TẠP CHÍ KHOA HỌC
TRƯỜNG ĐẠI HỌC QUY NHƠN

Xác định hàm lượng Aflatoxin B1 trong sản phẩm ngũ cốc dinh dưỡng cho trẻ em bằng phương pháp sắc ký lỏng siêu hiệu năng đầu dò khối phổ hai lần (UPLC-MS/MS) làm sạch bằng cột ái lực miễn nhiễm

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TÓM TẮT

Kết quả nghiên cứu quy trình phân tích độc tố Aflatoxin B1 (AFB1) với kỹ thuật sắc ký lỏng siêu hiệu năng đầu dò khối phổ (MS) hai lần (UPLC-MS/MS) trong nền mẫu sản phẩm ngũ cốc dinh dưỡng cho trẻ em được thể hiện trong nghiên cứu này. Theo kết quả nghiên cứu, phương pháp UPLC-MS/MS xác định Aflatoxin B1 với thời gian lưu 4,07 phút bằng cách sử dụng cột BEH C18 1,7 µm (2,1×150 mm), pha động ở chế độ ingredient 2 dung môi với tỷ lệ: 5 mM Amonium acetate/H_2O và 0,1% HCOOH/MeOH, dung môi chiết mẫu là hỗn hợp của metanol/nước tỷ lệ 70:30 (v/v). Điều kiện phân mảnh để định lượng AFB1: cặp ion định lượng m/z: 313,05 > 285 và cặp ion định tính (m/z) 313,05 > 241. Kết quả nghiên cứu cho thấy, hệ số xác định (R^2) của đường chuẩn đạt giá trị trên 0,9993; độ lặp lại (nồng độ ≤ 1 µg/kg) trong khoảng (6,72 - 9,00)%; độ tái lặp đạt trong khoảng (7,89 - 10,4)%; giới hạn phát hiện (LOD) là 0,03 µg/kg; giới hạn định lượng (LOQ) 0,10 µg/kg và độ không đảm bảo đo (U, Uncertainty) là ±17,1%.

Từ khóa: *Aflatoxin B1, UPLC-MS/MS (IAC), bột ngũ cốc cho trẻ nhỏ.*

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Determination of Aflatoxin B1 in cereal for infants using ultra-performance liquid chromatography to triple quadrupole mass (UPLC-MS/MS) cleaning up by an immunoaffinity column

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ABSTRACT

This work studied on developing the analytical procedure for determination of Aflatoxin B1 (AFB1) in cereal for infants by using ultra-performance liquid chromatography triple quadrupole mass spectrometry detector (UPLC-MS/MS). Under optimal conditions, the retention time of AFB1 is identified at 4.07 minutes as using BEH C18 1.7 µm (2.1×150 mm) column and mobile phase with two solutions gradient: 5 mM Ammonium acetate/H_2O and 0.1% HCOOH/MeOH, and ratio methanol/water is 70/30 for extraction. The mass-to-charge ratio (m/z) of AFB1 for quantitative and qualitative analyses are 313.05 > 285 and 313.05 > 241, respectively. Furthermore, the obtained results are validated. Typically, the statistical parameters are significant such as linear correlation coefficient of determination (R^2) ≥ 0.9993, repeatability (concentration ≤ 1 µg/kg) in range of (6.72 - 9.00)%, (7.89 - 10.4)% of reproducibility and uncertainty is ±17.1%, while the limit of detection (LOD) and the limit of quantitation (LOQ) are 0.03 µg/kg and 0.10 µg/kg, respectively.

Keywords: *Aflatoxin B1, UPLC-MS/MS (IAC), cereal for infants.*

1. INTRODUCTION

Currently, food safety has become the most important issue in protecting the health of consumers in Vietnam as well as in the world. Foods that contain viruses, bacteria, biological toxins and toxic chemicals (such as synthetic dyes, antibiotic residues and pesticides, etc.) are considered unsafe and can cause diseases from diarrhea to cancer.[1] They can be present in agricultural and food products such as peanuts, corn, wheat, and coffee, as well as in products that are made from these ingredients. *Aspergillus, Fusarium, and Penicillium* are the main fungi causing mycotoxins in agricultural products and foods. Up to now, more than 300 types of mycotoxins have been identified and documented. However, the groups of mycotoxins that frequently contaminate food and animal feed are aflatoxins, ochratoxins, fumonisin, patulin,

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zearalenone, deoxynivalenol, and T2-toxin.[2] Among them, the most influential and most concerned is aflatoxin (AF) which needs to be controlled. In high concentrations, aflatoxin can lead to acute poisoning (aflatoxicosis) and can be life-threatening, usually through liver damage. They can cause genetic changes, damage genes (DNA) and cause liver cancer in animals and humans.[3]

Until now, about 17 different types of aflatoxin have been recorded and studied. Of these, the four most common *bis*-furanocoumarin compounds are named B1, B2, G1, and G2, respectively. In terms of prevalence, aflatoxin B1 (AFB1) is the most abundantly found in nature and culture, followed by aflatoxin G1 (AFG1), aflatoxin B2 (AFB2), and then aflatoxin G2 (AFG2) and other substances have a rather low ratio.[4]

Cereals are important in providing primary nutrients for the growth and development of infants and children. Therefore, the identification and analysis of food contaminants have become a crucial concern due to the presence of mycotoxins and other common contaminants in cereals.[5] In Vietnam, the Ministry of Health has set the maximum allowable limit for aflatoxin (B1) at 0.1 µg/kg.[6]

Most of the AFB1 analytical methods used fluorescence detector high performance liquid chromatography (HPLC/FLD) using trifluoroacetic acid (TFA) derivatives to achieve sensitivity, meeting the allowable limit for AFB1 in food analysis.

However, in the case of nutritional foods for children, only TCVN 9522:2012[12] has a declared level of LOD = 0.05 µg/kg, which is lower than the regulation. However, theoretically, it is not easy to get the LOQ to 0.1 µg/kg when the LOD is 0.05 µg/kg. In the Annex to the standard, AFB1 concentrations at a quantitative threshold of LOQ = 0.05 µg/kg can be analyzed by increasing the HPLC/FLD injection volume to 1000µL. However, this injection volume is difficult to implement and is not suitable for practice. If injected at this volume, it will clog the column, cross-contaminate, waste solvent chemicals, take time to perform and clean.

Recent work by Nguyen Thanh Duy et al.[7] has presented a procedure for aflatoxin B1, B2, G1 and G2 analysis by UPLC-FLD fluorescence detector super-performance liquid chromatography for shows that the method is selective, the standard curve is linear in the range of 0.5 - 7.5 µg/L, the recovery ranges from 80.7 to 98.8% with the relative standard deviation (RSD) below 5% was obtained for each aflatoxin. The method detection limit (MDL) and method quantification limit (MQL) were 0.025 - 0.1 and 0.075-0.3 µg/kg, respectively. In which the MDL of aflatoxin B1 is 0.1 µg/kg, therefore the MQL will not meet the maximum allowable limit of 0.1 µg/kg.

In addition, compared with other analytical methods, the UPLC-MS/MS method applied to analyze AFB1 in nutritional cereals for children is evaluated to have the advantage of increasing selectivity and meeting current regulatory levels, operating more environmentally friendly, reducing analysis time, reducing operating pressure, reducing solvent costs, reducing environmental pollution.[8] Several papers reported that this method is sensitive, rapid, and durable enough for multiple mycotoxin determinations that fulfill international testing criteria.[9]

As an analytical method, this study has focused on the development and validation of a ultra-performance liquid chromatography with triple quadrupole mass spectrometer (UPLC-MS/MS) method for the determination of AFB1 toxin in the sample matrix namely cereal for infants.

2. APPARATUS, MATERIALS, AND METHODS

2.1. Apparatus

- The experiments were carried out at Khue Nam Science and Technology Service Co., Ltd – *address*: 2/17 Pham Van Bach, Ward 15, Tan Binh District, Ho Chi Minh City, Vietnam (https://khuenam.vn/nang-luc.php).

- The equipment used is the Acquity UPLC- MS/MS Xevo TQD by Waters supplier, and the analytical column is BEH C18 1.7 µm (2.1 × 150 mm).

- Other instruments and supporting equipment are required (solid phase extractor, centrifuge, ultrasonic machine, vortex...).

2.2. Chemicals

2.2.1. Reagents and standards

The chemicals in this study are methanol (MeOH), formic acid (HCOOH) of HPLC grade, NaCl with a purity of ≥ 99%, and phosphate buffer solution pH 7.4 (PBS), water of HPLC grade. The immunoaffinity column used is Aflatoxin with a column volume of 3 mL (IAC- AflaTest of Romer Lab). The stock solution of aflatoxin standard mixture consists of B1 (10 µg/mL), B2 (3 µg/mL), G1 (10 µg/mL), G2 (3 µg/mL) diluted in acetonitrile solvent.

2.2.2. Standard preparation

Intermediate mixed standard of 260 µg/L, 26 µg/L was prepared from a stock standard of 2600 µg/mL of acetonitrile (ACN) solvent, in which concentration of AFB1 standard is 1000 µg/L.

Working solutions were used to make a standard calibration curve of five points in AFB1 concentration of 0.2, 0.4, 0.8, 1.6, 3.2 µg/L. MeOH is solvent in preparing the calibration curve.

2.2.3. Solution preparation

MeOH/H_2O extraction solution (70/30) by volume: 150 mL of water was mixed with 350 mL of MeOH.

Phosphate buffer salt (PBS): Dissolve 8.0 g of sodium chloride, 1.2 g of anhydrous disodium hydrogen phosphate or 2.9 g of $Na_2HPO_4.12H_2O$, 0.2 g of potassium dihydrogen phosphate, and 0.2 g of potassium chloride in 900 mL of DI water. After dissolving, adjust the pH to 7.4 using hydrochloric acid or sodium hydroxide solution appropriately 5 mM ammonium acetate solution: Dissolve 0.385 g amonium acetate in 1.0 L of water in HPLC grade.

0.1% HCOOH/MeOH solution: Transfer 1.0 mL of formic acid into a 1.0 L volumetric flask and make up to the mark with MeOH.

2.2.4. Cereal sample preparation

The cereal powder sample was homogenized using a dry grinder. 10g of weighed powder was transferred to a centrifuge tube and 40 mL of MeOH/H_2O = 70/30 (v/v) was added. The mixture was vortexed for 1.0 minute and shaken for 30 minutes. It was sonicated for 30 minutes to extract the sample, then centrifuged at 3000 rpm for 5 minutes. The solution was filtered through a ∅110 mm diameter filter paper. 10 mL of the filtrate was collected and mixed with 20 mL of PBS buffer. The solution was gently mixed and passed through an IAC column. The column was washed twice with 20 mL of distilled water. The column was dried and eluted with 1.0 mL of MeOH. The eluate was filtered through a 0.22 µm PTFE membrane and analyzed using UPLC-MS/MS.[9]

2.3. Chromatographic conditions

To develop an analytical procedure for testing AFB1 in cereal powder using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) without using derivatives, the chromatographic conditions, including the mobile phase and fragmentation conditions, are listed in Tables 1 and 2.

Table 1. Mobile phase condition.

Time	Flow rate (mL/min)	%A	%B
Initial	0.30	90	10
4.00	0.30	0	100
5.00	0.30	0	100
6.00	0.30	90	10
7.00	0.30	90	10
Solution A (5 mM Ammonium acetate/H_2O); Solution B (0,1% HCOOH in MeOH)			

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Table 2. Fragmentation condition.

Analytes	Precursor ion (m/z)	Product ion (m/z)	Cone Volt (V)	Collision energy (eV)
Aflatoxin B1	313.05	241.00	50	30
Aflatoxin B1	313.05	285.00 (*)	50	37
(*) Quantitative ion				

3. RESULTS AND DISCUSSION

3.1. Determination of the linearity of the standard curve

Calibration curve for AFB1 was constructed based on AFB1 standard solution in MeOH with five specific standards of 0.2; 0.4; 0.8; 1.6; 3.2 µg/L, the results of the calibration curve are presented in Figure 1.

The statistical analysis was performed using the least-squares method, and the resulting linear equation was AUC = 1028.75×Conc. - 29.36 with a coefficient of determination, (R^2) ≥ 0.9993. Here, AUC represents the peak area and Conc. stands for the concentration of AFB1.

3.2. Method evaluation

To evaluate the analysis method for determining AFB1 content, the method parameters were analyzed and evaluated. The evaluated parameters include repeatability, reproducibility, recovery efficiency, measurement uncertainty, limit of detection, and limit of quantification, which are specifically presented in Table 3.

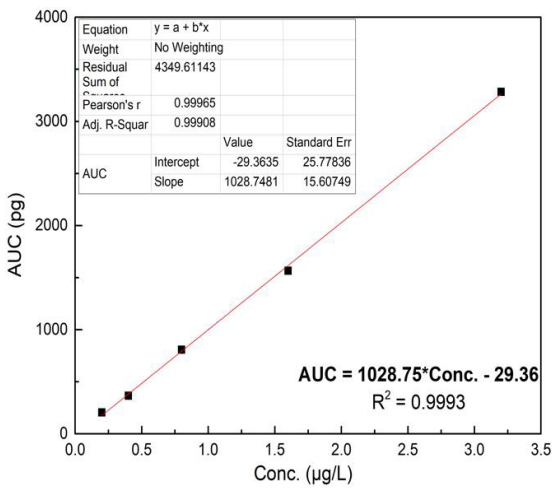

Figure 1. The calibration curve for determination of AFB1.

From this evaluation result, we certainly confirm that the UPLC-MS/MS method developed in this study can be used to determine the AFB1 content in the actual sample. That is the basis for studying and analyzing AFB1 on a sample of nutritious cereal powder for children in Ho Chi Minh City market.

3.3 Analysis of nutritional cereal powder samples for children in the Ho Chi Minh city market

To evaluate the AFB1 content on the actual sample, conduct an assessment of the AFB1 content of the sample "Supplementary Food Supplements Vanilla Organic Milk Powder with Vitamin B1 Babybio/Infant Cereals Vanilla/Babybio Céréales Vanille". Samples of weaning powder including cereals and milk for children over six months old, batch number: 3288131500102. According to the analytical procedure UPLC-MS/MS has been developed and evaluated as described in Section 3.1. and 3.2. The analysis results obtained for the specific sample are shown in Figure 4.

From the analytical results obtained on the chromatogram (Figure 2C), we can conclude that the proposed and validated method is UPLC-MS/MS, which is a highly accurate and sensitive analytical technique. The method's limit of quantitation (LOQ) is 0.10 µg/kg, which means that it is capable of detecting very low levels of AFB1 in a sample of Babybio/Infant Cereals Vanilla/Babybio Céréales Vanille.

Table 3. The results of the evaluation of the analytical method through the parameters.

No	Parameters	Criteria [11]	Result	Evaluation
1	Linearity of the standard curve	$R^2 \geq 0.99$	$R^2 \geq 0.9993$	Qualified
2	Repeatability (C ≤ 1µg/kg)	RSDr ≤ 30%	6.72 - 9.00	Qualified
3	Reproducibility (C ≤ 1µg/kg)	$RSD_R \leq 45\%$	7.89 - 10.4	Qualified
4	Recovery efficiency	40 -120%	73.6 - 110	Qualified
5	Limit of detection (LOD), µg/kg	0.03	0.03	Qualified
6	Limit of quantitation (LOQ), µg/kg	0.10	0.10	Qualified
7	Uncertainty of measurement (%)	-	± 17.1	-

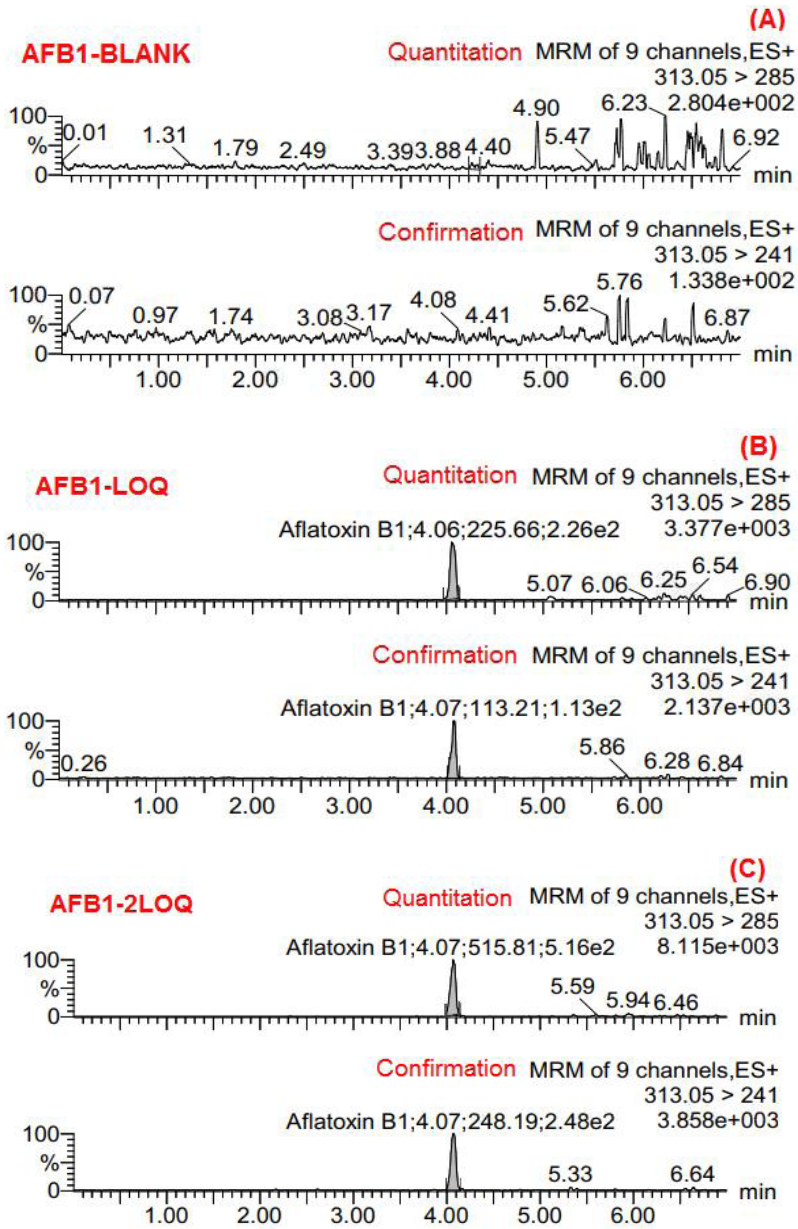

Figure 2. UPLC-MS/MS chromatograms of aflatoxins: (A) standard AFB1 blank sample (B) concentration control sample (< 0.1 g/kg) (C) supplemental food sample powdered organic milk powder with added vanilla flavor vitamin B1 Babybio/Infant Cereals Vanilla/Babybio Céréales Vanille.

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3.4 Quality assurance of test results by interlaboratory comparison

In order to verify the reliability of the analysis process, it is recommended to send an interlaboratory comparison sample to the Ho Chi Minh City Center for Laboratory Analysis Service (CASE), under the Department of Science and Technology in Ho Chi Minh City. The following steps should be followed: i) Take a sample of Babybio cereal powder that has not been analyzed for AFB1, spike the AFB1 concentration at 0.2 µg/kg and divide into three equal parts of 10g each in a centrifuge tube. ii) One tube sends to CASE, 1 tube to be made at the laboratory, 1 tube to store samples (Figure 3).

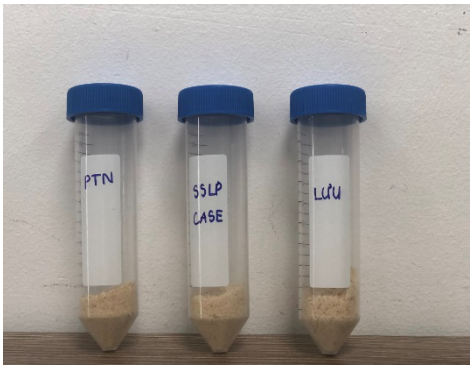

Figure 3. Samples of cereal powder after adding standards for quality assurance of test results.

Table 4. Results of analysis of AFB1 content in control laboratories.

Place of testing	Methods	AFB1 (µg/kg)	Recovery efficiency (%)	RSD_R (%)
In this work	AOAC 2005.08*	0.19	95%	2.1%
CASE	CASE.SK.0018**	0.22	110%	2.1%

* Validated method
** The method published by "Khue Nam" analytical center

Based on the analysis results presented in Table 4, it can be inferred that the method used for interlaboratory comparison was found to be satisfactory with a reproducibility of 2.1% and a recovery efficiency of 95 - 110%. Therefore, the method described in this work has been validated.

4. CONCLUSIONS

The analytical procedure for determining the content of Aflatoxin B1 by UPLC-MS/MS method has been developed in samples of nutritious cereal powder for children. The method of determining the content of Aflatoxin B1 by UPLC-MS/MS method has been validated in samples of nutritious cereal powder for children.

This method has the advantage of a simple sample processing technique, and the results of the verification of the analytical parameters are in line with the standards allowed by AOAC. Parameters such as selectivity, calibration curve, residual solvent, repeatability, reproducibility, recovery efficiency, measurement uncertainty, sample stability on UPLC-MS/MS with collection efficiency recovery ranged from 73.6 to 110% with a relative standard deviation of less than 15%, detection limit of 0.03 µg/kg and limit of quantification 0.1 µg/kg meeting the strict regulation of AFB1 in powders Weaning food for children from 6 months to 36 months according to QCVN 8:1:2011/BYT.

We have applied this developed analysis and appraisal process to analyze AFB1 in a sample of baby food powder. The product is a supplement that contains organic vanilla-flavored milk mixed with vitamin B1, meant for infants and is called Babybio/Infant Cereals Vanilla/Babybio Céréales Vanille. The result of the analysis is that AFB1 was not detected in the sample.

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