signal peaks of each compound were assigned correspondingly.

To perform a quantitative NMR analysis, each analyte must have a quantitative characteristic peak without any interference from others. In other words, the quantitative characteristic peaks of analytes should be no overlap with other peaks and the difference of chemical shift between the peaks should be large enough to avoid the error caused by integration. Figure 4 is the stacked NMR spectra of the 6 analytes. The characteristic peaks of NMN, NAD, NADH, NR, NA, and NAM were selected at chemical shift of 9.47, 9.40, 6.95, 9.62, 9.08, 7.60 ppm, respectively.

2.4 LODs, limits of quantitation and linearity of calibration curve method

To build the calibration curve for each analyte, first the NMR signals of each analyte were collected in triplicate by using 5 mm NMR test tube with 550 μL stock solution or working solutions of each analyte; then the integration ratio of the analyte characteristic peak and the internal standard TSP-d_4 peak (0 ppm) was set as *Y*-axis with the concentration of analyte as *X*-axis.

The LODs or limits of quantitation (LOQs) of each analyte in the proposed method were based on the signal intensities of characteristic peaks with the signal-to-noise ratio higher than 3 or 10 respectively under the optimal acquisition condition (60 s of delay time, 8 times of acquisition). Although a greater number of acquisition would improve the LOD and LOQ due to the decrease of the noise of baseline[37], it would greatly increase the acquisition time and decrease the efficiency of NMR method.

The LODs, LOQs and linearity of each analyte in the calibration curve method were summarized in table 1. The linear equation and correlation coefficients were also listed. The linearity of all analytes was fairly good in the range listed in table 1. Compared with the reported NAD analysis results by LC-MS method used in biological samples[12–13,15–18], which have LOQs low to several μmol/L or even to nmol/L, the sensitivity of this proposed NMR method is almost two magnitudes lower than that of reported LC-MS methods. However as mentioned before, the huge bias of the measurement from dilution for those LC-MS methods could

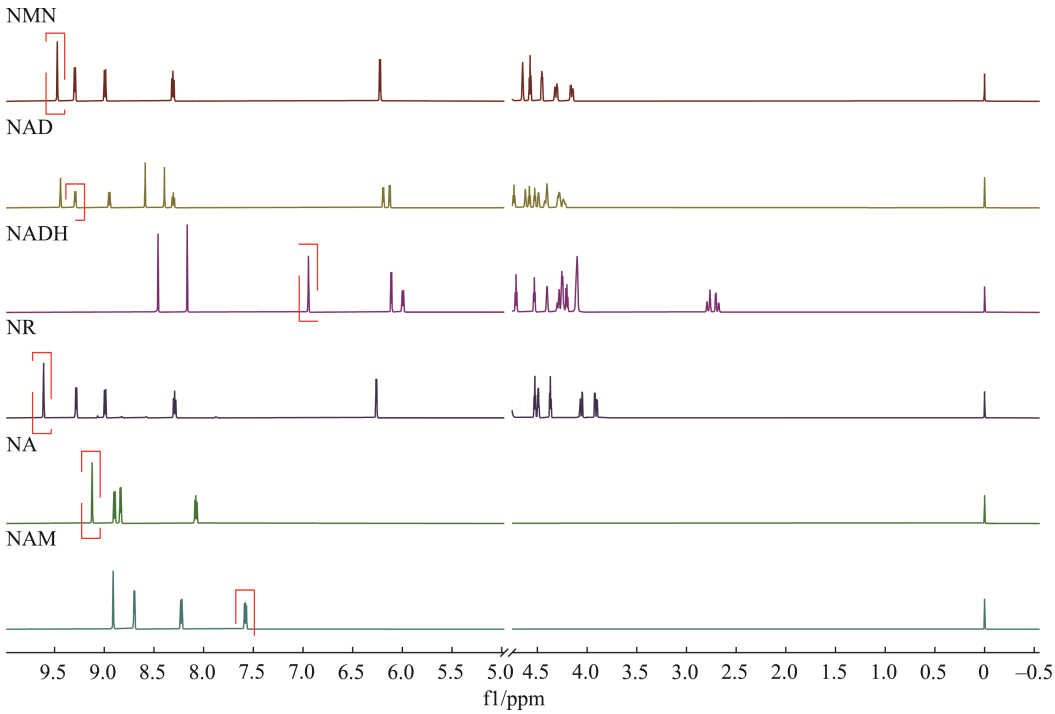


Fig.4 ^{1}H NMR of NMN (9.47 ppm), NAD (9.40 ppm), NADH (6.95 ppm), NR (9.62 ppm), NA (9.08 ppm), NAM (7.60 ppm) in deuterated water

Table 1　Linear ranges, linear equations, correlation coefficients (n=3)

analytes	chemical shift of characteristic peak (δ, ppm)	linear range/ (mmol/L)	linear equation	correlation coefficients (r^2)	LODs/(mmol/L)	LOQs/(mmol/L)
NMN	9.47	0.2–100.0	Y=0.0358X	1.0000	0.1	0.2
NAD	9.40	0.2–50.0	Y=0.0330X	1.0000	0.1	0.2
NADH	6.95	0.2–100.0	Y=0.0339X	0.9999	0.1	0.2
NR	9.62	0.2–100.0	Y=0.0335X	1.0000	0.1	0.2
NA	9.08	0.2–100.0	Y=0.0361X	1.0000	0.1	0.2
NAM	7.60	0.5–100.0	Y=0.0361X	0.9999	0.2	0.5

limit the use of those LC-MS methods in macronutrient analysis for food supplements since those methods are too sensitive. Another interesting observation is that for LC-MS method the reported LOQ and linear range are quite different for different NAD and precursors while for this proposed NMR method the LOQ and linear range are quite similar as shown in table 1. It's definitely demonstrated that NMR method is a relatively unified method due to the characteristics of NMR measurement. Contrarily the MS methods are quite selective methods and could be influenced by molecular weight, acidity/basicity, thermos-stability, and polarity of compounds. For examples, in LC-MS methods, it's reported that the LOQs for NR and NMN are 0.0195 μmol/L and 1.5 μmol/L, almost two magnitudes difference[12]; it's also reported that the linear range for NMN is from 0.2–20.0 μg/mL while the linear range for NAD^+ is from 5–500 μg/mL, over one magnitude difference[17]. Although high performance liquid chromatography-ultraviolet method was also used to quantify NAD^+ precursors with similar sensitivity of NMR method, it's only focused on one or two NAD^+ precursors to avoid the potential co-elution since the ultraviolet detector almost has no capability to dealing with co-elution[27,38].

2.5　Quantitative calculation

The spectra were acquired by Agilent DD2 600 MHz with Vnmrj 4.2, the spectra phase was automatically adjusted after the collection. The chemical shift was aligned based on TSP-d_4 as 0 ppm. The baseline correction and the peak area integration were conducted by MestReNova 11.0. Calibration curve and qNMR were applied for quantitative calculation of the content of NAD^+ and its precursors in food supplements as follows.

2.5.1　Quantification based on calibration curve method

The test sample was prepared by following the protocol for calibration curve method as described in section 2.3. From the established calibration curves, the linear equations were obtained as equation (1). Then, the concentration of analyte could be obtained by inputting the integration ratio of the analyte characteristic peak and TSP-d_4 peak into the linear equation (1). Next, the analyte content in the real sample would be calculated as equation (2):

$$y_i=a_ix_i+b_i \quad (1)$$

Wherein, y_i represents peak area ratio of the characteristic peak of analyte i and the peak of TSP-d_4; a_i is the slope of the calibration curve (L/mmol); x_i is the concentration of analyte i in NMR test solution (mmol/L), b_i is the intercept (forced to be zero):

$$W_i=\frac{x_i\times V\times M_i}{w_i}\times W \quad (2)$$

Wherein, W_i represents the content of target analyte i in each sample unit (mg); V is the volume of TSP-d_4 deuterated water solution (L); M_i is the molecular weight of the target analyte i (g/mol); w_i represents the weight of the testing sample (mg); W is the weight of sample unit (mg).

2.5.2　Quantification based on qNMR method

The sample was pretreated by following the protocol for qNMR method as described in section 1.3.3, after NMR signal acquisition, the content of target analyte in real sample was calculated according to equation (3):

$$W_i=\frac{I_i\times N_s\times Mi\times w_s}{I_s\times N_i\times Ms\times wi}\times P_s\times W \quad (3)$$

Wherein, W_i represents the content of target analyte i in each unit (mg); I_i represents the integration of target analyte i characteristic peak area; I_s represents the integration of TSP-d_4 peak area; N_s is the proton number of internal reference-TSP-d_4 peak; N_i is the corresponding proton number of the target analyte i characteristic peak; M_s is the molecular weight of internal reference-TSP-d_4(g/mol); M_i is the molecular weight of target analytes i (g/mol); w_s is the weight of internal standard-TSP-d_4 (mg); w_i is the weight of the testing sample (mg); P_s represents the purity of internal standard-TSP-d_4; W represents the weight of sample unit (mg).

2.6　Reproducibility

The inter-day and intra-day reproducibility of the calibration curve method was investigated on NMN and NAM, respectively, by monitoring the integration ratio of the analyte characteristic peak and the internal standard TSP-d_4 peak. The 5 mg of testing compounds were dissolved in 1 mL of 4 mmol/L TSP-d_4 deuterated water and the NMR signals were acquired six times in one day, the inter-day accuracies were 0.21% and 0.37% for NMN and NAM respectively. The measurements were also taken in triplicate for continuously 6 days, the intra-day accuracies were 1.03% and 1.44% respectively for NMN and NAM. The detail results were shown in table 2. Both the inter-day and intra-day reproducibility of the proposed method were quite good and reliable.

Table 2 Inter-day and intra-day reproducibility of NMN and NAM

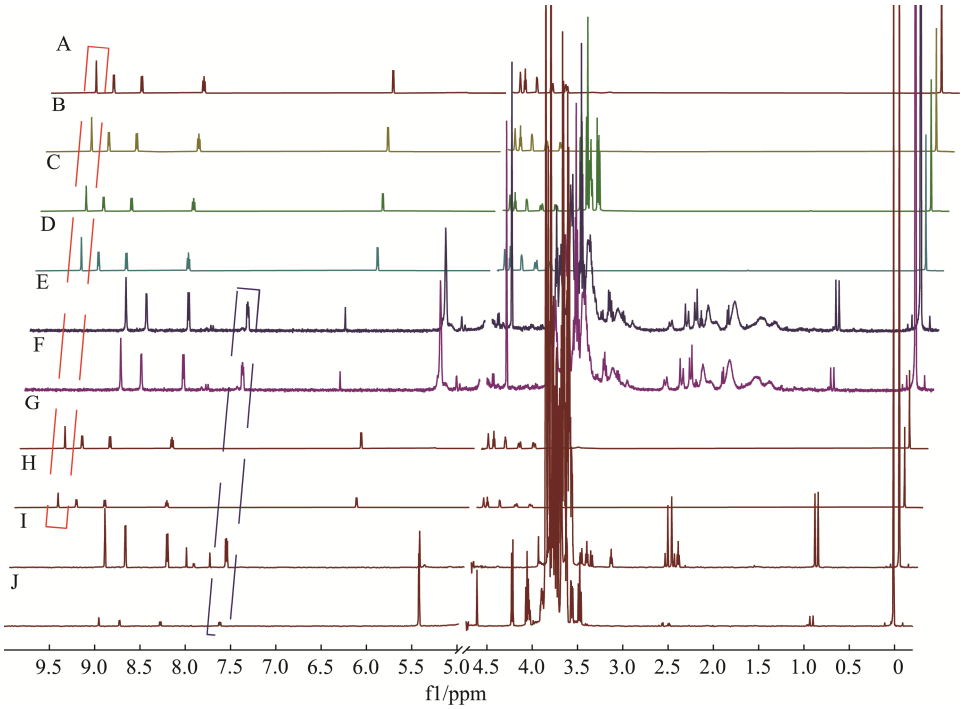
inter-day			intra-day (n=3)		
No	A_{NMN}/A_{TSP}	A_{NAM}/A_{TSP}	Day	A_{NMN}/A_{TSP}	A_{NAM}/A_{TSP}
1	0.465	1.327	1	0.468	1.315
2	0.467	1.322	2	0.468	1.317
3	0.466	1.316	3	0.467	1.323
4	0.467	1.330	4	0.478	1.348
5	0.465	1.321	5	0.472	1.354
6	0.467	1.322	6	0.477	1.356
avg	0.466	1.323	avg	0.471	1.335
RSD/%	0.21	0.37	RSD/%	1.03	1.44

Annotate: relative standard deviation (RSD).

2.7 Validation with commercial samples

Ten commercial samples were tested according to the proposed NMR methods, their ^{1}H NMR spectra were shown in figure 5, in which A-D, G and H were the spectra of NMN food supplements, E, F, I and J were the spectra of NAM food supplements. As shown in figure 5, the characteristic peaks of NMN and NAM were at chemical shift of 9.47 ppm and 7.60 ppm, and the quantitative analysis of these 2 kinds of compounds were based on the integration of the characteristic peaks correspondingly.

The content of target analytes in these 10 commercial samples were tested by both calibration curve method and qNMR methods in duplicate. The test results were summarized in table 3. For calibration curve method, the results of measurement were closely to the content marked on their labels except sample B and G which are obviously



Annotate: the Y-axis of sample E, F, I & J ware zoomed in 10 times.
Fig.5 ^{1}HNMR stack spectra of 10 commercial samples

Table 3 Information and test results of commercial samples (n=2)

sample	target analyte	contents on the label/(mg per unit)	calibration curve		qNMR		|error|/%
			test result/(mg per unit)	RSDs/%	test result/(mg per unit)	RSDs/%	
A	NMN	100	107.04	1.36	107.04	0.64	0
B	NMN	125	144.41	0.27	144.42	1.61	0.01
C	NMN	125	125.50	0.67	130.81	1.01	4.14
D	NMN	160	161.12	0.49	166.33	3.09	3.18
E	NAM	22	22.90	4.99	22.28	3.15	2.74
F	NAM	20.5	19.60	0	20.55	3.81	4.73
G	NMN	160	170.50	0.09	171.03	1.77	0.31
H	NMN	200	202.72	2.38	198.08	0.86	2.32
I	NAM	20	20.52	0.40	21.07	0.49	2.64
J	NAM	6.5	6.59	3.57	6.66	4.01	1.06

higher than the labeled value. All their RSDs were less than 5%. For qNMR method, the results were similar as those of calibration curve with RSDs less than 5% too. And the result of sample B and G are also higher than its label and almost identical to the result of calibration curve method. So, the actual content of NMN in sample B and D might be higher than it was labeled. The test results of calibration curve and qNMR methods were corroborated each other with good accuracy.

3 Conclusion

Two different NMR-based quantitative methods were established to simultaneously quantify NAD^+, NADH, and 4 kinds of precursors in food supplements. Both methods were applied for the measurements of commercial samples. The results of real samples measured were validated each other with reasonable error less than 5%. Without the need of calibration curves, the qNMR method has the advantages of less sample amount, less preparation and operation time over the calibration curve method. The investigations of the inter-day and intra-day reproducibility, the LOD, the LOQ, the linearity of calibration curve also indicated that the proposed NMR methods have good reliability and sensitivity and can be applied in production control and administrative regulation for food supplements of NAD^+ and its precursors.

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(责任编辑：张晓寒 郑 丽)

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