Application Note

Analysis of Total Aflatoxins in Food by HPLC and UHPLC

Introduction

Aflatoxins are a group of mycotoxins produced by microorganisms such as Aspergillus flavus, Aspergillus parasiticus and Aspergillus nomius living in tropical or subtropical regions and have a strong carcinogenic affect. It has been reported that higher levels of aflatoxins are often found which exceed the safe levels laid down by food safety agencies such as the FDA in wide variety of food products including fruits, grains and spices.

Present regulations require that total aflatoxins (sum of aflatoxin B1, B2, G1 and G2) must be lower than 10 µg/kg. A precolumn derivatization method that uses trifluoroacetic acid (TFA) in combination with HPLC with fluorescence detection offers greatly improved sensitivity for aflatoxins B1 and G1. In addition to the derivatization method, the utilization of multi-functional columns or immunoaffinity columns can be used to improve the reproducibility and recovery in the sample preparation procedure.

Jasco HPLC system

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Experimental

Conventional HPLC

Equipment	
Pump:	PU-2089
Autosampler:	AS-2057
Column Oven:	CO-2060
Detector:	FP-2020

Conditions	
Column:	YMC-Triart C18 (4.6 mm I.D. x 150 mmL, 5 !lm)
Eluent A:	Water/Methanol/Acetonitrile (60/30/10)
Eluent B:	Acetonitrile
Gradient Condition:	(A/B), 0.00 min (100/0) 15.00 min (100/0) 15.05 min (0/90) 20.00 min (0/90) 20.05 min (100/0) 1 cycle; 35.5 min
Flow Rate:	1.0 mL/min
Column Temperature:	40 °C
Wavelength:	Ex. 365 nm, Em. 450 nm, Gain x100
Injection Volume:	20 µL
Standard Sample:	Mixture of aflatoxin B1, B2, G1, and G2 (0.5~10 µlg/L each)

UHPLC

Equipment	
Pump:	X-LC 3185PU x 2
Degasser:	X-LC 3080DG
Mixer:	X-LC 3180MX
Autosampler:	X-LC 3159AS
Column Oven:	X-LC 3067CO
Detector:	X-LC 3120FP

Conditions	
Column:	X-PressPak V-C18-WL (3.0 mm I.D. x 75 mmL, 2 !lm)
Eluent A:	Water/Methanol/Acetonitrile (65/18/17)
Eluent B:	Acetonitrile
Gradient Condition:	(A/B), 0.00 min (100/0) 3.50 min (100/0) 3.55 min (0/90) 4.50 min (0/90) 4.55 min (100/0) 1cycle; 7.5 min
Flow Rate:	0.8 mL/min
Column Temperature:	40 °C
Wavelength:	Ex. 365 nm, Em. 450 nm, Gain x100
Injection Volume:	5 µL
Standard Sample:	Mixture of aflatoxin B1, B2, G1, and G2 (0.5~10 µlg/L each)

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Structure

Aflatoxin B1, B2, G1 and G2 are compounds that yield native fluorescence. However, the fluorescence intensity of B1 and G1 is much less in comparison to that of B2 and G2 and accordingly, the sensitivity of B1 and G1 must be improved by changing from the natural form into a hydroxidized form using TFA derivatization. The structure of aflatoxin B1, B2, G1 and G2 and the structure of derivatized B1 and G1 are shown in figure. 1.

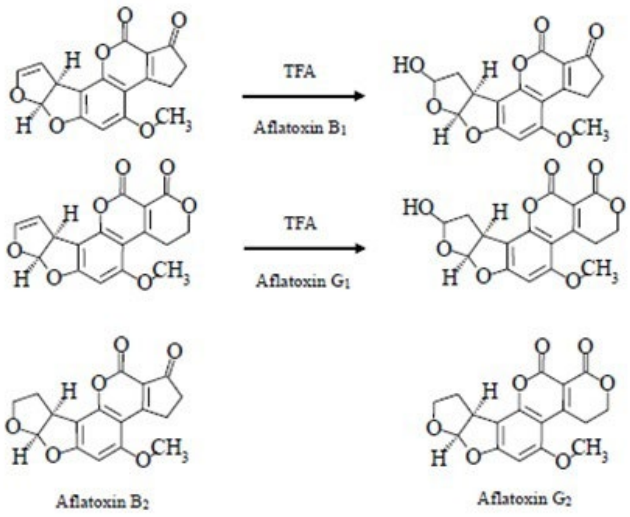

Figure 1. Structure of aflatoxin B1, B2, G1, G2 and TFA-derivatized B1 and G1

Derivatization

The procedures for TFA derivatization of the standard mixture of aflatoxins and actual sample are shown in Figure. 2.

Figure. 2. TFA derivatization

1.0 mL of standard mixture of aflatoxins (each of 0.5, 1.0, 5.0 and 10 µg/L) or actual sample solution, purified

↓ 0.1 mL TFA

Sample enrichment by solvent elimination using dry nitrogen gas at less than 45 °C.

↓ 0.9 mL Milli-Q water/acetonitrile (90/10)

Plug the vessel and mix thoroughly. Stand in a dark place at room temperature for 15 min. *1

↓

After mixing, inject an aliquot of the sample into the HPLC (Conventional HPLC: 20 µL, UHPLC: 5 µL)

*1 In order to avoid the adsorption of aflatoxins onto the inner wall of vessel, the vessel must be rinsed with acetonitrile and Milli-Q water, then dried.

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Sample Preparation

Corn grits and roasted peanuts were selected as test samples, and a multi-functional column was used for the sample preparation of corn grits, while an immune-affinity column was used for the preparation of roasted peanuts. The sample preparation procedures are shown in Fig. 3.

1. Corn Grits

Extraction

10.0 g of corn grits

↓ 40 mL acetonitrile/Milli-Q water (90/10)*1

Mix/shake for 30 minutes

↓

Centrifuge (4000 rpm, 10 min)

↓

The extract will be in the supernatant.

Purification

6 mL of supernatant

↓

Apply the supernatant to a multi-functional column (MycoSep 226 AflaZon +*2)

↓

Fractionate the first 2 mL of eluent as a purified solution with the flow rate at 1 mL/min.

↓

Apply purified solution to the TFA derivatizaton

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2. Roasted Peanuts

Extraction

20.0 g of roasted peanuts ground and homogenized

↓ 40 mL acetonitrile/Milli-Q water (90/10)*1

Mix/shake for 30 minutes

↓

Put 10 mL of supernatant into a 50 mL measuring flask and make up the volume with Milli-Q water to 50 mL

↓

Filter the solution using glass wool

↓

The extract will be in the eluent

Purification

Drain the preservation solvent from immune-affinity column (AFLAKING*3)*4 .

↓

Flush the column with 3 mL of PBS.*5,*6

↓

Apply 10 mL of eluent onto the column at flow rate of 1 drop/sec.*6

↓

Flush the column with 3 mL PBS, twice.*6

↓

Flush the column with 3 mL Milli-Q water, twice.*6

↓

Inject air into the column to completely drain the water out of column.*7

↓

Apply 1mL of acetonitrile onto the column by dripping and collect the eluent.

↓

Leave the column for 5 minutes.*8

↓

Apply 2 mL of acetonitrile onto the column by dripping and, collect the eluent

↓

Inject air into column to drain the acetonitrile out of column and collect a total of 3 mL acetonitrile solution as the purified solution.

↓

Apply the purified solution to the TFA derivatizaton (Fig.2.)

*1 In the recovery estimation test, the standard mixture of aflatoxins (each of 0.5 11g/L) in accetonitrile/Milli-Q water (90/10) will be used.

*2 Three separation modes (reverse phase, normal phase and ion-exchange) are available in the multi-functional column.(RomerLabs)

*3 Immunoaffinity column (HORIBA) utilizing unique binding capability between anti-aflatoxin monoclonal anti-body and aflatoxins

*4 Let the column equilibrate to room temperature. Punch a hole on the upper cap carefully so that that the air would not pass into the gel and then take the upper and the lower cap.

*5 Make up the Milli-Q water solution with 0.20 g potassium chloride,0.20 g of potassium dihydrogen phosphate, 1.16 g of disodium hydrogen phosphate and 8.0 g of sodium chloride (pH 7.4) to 1L.

*6 Flush the column carefully so that the air will not pass into the gel.

*7 Minimize the water content of the final eluent in the acetonitrile. If the water content is relatively high in the final elution, it will take a longer for solvent elimination, which may cause denaturation of aflatoxins.

*8 To release the interacted aflatoxins from gel completely.

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Results

The chromatograms of standard mixtures of TFA-derivatized aflatoxins (each of 5.0µg/L) are shown in the figure 4 [by conventional HPLC (upper), by UHPLC (lower)]. The separation is completed within 12 minutes by conventional HPLC and within the 3.5 minutes by the UHPLC.

The linearity of the standard mixtures of aflatoxins is in the range 0.5 to 10µg/L with excellent correlation achieved r >= 0.9997 for both conventional HPLC and UHPLC.

Good reproducibility including the TFA derivatization procedure (N = 6) was obtained for the conventional HPLC of better than 0.2% RSD and 3% RSD in peak retention time and area, respectively. The UHPLC also yielded good reproducibility with better than 0.2% RSD and 3.5% RSD in peak retention time and area, respectively. The 1.0ug/L of standard mixture of aflatoxins was used for this estimation.

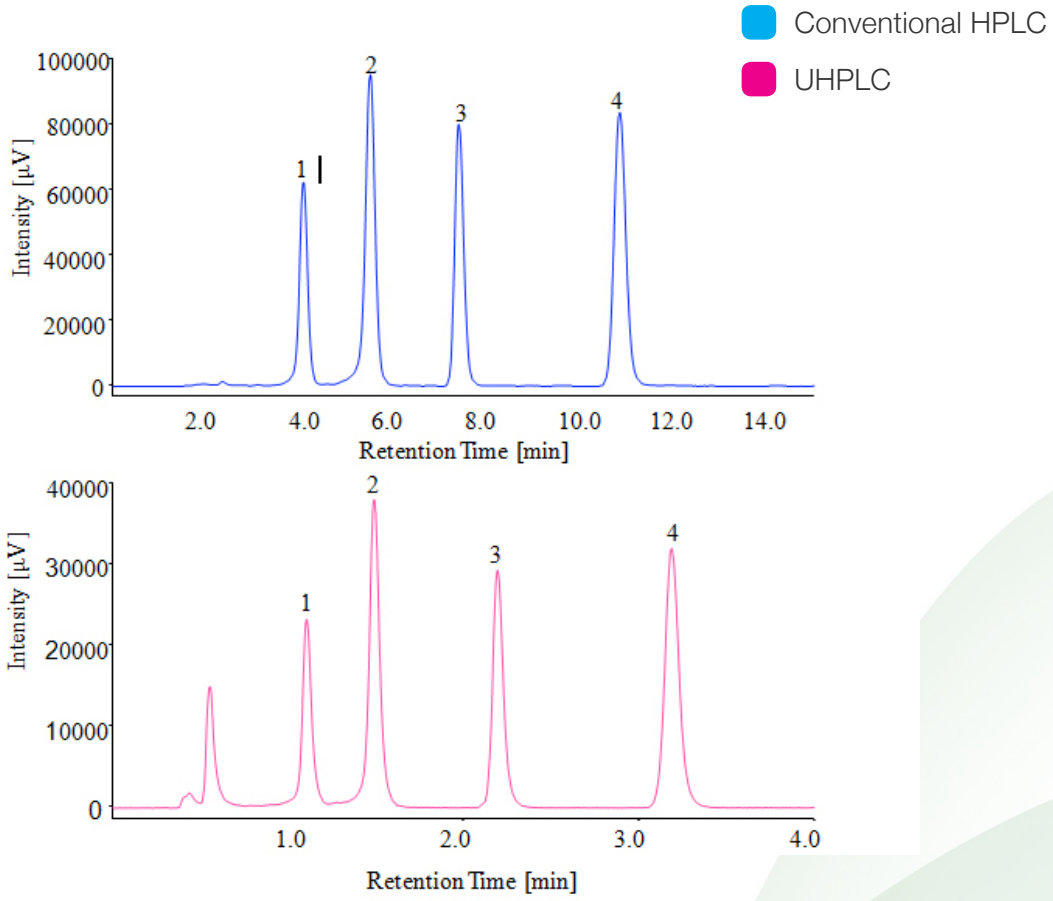

Figure. 4. Chromatograms of standard mixture of aflatoxins (5.0 µg/L each, TFA derivatized)
1=Aflatoxin G1, 2=Aflatoxin B1, 3=Aflatoxin G2, 4=Aflatoxin B2

Chromatograms of corn grits samples prepared using the multi-functional columns (MycoSep 226AflaZon+) in the sample preparation are shown in figure 5 (both the conventional HPLC and UHPLC). The samples from corn grits and those spiked with a standard mixture of aflatoxins were used for the recovery estimation of aflatoxins. Almost no contaminant peaks are observed in the chromatograms and good recovery of standard aflatoxins was obtained for both conventional HPLC and UHPLC as shown in Table 1.

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Chromatograms of the roasted peanuts sample prepared using the immunoaffinity column (AFLAKING) in sample preparation are shown in figure 6 (both the conventional HPLC and UHPLC). The samples from roasted peanuts and those spiked with the standard mixture of aflatoxins were used for the recovery estimation of aflatoxins. Almost no contaminant peaks are observed in the chromatograms and good recovery of standard aflatoxin was obtained for both conventional HPLC and UHPLC as shown in Table 2.

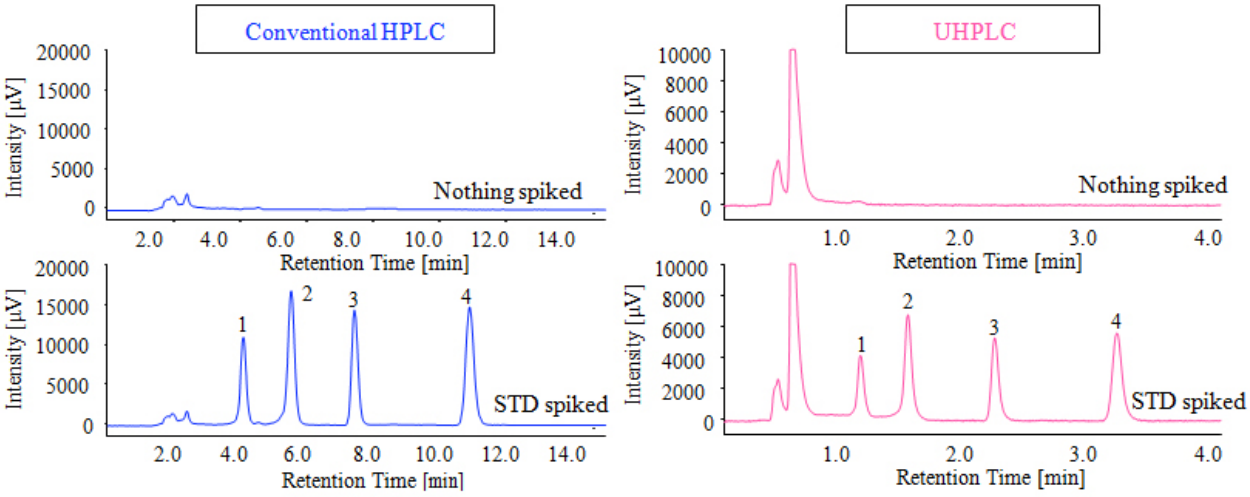

Figure 5. Chromatograms of purified solution from corn grits 1=Aflatoxin G1, 2=Aflatoxin B1, 3=Aflatoxin G2, 4=Aflatoxin B2

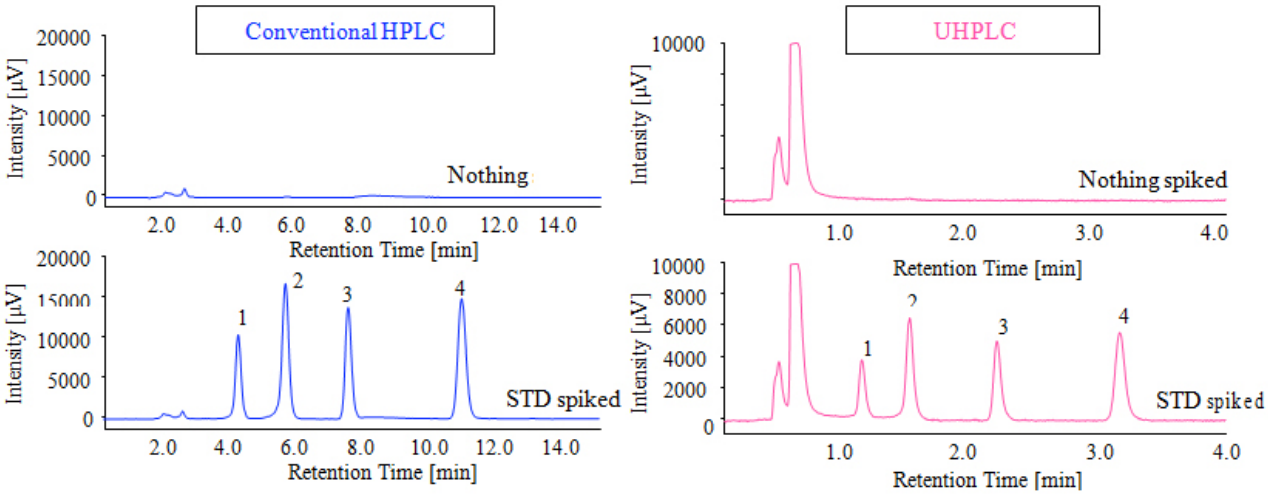

Figure 6. Chromatograms of purified solution from roasted peanuts 1=Aflatoxin G1, 2=Aflatoxin B1, 3=Aflatoxin G2, 4=Aflatoxin B2

Table 1. Recovery [%] of standard aflatoxins

	Corn Grits		Roasted Peanuts	
Aflatoxins	Conventional HPLC	UHPLC	Conventional HPLC	UHPLC
Aflatoxin G1	107	108	101	99
Aflatoxin B1	106	102	99	98
Aflatoxin G2	100	100	102	104
Aflatoxin B2	100	100	101	102

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