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Development of liquid chromatography-mass spectrometry method to determine biotin content in nutritional products and supplements

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Abstract

The study was carried out to develop a method for determining the biotin content in nutritional products and supplements by liquid chromatography-mass spectrometry (LC-MS/ MS) combined with a fast and simple pretreatment. The analyzed sample was hydrolyzed in acetate buffer (50 mM, pH 4) with the presence of ascorbate solution (10%) at 120 ± 1°C. After adding the internal standard ($^2H_4$-biotin), the mixture was analyzed on LC-MS/ MS system using C18 (100 mm × 2.1 mm; 1.7 µm) column and 0.1% formic acid and MeOH as mobile phases. The analyte was detected on an MS/MS system with an ESI (+) ionization source. The method was validated following the AOAC criteria. The method detection limit was 0.15 - 4.20 µg/100g, the method quantitative limit was in the range of 0.5 - 14.0 µg/100g, the recovery range was 81.3 - 107.3%, the repeatability was in the range of 2.00 - 7.30%, the reproducibility was 2.50 - 7.70%, which were in accordance with AOAC requirements. The method has been applied to analyze biotin content in 10 nutritional products and 10 supplements purchased in the market.

Keywords*: Biotin, LC-MS/MS, nutritional products, supplements.*

1. INTRODUCTION

Biotin (Vitamin H or B7) is an essential micronutrient, required in a number of metabolic reactions such as gluconeogenesis, fatty acid synthesis and amino acid metabolism [1]. In addition, biotin can keep skin and hair healthy. Beside the free biotin form, there are several derivatives such as biotin sulfoxide, dehydrobiotin, and natural conjugated forms such as biocytin. These compounds have strong activity with microorganisms, thus they usually not intended for animals or humans [1]. Biotin is found in fruits and vegetables in free form, while in meat and grains it is in the protein binding. In cow's milk, biotin is mainly found in free form and small amounts in the protein binding [1-2]. Almost the biotin content in natural foods is at low level, usually ranging from a few µg/kg in many vegetables to several hundred µg/kg in pork liver and egg yolk [1]. Biotin deficiency during pregnancy causes a significant increase in fetal malformations and mortality. Moreover, recent evidence

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indicated that biotin deficiency is associated with impaired glucose tolerance which can lead to the risk of diabetes [2]. Therefore, biotin supplementation through other sources such as nutritional products and health goods is very necessary for the human body.

To evaluate vitamin H content in nutritional products and supplements, it is required to develop feasible analytical methods. In the world, there have been a number of published studies on biotin content determination methods including: high performance liquid chromatography combined with different detectors such as: UV [3, 8], fluorescence with pre-column derivatization [4], mass spectroscopy [1-2 , 5, 6, 7]. Due to the poor absorption in the UV region and the inability of autoflourescense, these methods are not appreciated for the selectivity as well as the time saving of the derivatization processes. The standard method AOAC 2016.02 was published [8] to determine biotin content in nutritional products. Biotin in the sample was dispersed in PBS buffer and sterilized at 121°C for 25 min, cleaned through an immunoaffinity column, blow-dried and dissolved in 1 mL of water, analyzed by LC-UV at wavelength 200 nm. The method has some limitations such as complicated multi-step sample processing, high cost of immunoaffinity columns, wavelength 200 nm is not specific. The LC-MS/MS method has been chosen in many studies. However, the disadvantages of those studies are the limited number of sample matrices or the complicated, expensive processing and the enhancement of sample matrices without using internal standards. The aim of this study is to develop a fast, sensitive, and accurate analytical LC-MS/MS method with simple sample handling, saving time and solvents, to determine free biotin content in nutritional products and supplements.

2. MATERIALS AND METHODS

2.1. Research subjects

Research object was Biotin and tesearch samples were nutritional products such as powdered milk, liquid milk, yogurt, drinking yoghurt, cereals; supplements include: solid form (tablets, hard capsules), oil capsules, nuggets, and syrup.

2.2. Chemicals and standards

The chemicals used in the study are in analytical purity. Biotin standard (from Sigma Aldrich, purity of 99.0%); $^{2}H_4$ - biotin (from IsoSiences with 95.0% purity). Other chemicals: Methanol, acetonitrile, formic acid, ammonium hydroxide, ammonium acetate, acetic acid, ammonia, and sodium ascorbate from Merck.

Stock standard solution: Dissolve 20 mg of biotin standard (200 g/mL) in 3.6 mM ammoniac solution in a 100 mL flask. Store at -20°C for 6 months. Dissolve 1 mg of internal standard $^{2}H_4$ - biotin (100 g/mL) in 3.6 mM ammoniac solution into a 10 mL volumetric flask. Store at - 20°C for 6 months. Prepare a series of working standards with concentrations from 0.5 to 800 ng/mL from an intermediate standard of 10 μg/mL and 0.5 mL $^{2}H_4$ - biotin (4 μg/mL), add 20 mL of acetate buffer (50 mM, pH 4), 3 mL of ascorbate (10%), make up to the 50 mL mark with distilled water.

2.3. Instruments

The liquid chromatography-mass spectrometry (LC-MS/MS) system (XEVO TQ-XS, Waters) and chromatographic C18 (100 mm × 2.1 mm, 1.7 µm) column were used.

Other common laboratory equipment includes: analytical balance with 0.1 mg accuracy (MS-205DU, Mettler), vortex shaker (Genius, IKA), pH meter (Mettler), cabinet drying set at 120 ± 1°C.

Laboratory utensils include: Volumetric flasks, micropipettes, 50 mL centrifuge tubes, 0.2 µm sample filters, 0.2 µm mobile phase filters, 3 mL cylinders, beakers,...

2.4. Methods

2.4.1. Sampling

Nutritional products such as powdered milk, liquid milk, yogurt, drinking yogurt, cereals; supplements including: solid form (tablets, hard capsules), oil capsules, nuggets, syrup are randomly purchased in Hanoi market. In which, 10 samples are nutritional products and 10 samples are health protection goods (supplements).

2.4.2. Analytical methods

Referring to paper [4] for sample processing, samples are thoroughly homogenized before treatment step. Then, the sample was weighed an appropriate amount into a 50 mL centrifuge tube, added water to a volume of 25 mL (for solid samples), shaked for some minutes. After that, 3 mL of 10% ascorbate solution, 0.5 mL of $^{2}H_{4}$ - biotin (4 µg/mL) and 20 mL of acetate buffer (50 mM, pH = 4) was added into the sample, vortexed for 30 seconds then incubated at 120°C. After 30 minutes, samples were taken out and cool to room temperature, then centrifuged and transfered into 50 mL volumetric flasks and make up to 50 mL with water. The extraction was filtered through a 0.2 µm filter into the vial and injected into the chromatographic system.

LC-MS/MS conditions: chromatographic C18 (100 mm × 2.1 mm; 1.7 µm) column. Mobile phase A (formic acid 0.1%) and B (Methanol) with gradient program for the first minute, the ratio of channel A: channel B is 90 : 10 then increase to 60 : 40, hold for 2 minutes, from the 3rd minute switch to the first ratio. The total analysis time is 6 minutes and injection volume 5 µL.

Method was validated for some parameters such as specificity (based on retention times of standards, blanks, and increments), linear range, MDL, MQL, repeatability (n = 6), reproducibility (n = 10), recovery (at 3 spiked levels), measurement uncertainty. The method was evaluated on 6 samples including powdered milk, liquid milk, supplements in the form of tablets, oil capsules, nuggets, and syrup.

2.4.3. Data processing methods

Biotin content in the sample was calculated automatically based on the instrument software LC-MS/MS (MassLynx 4. 1). The method validation results were processed using Microsoft Excel 2010 software.

3. RESULTS AND DISCUSSION

3.1. Optimizing analytical processes

3.1.1. Investigation of analytical conditions on LC-MS/MS

By referencing some documents [2, 6] and the structure of biotin, the MS/MS conditions were investigated by electron spray ionization technique ESI with positive fragmentation (ESI+). 5 µL of biotin standard solution and $^{2}H_4$ - biotin 200 µg/L were injected directly into the mass spectrometer at automatic mode for optimizing mass spectrometry conditions. The information about parent ions and daughter ions for qualitative and quantitative purposes are shown in Table 1.

Table 1. *The optimized Fragmentation parameters*

Analyte	*Ionization mode*	*Molecular Mass*	*Precursor ion (m/z)*	*Production ion (m/z)*	*Cone potential (V)*	*Fragmentation Energy (Ev)*
Biotin	ESI+	245.100	245.100	97.100	20	30
				227.100*	20	15
$^{2}H_4$ - biotin	ESI+	248.990	248.990	96.810*	2	22
				169.760	2	26

Note: (*) are quantitative ion

Methanol and acetonitrile are the most commonly less polar organic solvents in reversed-phase liquid chromatography and formic acid is polar solvents commonly used in ESI-mode MS. Referring to some documents, a study was conducted in two mobile phases: mobile phase 1: formic acid 0.1% and methanol; mobile phase 2: formic acid 0.1% and acetonitrile. The results show that biotin signal using mobile phase 1 is higher than using mobile phase 2. Therefore, the mobile phase 1 was selected for further investigations.

The ion concentration in the mobile phase directly affects the ionization process and the signal of the analyte. Based on the ion fragmentation mechanism and references [2, 6], the signal of biotin, $^{2}H_4$ - biotin at 200 µg/L was affected by the concentration of formic acid in the mobile phase. The mobile phase survey results of formic acid concentrations in the range of 0.05 - 0.2% were shown in Figure 1.

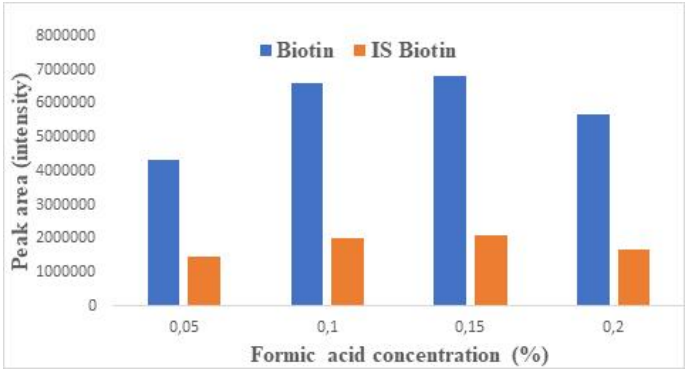


Figure 1. *Dependence of biotin, $^{2}H_4$ - biotin signal on formic acid concentration*

The analyte signals increased as the formic acid concentration increased, however, at the 0.2% formic acid, the analyte signal decreased, possibly due to ion competition. There was no significant difference between the analyte signals at the 0.1 and 0.15 % formic acid level. But the lower level of acidity was preffered to restrict the adversely effect to the separation column and LC-MS/MS system. Therefore, in this study, 0.1% formic acid mobile phase was selected.

3.1.2. Investigation of sample treatment conditions

Nutritional products were known as complicated matrices with many influencing components such as proteins, lipids, sugars, etc, which can obstructed the biotin determination. Therefore, it is necessary to have a sample processing procedure to limit the effects. According to AOAC 2016.02, [8] immunoaffinity columns were used in processing with high selectivity, but its weaknesses are many complicated steps, time wasting, moreover, expensive costs of immunoaffinity columns, thus it is not suitable for some laboratories in Vietnam. This study aims to build a simple and fast sample processing procedure to determine the free-form biotin content in nutritional products and health supplements. Vitamin B7 is stable over a wide pH range and is a relatively heat stable vitamin. Ascorbate solution was added to the sample to prevent oxidation of biotin during sample preparation. High-temperature pyrolysis is a common extraction technique used in biotin analysis to denature protein in milks, inactivate endogenous enzymes, and liberate biotin from complex bonds. Therefore, pyrolysis time and temperature directly affect the biotin content in the sample. An investigation was conducted to proof that effect and find the optimized pyrolysis conditions. Powder milk and soft capsule are complex samples representing the group of nutritional products and supplements which were selected for this research.

The pyrolysis times of 10, 15, 20, 25, 30, 35, 40, 45, 50 minutes and the fixed 120 ± 1°C temperature were conducted in the same milk powder samples and soft capsule samples. The results of the analysis are shown in Figure 2.

The results point that there is a change in the content of biotin obtained at different pyrolysis times. From 10 to 35 minutes, the amount of obtained biotin gradually increased. After that time, the content is gradually decreasing from 40 to 50 minutes due to the decomposition. The biotin content at 30 and 35 min was the same. Therefore, in this study, a time of 30 minutes was selected for time and energy saving.

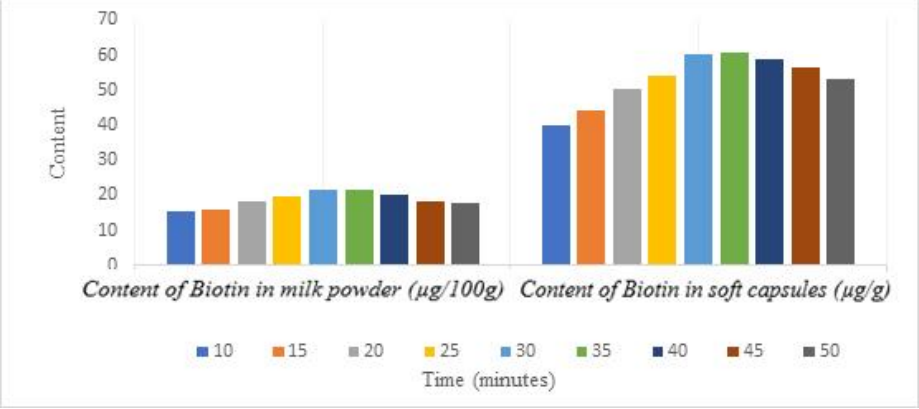


Figure 2. *Effect of hydrolysis time on biotin content of milk powder and supplements samples*

Other surveys were carried out at a number of different pyrolysis temperatures of 90, 110, 120, 130 ± 1°C on the same powder milk and soft capsule samples. The results of the analysis are shown in Figure 3.

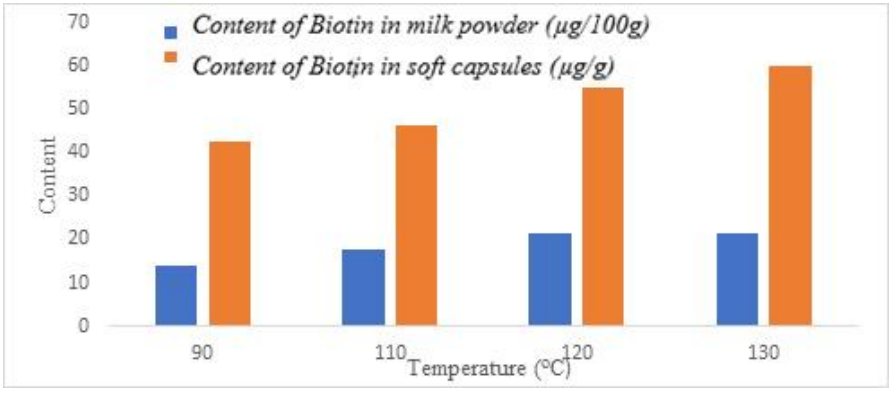


Figure 3. *Effect of pyrolysis temperature on the biotin content*

For samples in suspension such as reconstituted milk powder and softgel, the higher temperature, the better the layering ability and clean extract was obtained. In addition, high temperature also increases the solubility of the analyte. Therefore, biotin content gradually increased as the raise of pyrolysis temperature. The biotin contents at 120 and 130 ± 1°C were not significantly different. Therefore, in this study, the time selection is 120 ± 1°C to save energy.

3.2. Method validation

The analytical method was validated for the following parameters: specificity, linear, limit of detection/limit of quantification MDL/MQL, repeatability, reproducibility, recovery, and measurement uncertainty.

The specificity of the method was evaluated through analysis of blanks, standards and spiked standard samples. The same retention time of analyte signal in the standard and the increment sample and the disappearance in blank indicating that the method has good specificity.

The MDL and MQL were determine by analyzing 10 times the test sample (milk powder, tablet samples) of low concentration, calculating the mean, SD, and R value (from 4 to 10). The MDL and MQL of the method in nutrient product were 0.15 and 0.50 µg/100g, respectively. In supplements these values were 4.20 and 14.0 µg/100g, respectively.

Based on the optimal conditions, the biotin standard curve is linear in the range of 0.5 - 800 µg/L. The standard equation, correlation coefficient, repeatability (RSD_r), reproducibility (RSD_R), recovery (R), uncertainty (U) are presented in Figure 3, Figure 4 and Table 3.

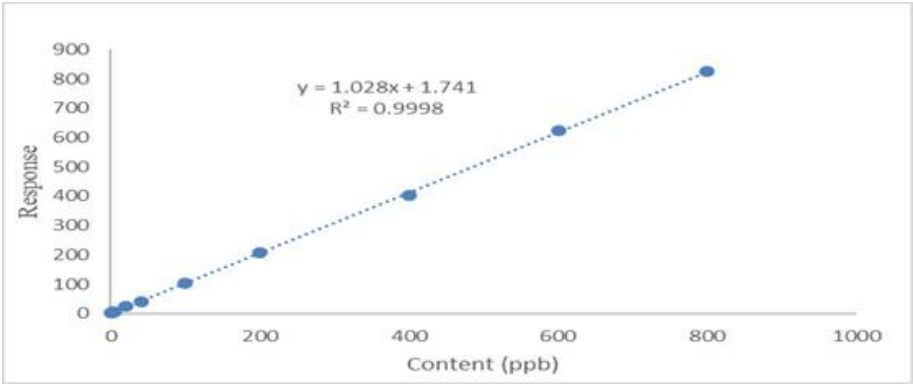


***Figure 3**. Linear relationship of biotin/internal standard signal ratio and concentration*

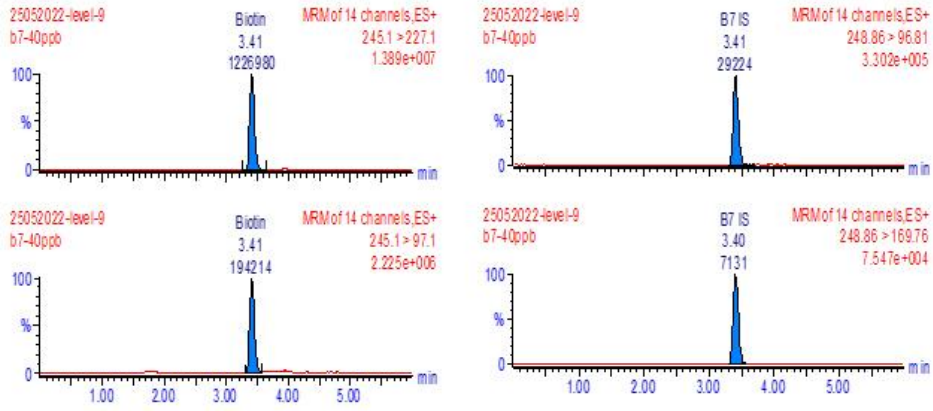


***Figure 4 .** Biotin chromatogram 40 µg/L, $^{2}H_{4}$ - biotin*

***Table 3.** Repeatability, reproducibility, recovery, and uncertainty on difference matrices*

Validation parameters	***Nutritional products***		***Supplements***			
	Liquid milk	***Milk powder***	***Nuggets***	***Soft capsules***	***Tablets***	***Syrup***
RSD_r (%)	2.20	4.60	6.60	3.40	2.00	7.30
RSD_R (%)	2.50	7.70	5.30	2.60	2.50	7.10
R (%)	95.5 - 101	96.4 - 102	81.3 - 97.9	94.8 - 107.3	92.2 - 103.9	85.5 - 99.1

U (%)	7.50	15.8	16.6	8.50	7.60	17.9

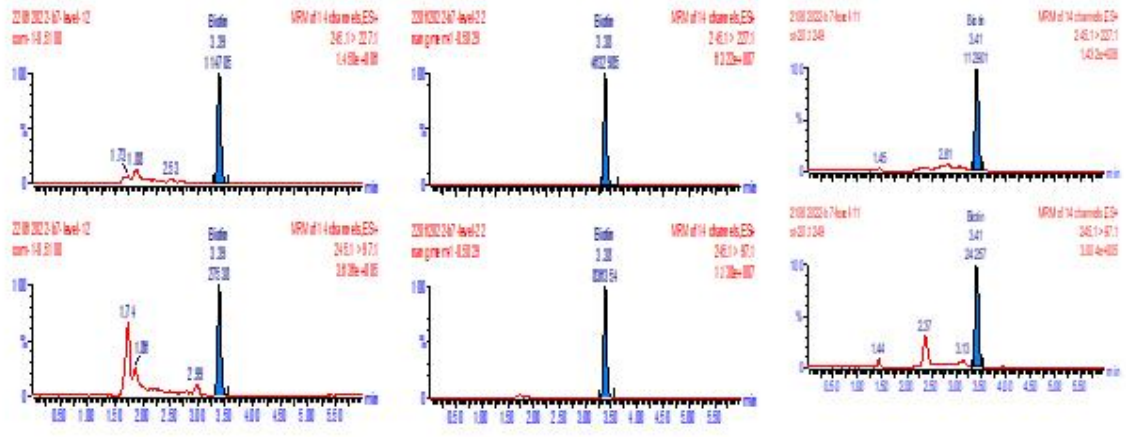
It can be seen that the coefficient of determination $R^2 > 0.99$; the repeatability, reproducibility, and recovery were all in accordance with AOAC. The method is completely applicable to the analysis of biotin content in the real sample.

3.3. Real sample analysis results

On the basis of the developed method, the content of biotin in 10 samples of nutritional products including powdered milk, liquid milk, yogurt, drinking yoghurt, cereals and 10 samples of healthy supplements including: tablets, hard capsules, syrup, soft capsules, nuggets collected in Hanoi were analyzed. The results were presented in Table 4 and Figure 5.

Table 4. *Real sample analysis results*

No	*Sample*	*Content biotin (µg/100g)*	*No*	*Sample*	*Biotin content (µg/g)*
1	Milk powder 1	35.1	1	Supplement- tablets 1	12.5
2	Milk powder 2	15.1	2	Supplement- tablets 2	5.19
3	Milk powder 3	20.3	3	Supplement- hard capsule 1	62.5
4	Cereals	7.15	4	Supplement- hard capsule 2	35.1
5	Liquid milk 1	2.15	5	Supplement- Syrup 1	10.1
6	milk 2	5.26	6	Supplement- Syrup 2	4.13
7	milk 3	4.55	7	Supplement- Nuggets 1	16.7
8	Yogurt	2.23	8	Supplement- Nuggets 2	25.1
9	Yogurt drink	3.42 _	9	Supplement- soft capsule 1	45.7
10	Raw milk	0.65 _	10	Supplement- soft capsule 2	33.1 _



a. Nuggets matrix *b. Soft capsule matrix* *c. Liquid milk matrix*

Figure 5. *Analytical chromatograms of biotin on some real samples*

From the obtained results, the biotin content in nutritional products ranges from 0.65 to 35.1 µg/100g. In which, the content of biotin in milk powder sample 1 was the highest at 35.1 µg/100g. The powder milk samples all had higher biotin concentrations than the liquid samples. In solid samples (milk powder and cereals) with concentrations ranging from 7.15 - 35.1 µg/100 g. Liquid samples had biotin content from 0.65 - 5.26 µg/100 g. Biotin content in supplement samples range from 4.13 - 62.5 µg/g. In hard capsule 1, the biotin level was the highest at 62.5 µg/g, in syrup sample 2 had the lowest biotin content of 4.13 µg/g. Biotin content in syrup and nuggets samples was lower than that in hard capsule, tablet and softgel samples. In the 20 actual samples, 13 samples (65% of the total samples) had an analyte content compared on the product label in the range (80 - 120%) of the declared label, in the remaining 7 samples, there was 1 sample of raw fresh milk without the declared label and 06 samples (30% of the total samples) with a content of only 70% compared to the product declared label. The results of the study have preliminarily reflected the current status of biotin- fortified products on the market. Furthermore, the method is also applied to analyze the infant formula product sample (DDP -Infant Formula Powder) - an international multi-laboratory testing organized by Nestlé with good results (Z-core of 0.3; 0.6; 0.2, respectively in three consecutive rounds from 2021 to 2022) that contributed confirm the accuracy of the method.

4. CONCLUSION

The study was carried out with the goal of developing a liquid chromatography-mass spectrometry (LC-MS/MS) method to determine biotin content in nutritional products and suppplements. The advantage of the method is the fast, simple processing which is applicable to many sample matrices. The accuracy of the method is evidenced through the validation parameters which satisfy the AOAC requirements and the results of participation in international proficiency testing programs.

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Phát triển phương pháp sắc ký lỏng khối phổ xác định hàm lượng biotin trong sản phẩm dinh dưỡng và thực phẩm bảo vệ sức khỏe

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Tóm tắt

Nghiên cứu được thực hiện với mục tiêu phát triển phương pháp xác định hàm lượng biotin tự do trong sản phẩm dinh dưỡng và thực phẩm bảo vệ sức khỏe bằng phương pháp sắc ký lỏng khối phổ LC-MS/MS với quy trình xử lý mẫu nhanh, đơn giản. Mẫu phân tích được thủy phân trong dung dịch đệm acetat (50 mM, pH = 4); dung dịch ascorbat (10%) ở 120 ± 1°C và phân tích trên LC-MS/MS, sử dụng nội chuẩn $^{2}H_{4}$- biotin. Các điều kiện LC-MS/MS bao gồm: cột C18 (100 mm × 2,1 mm; 1,7 µm) và pha động acid formic 0,1% và MeOH. Chất phân tích được phát hiện trên hệ khối phổ hai lần MS/MS với nguồn ion hoá ESI (+). Phương pháp đã được thẩm định theo các tiêu chí của AOAC. Giới hạn phát hiện của phương pháp là 0,15 - 4,20 µg/100 g, giới hạn định lượng của phương pháp là 0,5 - 14,0 µg/100g, độ thu hồi trong khoảng 81,3 - 107,3%, độ lặp lại RSD trong khoảng 2,00 - 7,30 %, độ tái lập nội bộ trong khoảng 2,50 - 7,70% đều đạt theo yêu cầu của AOAC. Phương pháp đã được áp dụng để phân tích hàm lượng biotin trong 10 mẫu sản phẩm dinh dưỡng và 10 mẫu thực phẩm bảo vệ sức khỏe.

***Từ khóa**: Biotin, LC-MS/MS, sản phẩm dinh dưỡng, thực phẩm bảo vệ sức khỏe.*