Determination of Aflatoxins (B1, B2, G1 and G2) in Corn and Peanut Butter by HPLC-FLD Using Pre-column Immunoaffinity Cleanup and Post-Column Electrochemical Derivatization

Application Note

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Abstract

A method for simultaneous determination of four aflatoxins B1, B2, G1 and G2 in corn and peanut butter using HPLC-FLD was developed in the work. After extracted by methanol/water (60/40, v/v), the sample was cleaned up and concentrated on an immunoaffinity column and then the sample solution was separated and quantified by HPLC-FLD with KOBRA cell for derivatization. Each aflatoxin got linear range from 0.1 ~ 10 µg/L with the square of correlation coefficient (R^2) > 0.99998. The limit of detection (LOD, S/N = 3) was in the range of 0.004 ~ 0.007 µg/L (equivalent to 0.008 ~ 0.014 µg/kg content in samples). The precision was validated by six sequential injections of 10 µg/L standard solution, the relative standard deviations (RSDs) obtained of retention time and peak area were less than 0.1% and 0.3% for each aflatoxin, respectively. It was proved that the proposed method could be utilized to determine aflatoxins in corn and peanut butter effectively and accurately.

Introduction

Aflatoxins are produced as metabolites by the Aspergillus Flavus and Aspergillus Parasiticus and exist in nature world widely. The common aflatoxins are B1, B2, G1 and G2. Among these mycotoxins the aflatoxin B1 is of most toxicity followed by G1, the toxicities of B2 and G2 are relative weak. Due to the high toxicity and carcinogenicity to human and animals, many countries and regulatory agencies impose strict limits on aflatoxins. The European Commission has set maximum levels for aflatoxin B1 between 2.0 and 8.0 µg/kg and for the sum total of all four aflatoxins (B1, B2, G1 and G2) between 4.0 and 15.0 µg/kg in crops such as nuts, grains and dried fruits [1]. The action level was set of 20 and 300 µg/kg for human food and animal feed by U.S. FDA, respectively [2]. Therefore, it is important to develop an accurate and effective method for aflatoxins determination.

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HPLC with fluorescence detection was utilized for aflatoxins determination in many matrices increasingly. HPLC analysis was frequently required with immunoaffinity clean up for sample preparation and derivatization for high sensitivity detection due to the influence of matrix components and the trace level of target compounds. In this work, a fast HPLC-FLD method with immunoaffinity clean up and post-column electrochemical bromo-derivatization was developed considering combined influences of post-column band broadening, analysis speed, resolution and sensitivity and applied to determine contaminated corn and peanut butter successfully.

Experimental

Instrument and Equipment

Agilent 1260 Infinity system, consisting of solvent rack, quaternary pump (with built-in degasser), standard autosampler, column compartment and fluorescence detector, was used for separation and quantification. R-Biopharm electrochemical derivatization kit, including KOBRA® cell, variable control current source, 0.5 mm i.d. peek tubing (at least 34 cm long) and so on, was employed for derivatization.

HPLC Conditions

Column:	Zorbax Eclipse Plus C18, 4.6 x 150 mm x 5 µm
Column Temp.:	40 °C
Mobile Phase A:	1L water containing 238 mg KBr and 700 µL 4M HNO_3
Mobile phase B:	MeOH
Isocratic:	A：B = 50 : 50, 12min
Flow rate:	1.0 mL/min
Detection:	Ex: 362 nm, Em: 455 nm, gain = 15
Injection:	20 µL
Electrochemical Current:	100 µA setting
Reaction coil:	0.5 mm i.d.*34 cm long peek tubing (from the exit of KOBRA cell to the entrance of FLD)

Sample Preparation

Weigh 25g of sample and 2g of sodium chloride into high-speed blender jar. Add 125ml of HPLC Grade methanol:distilled water (60:40, v:v) into the jar, cover and blend for 1 minute at high speed. Dilute the extract with 125ml of distilled water. Mix well by swirling followed by filtering approximately 40-50 ml of sample extract through Whatman No. 4 filter paper immediately. Transfer 10ml of the filter (equivalent to 1g of sample) into the glass syring barrel for passage through the prepared immunoaffinity column (ref. to AFLAPREP® column preparation manual)[3] at a flow rate of 2-3 ml/min. Then add 10ml of distilled water to the glass syring for washing the column. Expel the residual water from the column and transfer accurate 1ml of HPLC-grade methanol to elute aflatoxins from the column. It could be back-flushed with the methanol solution for completely release of aflatoxins from the monoclonal antibody if necessary. Collect all of the methanol elution and dilute with 1 ml of distilled water before injection into HPLC system.

Method Linear Range, Limit of Detection and Precision

Prepare a series of mixture solutions with the concentration of each aflatoxin at 0.1、0.25、0.5、1.0、5.0、10.0 µg/L with $MeOH/H_2O$=50/50 as dilution. Each solution was injected as HPLC conditions described above and the peak area of each aflatoxin obtained was plotted against concentration (See Figure 1). The linearity was maintained over the concentration range of 0.1 ~ 10.0 µg/L for each aflatoxin with the square of correlation coefficient (R^2) > 0.99998. The limit of detection (LOD), defined as signal-to-noise (S/N) = 3, was 0.007、0.004、0.006 and 0.005 µg/L for B1、B2、G1 and G2, respectively. Method precision was validated with relative standard deviation (R.S.D.), which was <0.3% of peak area and <0.1% of retention time for each aflatoxin by six sequential injections of 10 µg/L standard solutions. The overlay of six injections of 10 µg/L standard solutions and the chromatogram of 0.1 µg/L standard solution were shown in Figure 2. The LODs of this work and frequently-used Chinese criterions were compared in Table 1. It was evident that the method proposed in the work could provide lower LOD than that of current Chinese SN and GB (/T)s.

Table 1. Comparison the LODs in this work with current Chinese SN and GB(/T)s

Method in	Aflatoxins	Method Abstraction	LOD (μg/kg)
This work	B1、B2、G1、G2	Post-column electrochemical bromo-derivatization HPLC-FLD	0.008 ~ 0.014
SN 0277-93	B1、B2、G1、G2	HPLC-FLD	0.12 ~ 0.50
GB/T 5009.22-2003	B1	Thin layer chromatography (the primary method); ELISA (the secondary method)	5.0; 0.01
GB/T 18979-2003	B1、B2、G1、G2	Post-column derivatization (I2)-HPLC-FLD	1 (total sum)
GB/T 5009.23-2006	B1、B2、G1、G2	Pre-column derivatization (TFA)-HPLC-FLD (the third method)	0.05 ~ 0.5
GB/T 23212-2008	B1、B2、G1、G2、M1、M2	Post-column derivatization (PBPB)-HPLC-FLD	0.001 ~ 0.003*
GB 5009.24-2010	B1、M1	Thin layer chromatography	0.1 ~ 0.5

*：Injection volume was 100 μL.

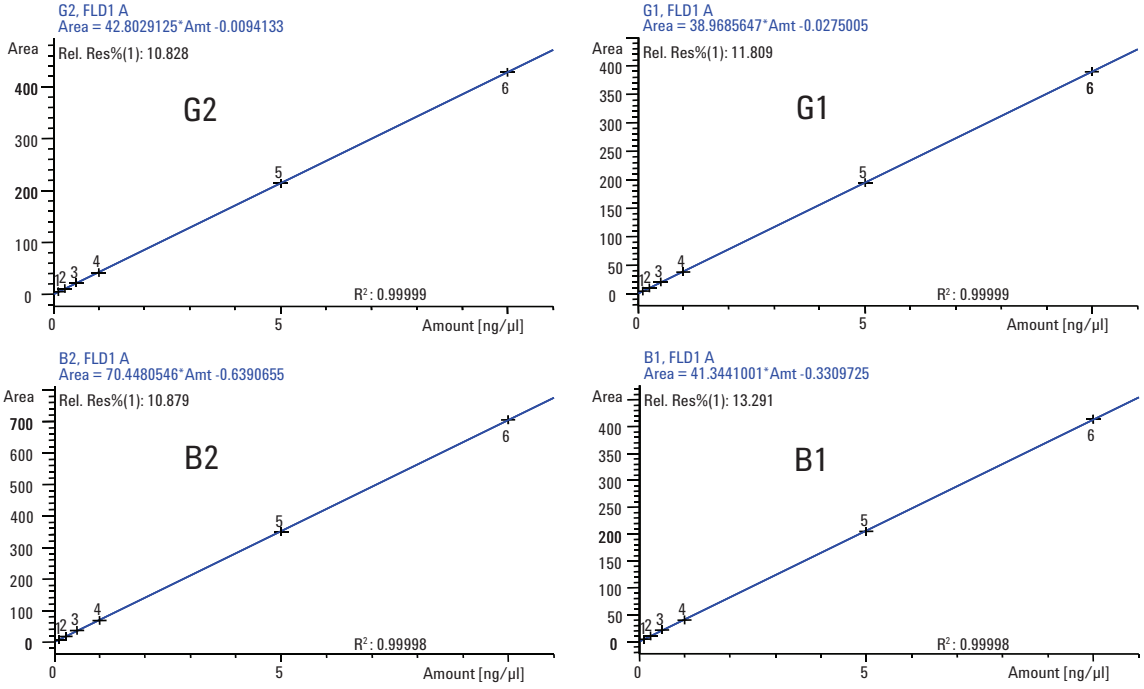

Figure 1. The linear fitted curve and the relative coefficient of each aflatoxin

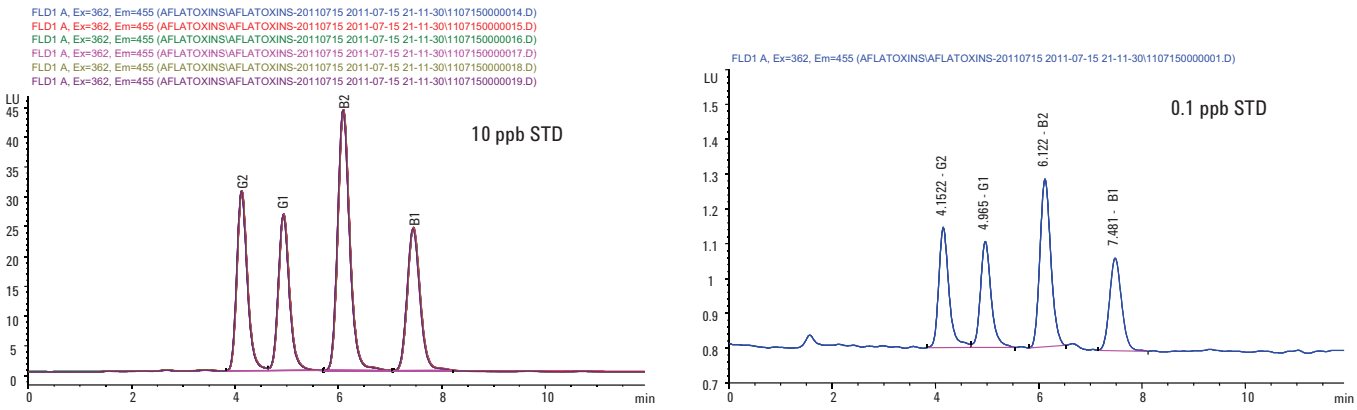

Figure 2. The overlay of six sequential injections of 10 µg/L standard solution (Left) and the chromatogram of 0.1 µg/L standard solution (Right)

Sample Analysis

Aflatoxins in Corn and peanut butter were determined by the method after using described sample preparation procedure for cleanup. Chromatograms were shown in Figure 3.

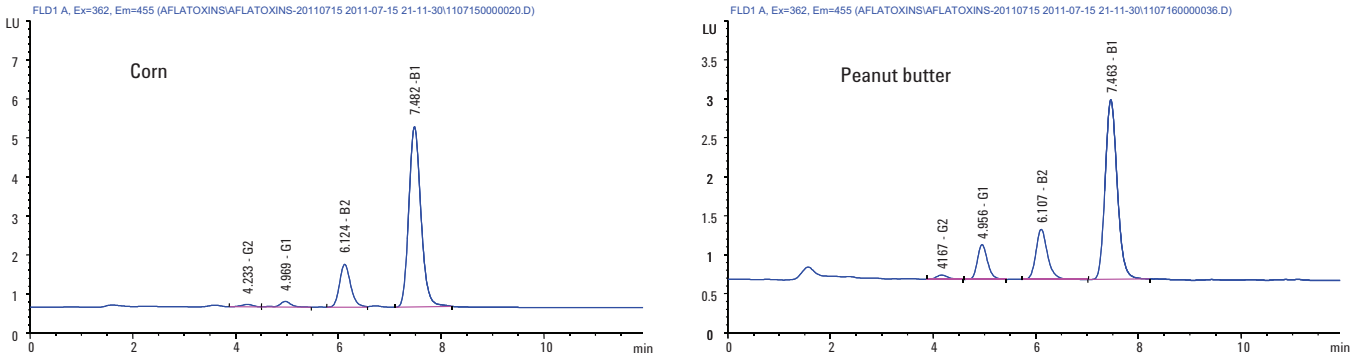

Figure 3. Chromatograms of corn (left) and peanut butter (right)

Conclusion

The limit of detections of aflatoxin G1 and B1 were improved by post-column electrochemical bromo-derivatization and fluorescence detector. The analysis was shortened into 8 minutes without compromise of resolution of aflatoxins (in order to completely elute all the components in sample the analysis time was set for 12 minutes). Meanwhile, it was shown from the chromatograms for real samples that the cleanup of sample was effective by pre-column immunoaffinity column. It was proved that the method in the work could be applied for determination of aflatoxins at trace level in real samples, and method is fast and accurate.

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