An Innovative IAC-HPLC-FLD Internal Standard Method Based on AFB1 Structural Modifications

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Abstract. An innovative immunoaffinity column-based high-performance liquid chromatography (IAC-HPLC) approach was established, incorporating the synthesized structural analog AFB1-OH as an internal standard for the quantitative analysis of aflatoxin B1 (AFB1). AFB1-OH was synthesized via oximation and employed to quantify AFB1 levels in grain samples collected from different regions. Grain samples were extracted with a methanol-ultrapure water mixture (7:3, v/v), purified using IAC, and analyzed via HPLC with internal standard quantification. The method demonstrated outstanding linearity across a concentration range of 0.0625–50 ng/mL, achieving a correlation coefficient (R^2) greater than 0.9998. The detection limit (LOD) and quantification limit (LOQ) were established at 0.55 ng/mL and 1.29 ng/mL, respectively. Recovery experiments indicated that the average recovery rates of AFB1 ranged between 95.09% and 106.54%, with variation coefficients (CVs) maintained below 5.76%. These findings highlight that AFB1-OH exhibits excellent stability and cost efficiency, while the developed method offers simplicity, high sensitivity, accuracy, and precision. This robust approach provides a reliable solution for determining AFB1 in diverse grain samples.

Keywords: AFB1; Immunoaffinity Column; High-Performance Liquid Chromatography (HPLC); Internal Standard Method

1 Introduction

Aflatoxins (AFs), produced by fungi like *Aspergillus flavus*, are potent carcinogens, with aflatoxin B1 (AFB1) categorized by IARC as a Group 1 human carcinogen[1-6]. AFB1 contamination, particularly in tropical regions, poses health and economic risks, prompting stringent limits like the EU's 0.1 μg/kg for infant foods [7, 8].

AFB1 detection methods, such as ELISA, TLC, and HPLC, are widely used, with HPLC being the most sensitive. However, complex food matrices impact accuracy, which can be addressed using synthetic internal standards like AFB1-OH to improve precision [9].

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2 Materials and Methods

2.1 Chemicals and Reagents

Standard aflatoxin solutions (10 μg/mL AFB1 and AFB2, purity ≥98%) and solid aflatoxins (5 mg AFB1, 1 mg AFB2) were obtained from Pribolab (Qingdao, China). IACs were supplied by Shenzhen Bioeasy Biotechnology Co., Ltd. Methanol and acetonitrile of chromatographic grade were obtained from ScharLab (Barcelona, Spain), while HPLC-grade water was produced using a MicroPure UV/UF purification system (Thermo Electron LED, Germany). Other reagents, including 50% hydroxylamine solution, pyridine, sodium chloride, and HRP goat anti-mouse IgG, were from Aladdin (Shanghai, China). Grain samples (corn, rice, barley) were collected from local supermarkets and online sources (Taobao) across three regions, with a minimum of 1.0 kg per sample.

2.2 HPLC-FLD System

HPLC analysis was performed using an Agilent 1260 Infinity II system equipped with a Pribolab C18 column (5 μm, 4.6 × 250 mm) maintained at 40°C. The mobile phase consisted of 55% ultrapure water (solvent A) and 45% methanol-acetonitrile mixture (70:30, solvent B) at a flow rate of 1 mL/min, with an injection volume of 10 μL. Fluorescence detection parameters were set at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The column was equilibrated with the mobile phase prior to analysis.

2.3 Synthesis of the Internal Standard AFB1-OH

AFB1-OH was synthesized by reacting 5 mg AFB1 with 1.2 mL hydroxylamine in 500 μL pyridine at 27°C for 8 hours. After freeze-drying to remove pyridine, the residue was washed with anhydrous THF and water, centrifuged, and vacuum-dried to obtain AFB1-OH as an internal standard. The synthesis process is shown in Figure 1.

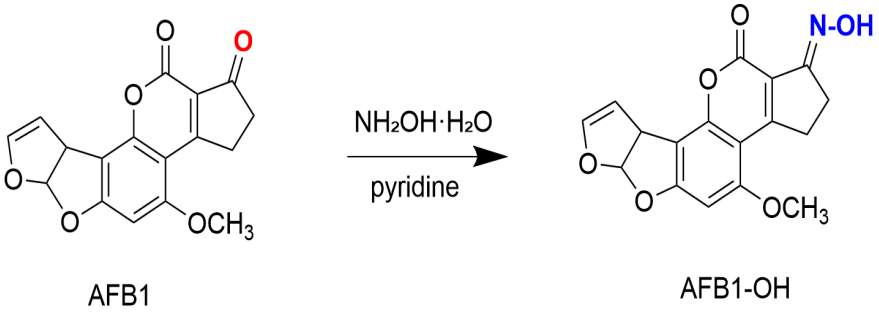

Fig. 1. Schematic diagram of the synthesis of the structural analog AFB1-OH

2.4 Characterization of Internal Standard AFB1-OH

AFB1-OH was characterized by UV-Vis and fluorescence spectrophotometry. UV-Vis spectra (200–800 nm in methanol) were recorded using an Agilent Cary 60 spectrophotometer, while fluorescence spectra (excitation at 360 nm, emission 400–600 nm) were obtained with a Hitachi F-2700 spectrophotometer. HPLC-MS/MS analysis (Agilent 6550 QTOF, Thermo Orbitrap Q-Exactive) under negative ionization confirmed AFB1-OH's identity through its fragmentation pattern.

2.5 Sample Preparation and Purification

Grain samples were categorized by type and region, ground to <2 mm, and homogenized (100 g per sample stored). Following standard protocols, each 5 g sample was combined with 100 μL of 150 ng/mL AFB2-OH (internal standard) solution and subjected to extraction using 20 mL of methanol-water (70:30, v/v) on a shaker for 20 minutes. The mixture was centrifuged at 6000 rpm for 10 minutes, and the resulting supernatant was passed through a 0.22 μm membrane filter before purification with AFB1 IAC columns, operated at a controlled flow rate of one drop per second. The columns were rinsed with ultrapure water, and the target analytes were recovered using 2 mL methanol, dried under nitrogen at 55°C, and dissolved in the mobile phase for HPLC analysis.

2.6 Standard Curves and Sensitivity Determination

Standard solutions of AFB1 (0.25–50 ng/mL) and AFB1-OH (150 ng/mL) were utilized to construct external and internal standard calibration curves by correlating the AFB1 peak area with its concentration and the ratio of AFB1 to AFB1-OH peak areas, respectively. Linear regression equations and correlation coefficients (R^2) were calculated. Detection (LOD) and quantification (LOQ) limits were established based on signal-to-noise ratios ($S/N \geq 3$ for LOD, $S/N \geq 10$ for LOQ) through triplicate analyses.

2.7 Spike Recovery Experiment

Grain samples (corn, rice, barley) were spiked at low (0.5 ng), medium (10 ng), and high (50 ng) AFB1 concentrations. Each spiked sample included 15 ng of AFB1-OH as the internal standard. After extraction and purification, the samples were analyzed by HPLC. Recovery rates and coefficients of variation (CVs) were calculated to assess the method's accuracy and precision.

2.8 Detection of AFB1 in Grain Samples

AFB1 levels in grain samples collected from various locations were quantified using the IAC-HPLC method with AFB1-OH as the internal standard. Following IAC purifi-

cation, the samples underwent HPLC analysis. The AFB1 concentration was determined from the ratio of AFB1 to AFB1-OH peak areas and validated against the external standard calibration curve.

3 Results

3.1 Spectral and Mass Spectrometric Characterization of AFB1-OH

Fluorescence detection (Figure 2a, 2b) revealed comparable optical properties between AFB1 and AFB1-OH, showing excitation at 365 nm and emission at 430 nm. UV-Vis spectra (Figure 2c) revealed a prominent absorption peak at 365 nm, consistent with their dihydrofuran coumarin structure. Mass spectrometry (Figure 2c) validated AFB1-OH's identity through the detection of a deprotonated molecular ion at m/z 326.0672, matching the predicted structure. These shared spectral properties enable simultaneous detection of AFB1 and AFB1-OH using HPLC-FLD for unified quantitative analysis.

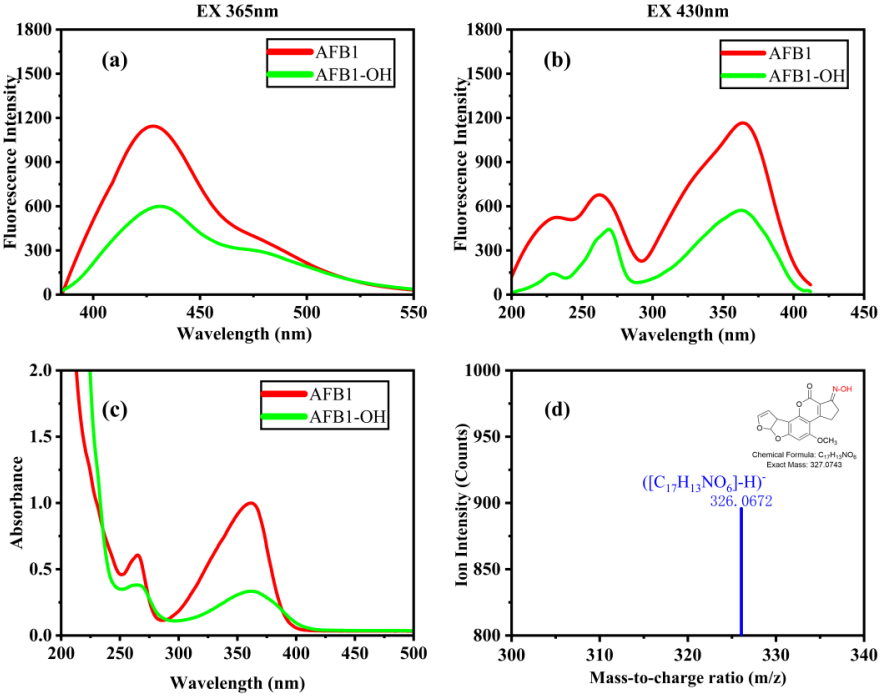

Fig. 2. Spectral and Mass Spectrometric Characterization of AFB1 and AFB1-OH

3.2 Calibration Curve, Limit of Detection, and Limit of Quantification

The calibration curves for AFB1 exhibited excellent linearity, covering a concentration range of 0.25–50 ng/mL for the external standard ($y = 2.1872x + 0.1692$, $R^2 = 0.9999$) and for the internal standard ($y = 0.3386x + 0.0531$, $R^2 = 0.9998$), indicating a robust

correlation between AFB1 concentration and peak area. The method achieved a detection limit (LOD) of 0.55 ng/mL and a quantification limit (LOQ) of 1.29 ng/mL, highlighting its superior sensitivity and precision.

3.3 Spiking Recovery Results

AFB1 standard solutions (0.5, 10, and 50 ng) were spiked into corn, rice, and barley samples with AFB1-OH as the internal standard. Recovery rates ranged from 99.24% to 112.05%, with RSDs between 0.54% and 5.76%, confirming excellent accuracy and precision (Table 1).

Table 1. Detection Results and Statistical Analysis of AFB1 Spiked in Different Samples (n=6)

AFB1 Spiking Amount(ng)	Sample Type	Internal Standard Method Average Recovery (%)	ISM RSD %	External Standard Method Average Recovery (%)	ESM RSD %
0.5	Corn	112.05	5.76	89.07	7.27
	Rice	102.72	3.24	86.59	3.08
	Barley	101.19	0.84	85.01	2.60
10	Corn	99.66	2.63	81.98	1.27
	Rice	100.78	0.74	84.04	1.46
	Barley	99.53	0.83	84.72	1.52
50	Corn	101.39	0.72	94.10	0.68
	Rice	99.24	0.62	86.42	0.67
	Barley	99.96	0.69	83.98	0.47

3.4 Detection of AFB1 in Grain Samples

Grain samples from different regions were analyzed using internal and external standard methods. In Kunming corn, AFB1 was detected at 4.23 ng (RSD = 0.31%) by the internal standard method and 3.95 ng (RSD = 1.99%) by the external standard method. Samples from Fanshi, Shanxi, and Harbin, Heilongjiang, had AFB1 concentrations below the detection limit (Table 2), indicating regional contamination variation.

Table 2. Detection Results of Grain Samples from Different Origins (n=6)

Sample Type	Origin	Average Detected Amount by ISM (ng)	ISM RSD %	Average Detected Amount by ESM (ng)	ESM RSD %
Corn	Yunnan	4.23	0.31	3.95	1.99
	Shanxi	/	/	/	/
	Heilongjiang	/	/	/	/
Barley	Henan	/	/	/	/
	Inner Mongolia	/	/	/	/

	Henan	/	/	/	/
	Xinjiang	/	/	/	/
Rice	Henan	/	/	/	/
	Shanxi	/	/	/	/

(Note: "/" indicates below the detection limit)

4 Discussion

This study developed an IAC-HPLC method with AFB1-OH as a synthetic internal standard for detecting AFB1 in grain samples, demonstrating high sensitivity, accuracy, and precision.

4.1 Comparison of Internal Standard Method and External Standard Method

Table 1 shows that the internal standard method (ISM) achieved higher recovery rates and lower RSD values than the external standard method (ESM), particularly at low concentrations. For example, AFB1 in Kunming corn was 4.23 ng/g (RSD = 0.31%) by ISM, compared to 3.95 ng/g (RSD = 1.99%) by ESM, highlighting ISM's superior reliability and consistency.

4.2 Comparison with Other Methods

Compared to HPLC-MS/MS [10], which involves complex solid-phase extraction, the IAC-HPLC method is simpler, reducing pretreatment time and costs while maintaining high accuracy. Its LOD (0.55 ng/kg) and LOQ (1.29 ng/kg) outperform standard HPLC [11]. The synthetic internal standard is also more cost-effective than isotopic standards, enhancing practicality [12].

5 Conclusion

This study developed a cost-effective IAC-HPLC method using AFB1-OH as a synthetic internal standard, achieving high accuracy and precision for AFB1 detection in grain samples. The method showed excellent linearity ($r > 0.999$) over 0.25–50 ng/mL, with an LOD of 0.55 ng/kg and LOQ of 1.29 ng/kg. Recovery rates ranged from 95.09% to 106.54% (RSD < 5.76%). It simplifies complex matrix analysis without requiring MS/MS, offering a reliable and economical solution for food contaminant detection.

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