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Simultaneous determination of nicotinamide mononucleotide, niacinamide, and nicotinic acid in health supplements using liquid chromatography

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Abstract

This study aimed to develop and validate an HPLC-PDA method for simultaneous quantification of three compounds: nicotinamide mononucleotide (NMN), nicotinic acid, and niacinamide in dietary supplements. The sample preparation procedure was simple, fast, and used environmentally green extracted solvents. The HPLC conditions were as follows: C18 column (250 mm x 4.6 mm, 5 µm); mobile phase: 10 mM phosphate buffer pH = 3: methanol (90:10, v/v), UV detection at wavelength 261 nm. The method met the AOAC criteria for validation, including selectivity, linearity (R^2> 0.999 for all analytes in the range of 0.1 - 50.0 µg/mL), sensitivity (LOD: 0.4 - 0.45 mg/kg for NMN, 0.1 - 0.61 mg/kg for nicotinic acid, and 0.1 - 0.6 mg/kg for niacinamide; LOQ: 1.3 - 1.5 mg/kg for NMN, 0.49 - 2.00 mg/kg for nicotinic acid, and 0.47 - 1.90 mg/kg for niacinamide), accuracy (recovery: 95.5 - 103.0 % for NMN, 92.5 - 104.1 % for nicotinic acid, and 95.3 - 103.8 % for niacinamide), precision (RSD_r: 0.54 - 3.79 % for NMN, 0.39 - 4.82 % for nicotinic acid, and 0.81 - 5.14% for niacinamide; RSD_R: 1.03 - 3.70 % for NMN, 1.75 - 4.10 % for nicotinic acid, and 1.83 - 4.48 % for niacinamide). The method was successfully applied to analyze 20 supplement products collected in Hanoi.

Keywords: *Nicotinamide mononucleotide, NMN, niacinamide, nicotinic acid, HPLC-PDA.*

1. INTRODUCTION

The global population is aging rapidly. Worldwide data show that the number of people aged 60 and over will increase from 901 million in 2015 to more than 2.1 billion by

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2050 [1]. This trend poses health challenges, as aging reduces the function of vital organs and raises the risk of age-related diseases. As a result, anti-aging products are in high demand. One of the most promising products is NMN, which has attracted considerable interest from both consumers and researchers.

NMN stands for nicotinamide mononucleotide, a key precursor of nicotinamide adenine dinucleotide (NAD+) - a crucial enzyme for various essential cellular functions, such as metabolism, DNA repair, and aging regulation [2]. NAD+ levels decline with age, so supplementing NAD+ with NMN can help prevent or delay aging-related impairments in humans. Moreover, NMN has other benefits, such as skin rejuvenation, nerve function improvement, heart protection from ischemia, obesity and type II diabetes prevention, and cancer treatment support [3]. In November 2022, the FDA announced that NMN is undergoing further clinical trials to be approved as a drug rather than a dietary supplement, due to its remarkable effects [4].

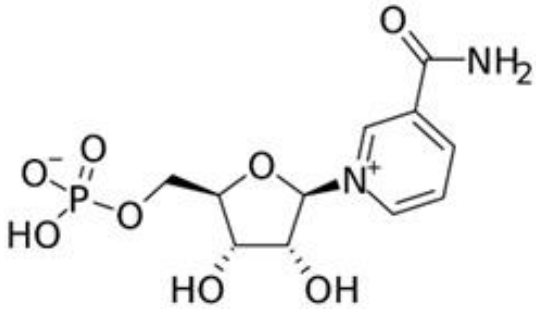

***Figure 1**. The structural formula of NMN*

The demand for NMN products is growing rapidly in the dietary supplement and cosmetics markets, especially in North America, Europe, and China. According to a report, the global NMN market size was 253 million USD in 2020 and is projected to reach 386 million USD by 2027 [2]. NMN products are usually combined with vitamin B3, which has two forms: nicotinic acid and nicotinamide. Nicotinic acid lowers cholesterol levels and prevents cardiovascular disease. Nicotinamide is soluble in water and enhances skin health by strengthening the skin barrier, repairing skin damage, reducing hyperpigmentation, and smoothing skin texture [5]. Vitamin B3 is not stored in the body and needs to be obtained from food sources. The daily recommended intake of vitamin B3 is 12 - 16mg/day [6].

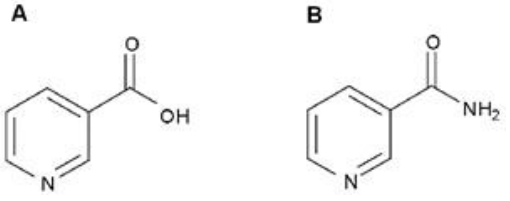

***Figure 2**. Structural formula of nicotinic acid (A) and niacinamide (B)*

NMN, nicotinic acid, and nicotinamide are polar compounds that dissolve and remain stable in water at normal temperatures [2]. They also have an aromatic ring that absorbs UV-Vis light at 261 nm. We found no previous studies that quantified all three compounds

together and only a few studies that measured NMN. Previous studies used UPLC or HPLC with MS/MS detection [7-9] to analyze NMN in biological fluids and vitamin B3 in food and milk powder [10-13]. Although these methods had low detection limits, fast analysis times, high sensitivity and precision, but in supplements with high concentrations of analytes, it requires many dilution steps causing high error. Another study used a microbiological assay to measure NMN, but this method was costly, time-consuming, and inaccurate. Laura et al. used HPLC with FLD detection to quantify NMN [14], and HPLC FLD was also adopted by Vietnamese standards for vitamin B3 in food and cereals [15]. This method had high sensitivity and selectivity, but it was affected by the complex pre-column derivation step that took longer preparation time. In this study, we developed a simple and common HPLC-PDA method to simultaneously and rapidly analyze NMN, nicotinic acid, and nicotinamide in functional products and health foods in Hanoi with high reliability, the method was also used in a study for quantitative analysis of NMN consumer products [16].

2. MATERIALS AND METHODS

2.1. Equipment

HPLC system (Shimadzu, model: 20A) equipped with high-pressure pump, autosampler, PDA detector and GL Sciences InertSustain C18 column (250 mm x 4.6 mm, 5 µm) and corresponding pre-column; Hermlet Z383K centrifuge; analytical balance (Mettler Toledo), ultrasonic bath (Germany), vortex shaker (IKA), micropipette (Germany).

2.2. Chemicals and materials

The reference standards included nicotinamide mononucleotide (Sigma) purity > 99%, nicotinamide, and nicotinic acid (Sigma) with purities of higher than 99%. Other solvent-type reagents in HPLC analysis include acetonitrile (ACN), methanol (MeOH) sodium dihydrogen phosphate (NaH_2PO_4) from Merck, and orthor-phosphoric acid 85%. Ultrapure water was prepared using a Milli-Q water system (Millipore, Billerica, MA, USA).

2.3. Experiments

2.3.1. Preparation of standard solution

Standard solutions of NMN, nicotinic acid, and niacinamide were prepared in water at 1000 mg/L. Intermediate and working standard solutions were prepared for use during the day.

2.3.2. Sample treatment

Accurately weigh about 0.3 - 2.0 g of the homogenized sample into a 50 mL polypropylene centrifuge tube. Add 20 mL of water to the tubes. Mechanical shake for 20 minutes and centrifugate at 6000 rpm for 5 minutes. Decant the extract and transfer it to a 25 mL volumetric flask and fill up with water. The extract was filtered through a 0.2 µm PTFE syringe filter before being analyzed by HPLC-PDA.

2.3.3. Chromatography conditions

The PDA detector was measured at a wavelength of 261 nm. GL Sciences InertSustain C18 chromatography column (250 mm x 4.6 mm, 5 µm) was used with the mobile phase containing 10 mM phosphate buffer pH 3: methanol (90:10), at the flow rate of 1.0 mL/min. The injection volume was 20 µL.

2.3.4. Method validation

The specificity, linear range, sensitivity, precision, accuracy, and uncertainty were validated for the method proficiency. The sensitivity of the method was evaluated by LOD and LOQ values. The blank samples were spiked at low concentration levels (1 - 4 mg/kg) and analyzed 10 times (n=10). Calculate LOD by multiplying 3 times of SD value and 10 times for LOQ.

In this method, specificity was determined by comparing the retention time of analytes in blank, standard, and spiked samples. To assess the accuracy, blank samples were spiked at three different standard concentration levels within the working concentration range (50 - 400 µg/g for NMN, 9 - 36 µg/g for nicotinic acid, and 10 - 35 µg/g for niacinamide). Prepare three samples for each level. Inject the samples into the instrument and calculate the percentage of the recovery rate for each test sample at each concentration level. Repeatability was estimated by testing samples six times by one person (n=6) and by another person after 1 day (n=4) to estimate the reproducibility. Calculate the standard deviation SD and relative standard deviation RSD. The uncertainty was calculated by top-down method through precision, recovery, and uncertainty of standards.

2.3.5. Sample collection

20 samples of functional products and health protection supplements containing these analytes in five different dosage forms: hard capsules, soft capsules, tablets, granules, and syrup were collected in Hanoi and stored at room temperature. Samples were thoroughly homogenized before analysis.

3. RESULTS AND DISCUSSION

3.1. Optimize LC conditions

The mobile phase affects analyte elution (retention time), separation column efficiency, selectivity, and chromatographic peak width (band broadening). Referring to the mobile phase used in papers [7, 13] and the polar characteristics of these substances, the mobile phase phosphate buffer (A) and Methanol or Acetonitrile (B) were investigated.

Analyze the mixed standard solution (NMN, nicotinic acid, and niacinamide) at a concentration of 20 µg/mL. The results showed that the Acetonitrile mobile phase had a fast elution time of three analytes, but many background impurities led to the phenomenon of background rise and uneven peak base. With methanol, the chromatographic peak is more symmetrical. Therefore, the buffer mobile phase Methanol was selected for further investigation.

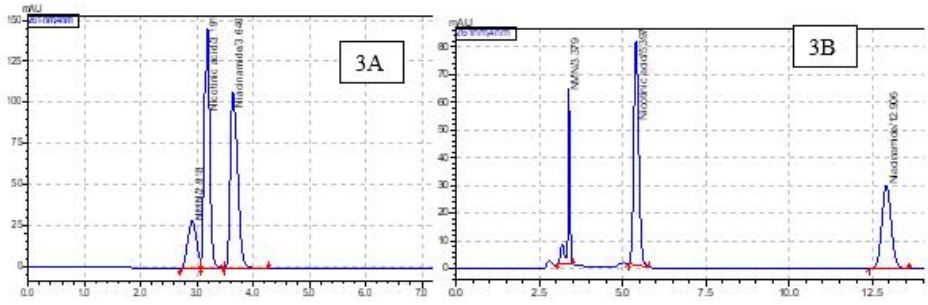

***Figure 3**. Chromatogram of three analytes at mobile phase phosphate buffer pH 2 (3A) and pH 5.3 (3B)*

The pH of the mobile phase affects ionizable substances (weak acids), so it is necessary to minimize the ionization of the analyte, which will affect the retention time and separation efficiency. Analyze the mixed standard solution at a concentration of 20 µg/mL with the phosphate buffer pH at 3 points pH 5.3; pH 3; pH 2 (pKa of acid nicotinic = 2.4, pKa of niacinamide = 3.35 and pH of sodium dihydro phosphate 10 nM solution of 5.3). The results showed that at pH 2, the separation efficiency was poor, and the analytical signal was low (Figure 3A). At pH 5.3, the analysis time was prolonged, there were many background impurities, and the chromatographic peak was split (Figure 3B). Therefore, choose mobile phase pH 3 for sharp peaks, good separation coefficient, short retention time, high analytical signal, and limited background influence.

In this study, mobile phase NaH_2PO_4 pH 3 was investigated with concentrations of 5 mM, 10 mM, 20 mM with the same standard solution concentration as above. At buffer concentration of 5 mM, nicotinic acid and niacinamide are almost impossible to separate (Figure 4), at concentrations of 10 mM and 20 mM give high analytical signals but a concentration of 10 mM gives a better separation effect and sharp peaks.

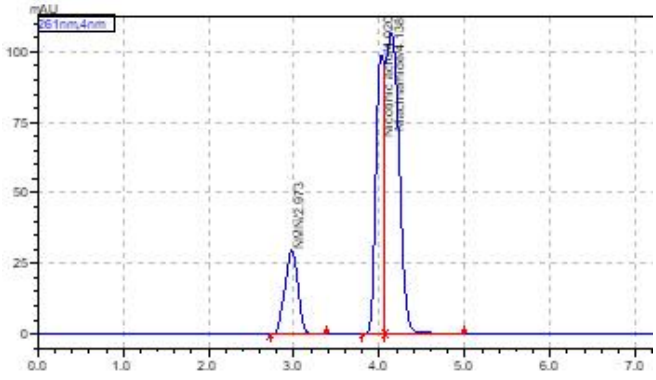

***Figure 4**. Chromatograms of three analytes at mobile phase 5 mM phosphate buffer pH 3*

After choosing methanol and phosphate buffer of 10 mM and pH 3, the mobile phase ratio was optimized in further study. With the mobile phase 10 mM NaH_2PO_4 pH 3 buffer solution - MeOH (70:30, v/v) and 10 mM NaH_2PO_4 pH 3 buffer solution:MeOH (80:20, v/v), the analysis time was reduced, but the peaks were eluted too quickly, causing the

phenomenon of overlapping peaks. Therefore, in this study, the mobile phase 10mM NaH_2PO_4 pH 3:MeOH (90:10, v/v) was chosen because of its short analysis time, symmetrical sharp peak shape, and good separation coefficient.

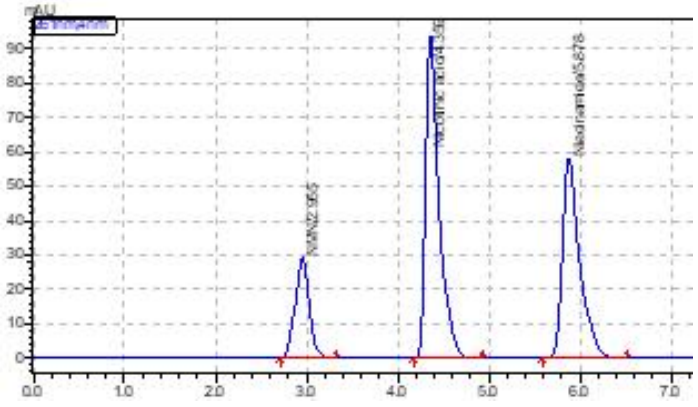

***Figure 5**. Chromatograms of three analytes at investigating the ratio of 10 mM phosphate buffer pH 3,0 : MeOH (90 : 10, v/v)*

3.2. Optimize sample processing

After homogenizing the sample, accurately weigh about 0.3g-2.0g of the test sample to investigate and select the extraction solvent. All three analytes are polar so we chose to investigate in two extracted solvents: H_2O and 4% TCA solution. The results show that the extraction efficiency in water solvent is higher than in 4% TCA solvent (Figure 6). Furthermore, water is an environmentally and green solvent that is suitable for formulation as a routine method.

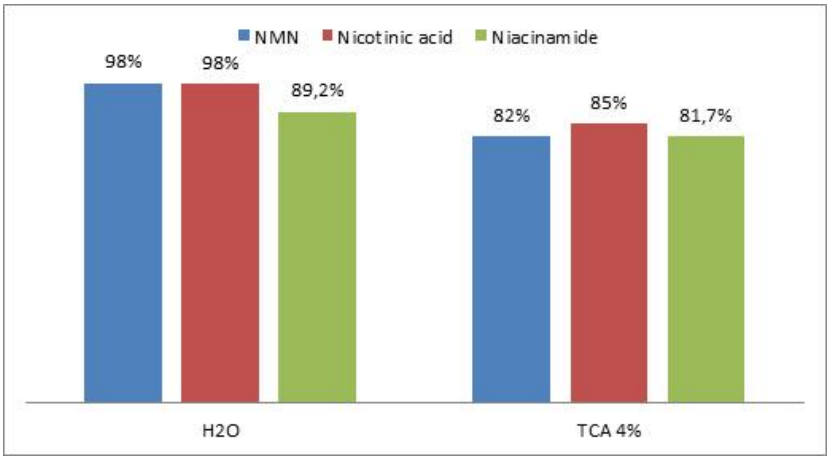

***Figure 6**. Content (%) of NMN, nicotinic acid, and niacinamide obtained from the extraction solvent*

3.3. Method validation

The specification of the method was approved with no statistically significant difference in the retention time of each analyte. The chromatogram of blank (Figure 7A), standard (Figure 7B) and spiked sample (Figure 7C).

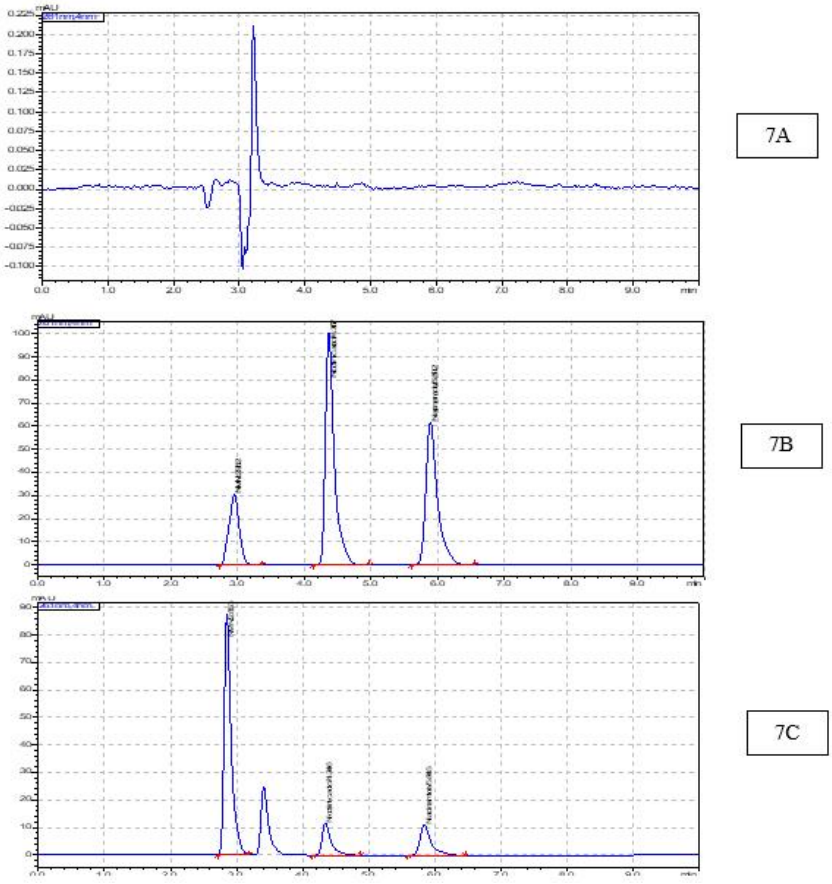

***Figure 7**. The chromatogram of blank (7A), standard (7B), and spiked sample (7C)*

The results of LOD, LOQ, and the accuracy at LOQ are shown in Table 1.

***Table 1**. LOD and LOQ results of three substances in five sample matrices*

Analyte	*Parameter*	*Dietary supplement*				
		Tablets	*Soft capsules*	*Hard Capsules*	*Granules*	*Syrup*
NMN	LOD (mg/kg)	0.40	0.45	0.41	0.43	0.40
	LOQ (mg/kg)	1.30	1.50	1.40	1.40	1.30
Nicotinic acid	LOD (mg/kg)	0.60	0.61	0.4	0.10	0.2
	LOQ (mg/kg)	1.90	2.00	1.5	0.49	0.53
Niacinamide	LOD (mg/kg)	0.49	0.60	0.40	0.10	0.20
	LOQ (mg/kg)	1.6	1.90	1.40	0.47	0.50

Linear range and calibration curve: the purpose of the survey is to find the linear working range of the analyte with high reliability for the analytical process. Prepare 8 different concentrations: 0.1; 0.2; 1; 2; 5; 10; 20; 50 µg/mL to determine the linear range. The result meets the standard if the values of the correlation coefficient were $R^2 > 0.999$ and bias (%) $\leq \pm 15\%$.

***Table 2**. Calibration equations for three analytes*

Analyte	Calibration equation	*Bias (%)*	R^2
NMN	y = 16272x – 17,50	-0.83-4.28	1
Nicotinic acid	y = 47216x – 25,86	-0.42-3.12	1
Niacinamide	y = 37371x + 34,57	-0.64-1.87	1

The correlation coefficients (R^2) of the three substances are higher than 0.999, demonstrating high linearity between peak areas and concentrations of substances and small bias (Table 2). Thus, they met linearity requirements.

The results of accuracy, repeatability, reproducibility, and measurement uncertainty are presented in Table 3.

***Table 3**. Summary of results of accuracy, repeatability, reproducibility, and measurement uncertainty of the method*

Analytes	*Parameter*	*Dietary supplement*				
		Tablets	*Soft capsules*	*Hard Capsules*	*Granules*	*Syrup*
NMN	RSD_r (%)	2.56	0.54	1.84	3.79	2.23
	RSD_R (%)	2.14	1.03	2.44	3.70	1.82
	R (%)	95.5-102	95.8-98.7	97.0-103.0	96.8-103	96.3-101
	U (%)	6.68	6.32	6.50	8.37	8.11
Nicotinic	RSD_r (%)	2.65	0.39	2.50	4.82	2.46
acid	RSD_R (%)	2.31	1.75	2.65	4.10	2.00
	R (%)	92.5-100.0	96.5-102.0	95.5-104	96.1-101	98.3-103
	U (%)	9.51	6.73	7.55	7.30	4.98
Niacinamide	RSD_r (%)	3.07	0.81	1.58	5.14	2.37
	RSD_R (%)	2.93	1.83	3.00	4.48	1.85
	R (%)	96.2-101	98.4-104.0	95.5-101	95.3-98.3	96.0-100
	U (%)	6.02	7.18	7.56	8.69	7.16

Note: RSD_r %: repeatability; RSDR%: reproducibility; R %: recovery; U %: uncertainty

The results show that the method has good repeatability and reproducibility; high accuracy, and measurement uncertainty which all meet the AOAC requirement standards. The above validation results prove that the HPLC-PDA method is suitable for simultaneously analyzing three substances in dietary supplements and health protection supplements.

3.4. Analysis of real samples

The developed method analyzed 20 samples of functional and health supplements containing analytes collected in Hanoi. The results are shown in Table 4 and Figure 8.

***Table 4**. Results of quantified active ingredient content compared to the label content of 20 products*

Samples	*NMN (%)*			*Nicotinic acid (%)*			*Niacinamide (%)*		
	Product label claim (mg/serving)	*Result (mg)*	*Content (%)*	*Product label claim (mg/serving)*	*Result (mg)*	*Content (%)*	*Product label claim (mg/serving)*	*Result (mg)*	*Content (%)*
Hard Capsules	250	238.4	95.4	nd	-	-	nd	-	-
Hard Capsules	125	0.237	0.2	nd	-	-	nd	-	-
Hard Capsules	250	247.2	98.9	15	13.19	88.0	nd	-	-
Hard Capsules	200	7.01	3,5	10	5.51	54.5	5	3.74	74.7
Soft capsules	354	303.42	85.7	nd	nd	nd	nd	-	-
Soft capsules	200	1.59	0.8	nd	nd	nd	nd	-	-
Soft capsules	20	18.28	91.6	nd	nd	nd	10	9.67	96.7
Soft capsules	50	nd	nd	nd	nd	nd	nd	-	-
Tablets	1000	340.9	34.1	25	5.395	21.6	nd	-	-
Tablets	500	443.7	88.7	10	9.007	90.1	10	8.417	84.2
Tablets	125	nd	nd	nd	-	-	nd	-	-
Tablets	500	nd	nd	nd	-	-	nd	-	-
Granules	300	156.23	52.1	20	19.46	97.3	10	9.819	98.2
Granules	500	505.8	101.2	nd	-	-	nd	-	-
Granules	250	nd	nd	nd	-	-	nd	-	-
Granules	300	nd	nd	10	1.538	15.4	nd	-	-
Syrup	200	185.6	92.8	nd	-	-	nd	-	-
Syrup	200	47	23.5	5	2.25	44.9	5	3.82	76.3
Syrup	500	337	67.4	30	22.13	74.8	nd	-	-
Syrup	500	nd	nd	nd	-	-	nd	-	-

Note: "nd": not detected, "-" is not included in the product's ingredient list

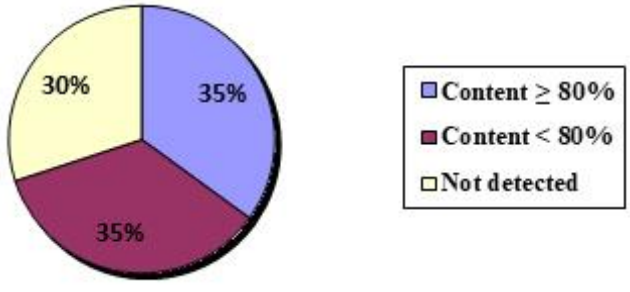

***Figure 8**. Analytical results of analytes in 20 products compared to the content stated on the label*

The numbers above display the results of analyzing 20 product samples that contain NMN alone or in combination with other ingredients. The analysis reveals that the quality of NMN products on the market varies widely (Figure 8). Only 7 product samples (35%) have an NMN content that is ≥ 80% of the label content, with one product exceeding the label content by 1.2%. However, 6 product samples (30%) have no detectable NMN content or have an NMN content below the detection limit. The product samples with three substances show good separation and resolution capabilities. The study indicates that the quality management of NMN products on the market needs to be improved to ensure consumer satisfaction and safety.

4. CONCLUSION

A method for the simultaneous quantification of NMN, nicotinic acid, and niacinamide in health supplements was developed and validated following the AOAC guidelines for specificity, linearity, accuracy, repeatability, reproducibility, LOD, LOQ, and measurement uncertainty. The sample preparation procedure involves minimal steps and uses eco-friendly solvents. The method is suitable for the simultaneous analysis of three analytes in 20 different food products and can serve as a standard method for the determination of NMN, nicotinic acid, and niacinamide in supplements.

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Phương pháp sắc ký lỏng xác định đồng thời nicotinamide mononucleotide, niacinamide và nicotinic acid trong thực phẩm bảo vệ sức khoẻ

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Tóm tắt

Nghiên cứu được thực hiện với mục tiêu là xác định đồng thời 3 hợp chất sử dụng phương pháp HPLC-PDA trong thực phẩm bổ sung, thực phẩm bảo vệ sức khoẻ. Quy trình xử lý mẫu nhanh, đơn giản, dung môi thân thiện với môi trường. Các điều kiện HPLC bao gồm: Cột sắc kí C18 (250 mm x 4,6 mm, 5 µm); pha động: 10 mM đệm phosphate pH 3: methanol (90:10, v/v), bước sóng phát hiện 261 nm. Phương pháp đã được thẩm định đạt các tiêu chí của AOAC. Các tiêu chí độ chọn lọc; đường chuẩn của 3 chất phân tích được xây dựng trong khoảng nồng độ 0,1 - 50,0 µg/mL có hệ số tương quan (R^2> 0,999); LOD (NMN: 0,4 - 0,45 mg/kg, nicotinic acid: 0,1 - 0,61 mg/kg, niacinamide: 0,1 - 0,6 mg/kg); LOQ (NMN: 1,3-1,5 mg/kg, nicotinic acid: 0,49-2,00 mg/kg, niacinamide: 0,47 - 1,90 mg/kg); độ đúng R (với độ thu hồi của NMN: 95,5 - 103,0 %, nicotinic acid: 92,5 - 104,1% và niacinamide: 95,3 - 103.8%); độ lặp lại RSD_r (NMN: 0,54 - 3,79%, nicotinic acid: 0,39 - 4,82% và niacinamide: 0,81 - 5,14%); độ tái lặp RSD_R (NMN: 1,03 - 3,70%, nicotinic acid: 1,75 - 4,10% và niacinamide: 1,83 - 4,48%) đều đạt yêu cầu của AOAC. Phương pháp được áp dụng để phân tích đồng thời ba chất phân tích với 20 sản phẩm thu thập trên địa bàn Hà Nội.

Từ khóa: *Nicotinamide mononucleotide, NMN, niacinamide, nicotinic acid, HPLC-PDA*