Application Note

630005HX

Analysis of Total Aflatoxins in Food by HPLC and UHPLC

Introduction

Aflatoxins are one of the mycotoxins which are produced from the microorganisms such as *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* living in tropical or subtropical regions and, they have a strong carcinogenic action. It has been reported that the higher level of aflatoxins than the regulated level in Food Sanitation Act has been detected mainly from imported grain, pulse and spice. Recently, it is also reported that the aflatoxins have been detected even from the Japanese domestic foods.

The Japanese Government has announced on the 31st of March, 2011 that the regulation in Food Sanitation Act related to the aflatoxins will be strengthened and, the new act will become mandatory from the 1st of October, 2011. In the previous regulation, the quantity of aflatoxin B_1 giving the strongest carcinogenic effect in the natural compounds available, must be regulated to be lower than 10 µg/kg. However, in the present regulation, it is strengthened so that the total aflatoxins (sum of aflatoxin B_1, B_2, G_1 and G_2) must be lower than 10 ug/kg. In the new procedure of the present regulation, the derivatization method by using of trifluoroacetic acid (TFA) in combination with HPLC including fluorescence detector has been recommended in order to improve the sensitivity of aflatoxin B_1 and G_1 .In addition to the derivatization method, the utilization of multi-functional columns or immunoaffinity colums has been recommended to improve the reproducibility and recovery in the sample preparation procedure.

In this application note, the new procedure has been applied to both conventional HPLC and UHPLC.

Keyword : total aflatoxins, TFA derivatization, fluorescence detector, UHPLC

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 µm)
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 B_1, B_2, G_1, and G_2 (0.5~10 µg/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 µm)
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 B_1, B_2, G_1, and G_2 (0.5~10 µg/L each)

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[Structure]

The aflatoxin B_1, B_2, G_1 and G_2 are the compounds yielding natural fluorescence. However, the fluorescence intensity of B_1 and G_1 is not enough in comparison to that of B_2 and G_2 and accordingly, the intensity of B_1 and G_1 needs to be improved by changing their natural form into hydroxidized form by TFA derivatization.

The structure of aflatoxin B_1, B_2, G_1 and G_2 and the structure of derivatized B_1 and G_1 are shown in Fig. 1.

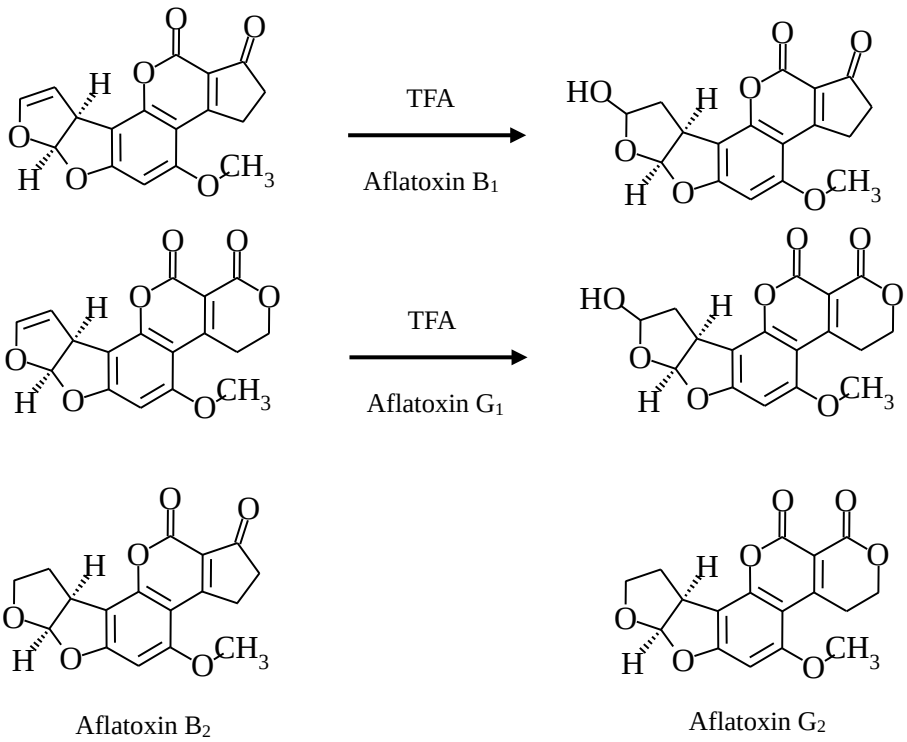

Fig. 1. Structure of aflatoxin B_1, B_2, G_1, G_2 and TFA-derivatized B_1 and G_1

[Derivatization]

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

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

↓

Enrich/eliminate solvent by nitrogen gas stream at less than 45 °C.

↓ ← 0.1 mL TFA

Plug the vessel, then shake it strongly. Leave it at dark place at room temperature for 15 min. *1

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

After mixing, inject the solution into 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 inside must be flushed by acetonitrile and Milli-Q water, then dried.

Fig. 2. TFA derivatization

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[Sample preparation]

Corn grits and roasted peanuts were selected as actual sample, and the multi-functional column was applied to the sample preparation of corn grits, while the immunoaffinity column was applied to the preparation of roasted peanuts. Each of sample preparation procedure is 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 onto muluti-functional column (MycoSep 226 AflaZon +*2)

↓

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

↓

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

*1 In the recovery estimation test, the standard mixture of aflatoxins (each of 0.5 µg/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.(Romer Labs)

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

*4 Let the temperature of column inside be equivalent to the room temperature. Make a hole on the upper cap carefully so that that the air would not come into the gel and then, take the upper and the lower cap.

*5 Adjust Milli-Q water solution including 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 be 1 L.

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

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

*8 To release the interacted aflatoxins from gel completely.

(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

↓

Centrifuge (4000 rpm, 10 min)

↓

Put the 10 mL of supernatant into the 50 mL of measuring flask and, fix the volume by Milli-Q to be 50 mL.

↓

Filtrate the solution by glass wool.

↓

The extract will be in the eluent.

<Purification>

Drain the preservation solvent from immunoaffinity column (AFLAKING*3)*4 .

↓

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

↓

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

↓

Inject air into column to drain the water out of column completely.*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 the total of 3 mL acetonitrile solution as the purified solution.

↓

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

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Results

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

Regarding the linearity of standard mixtures of aflatoxins in the range from 0.5 to 10ug/L each of aflatoxin, the excellent correlation coefficient over 0.9997 was obtained for both conventional HPLC and UHPLC.

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

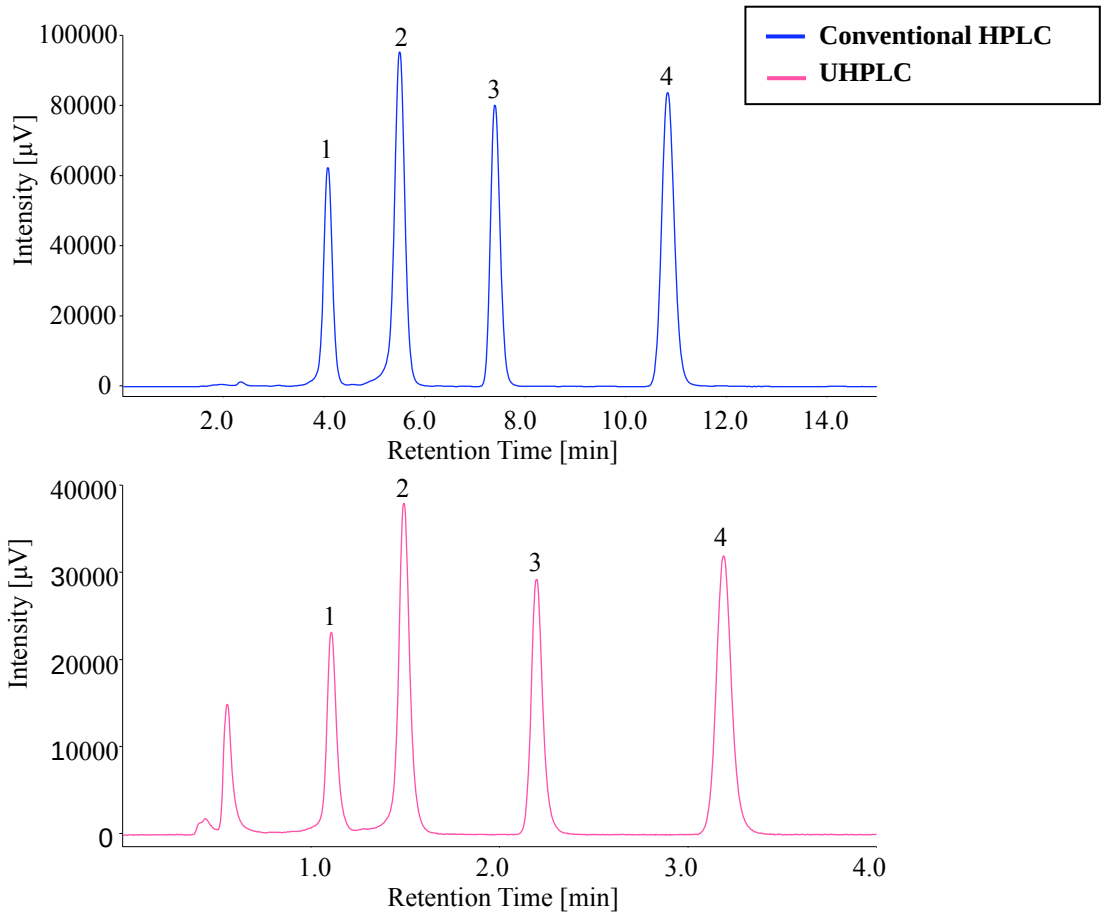

Fig. 4. Chromatograms of standard mixture of aflatoxins (5.0 µg/L each, TFA derivatized)
1=Aflatoxin G_1, 2=Aflatoxin B_1, 3=Aflatoxin G_2, 4=Aflatoxin B_2

The chromatograms of the purified solution from corn grits utilizing multi-functional column (MycoSep 226 AflaZon+) in sample preparation are shown in Fig. 5 (by both the conventional HPLC and UHPLC). The samples from corn grits and those spiked with standard mixture of aflatoxins, were used for the estimation of recovery 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 1.

The chromatograms of the purified solution from roasted peanuts utilizing immunoaffinity column(AFLAKING) in sample preparation are shown in Fig. 6 (by both the conventional HPLC and UHPLC). The samples from roasted peanuts and those spiked with standard mixture of aflatoxins, were used for the estimation of recovery of aflatoxins. Almost the 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.

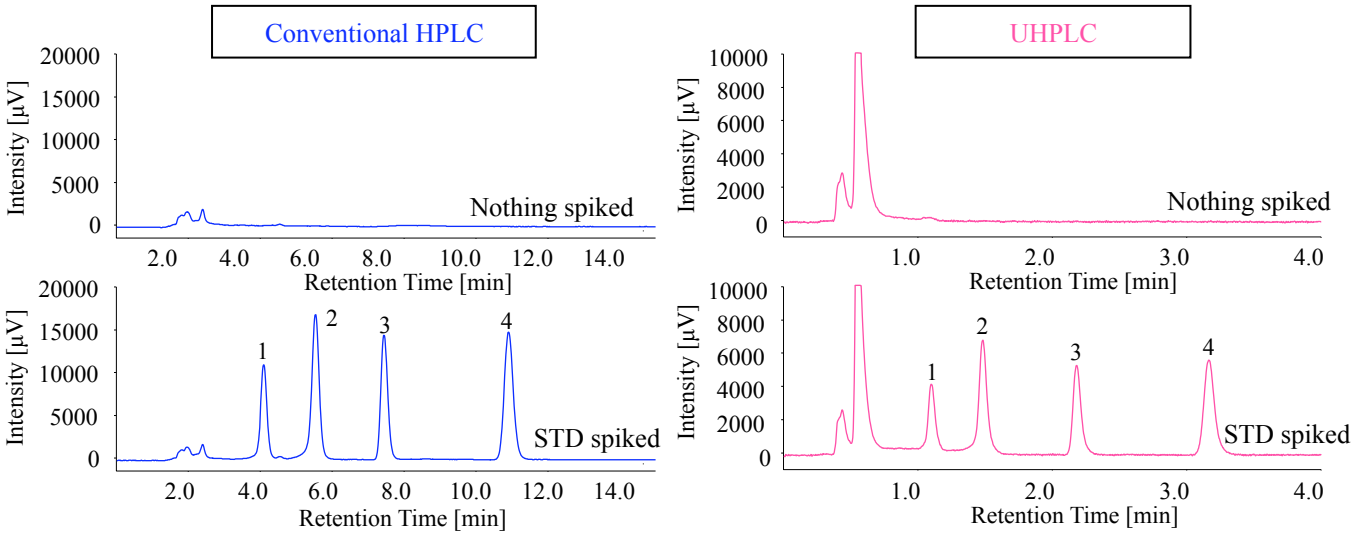

Fig. 5. Chromatograms of purified solution from corn grits
1=Aflatoxin G_1, 2=Aflatoxin B_1, 3=Aflatoxin G_2, 4=Aflatoxin B_2

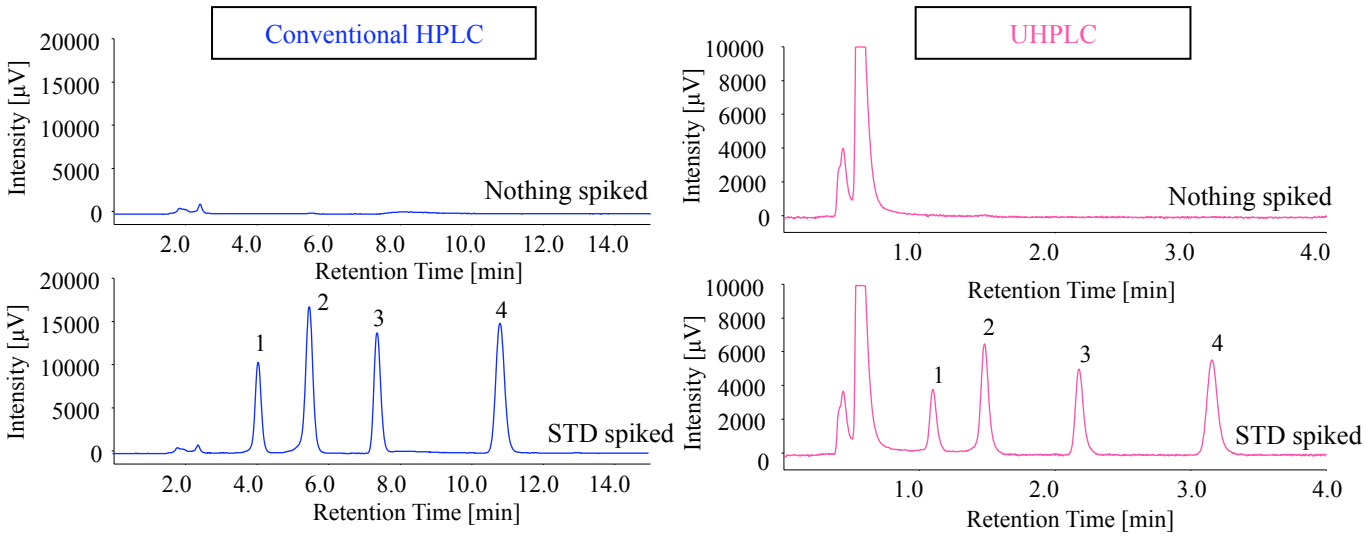

Fig. 6. Chromatograms of purified solution from roasted peanuts
1=Aflatoxin G_1, 2=Aflatoxin B_1, 3=Aflatoxin G_2, 4=Aflatoxin B_2

Table 1. Recovery [%] of standard aflatoxins

Aflatoxins	Corn grits		Roast peanuts	
	Conventional HPLC	UHPLC	Conventional HPLC	UHPLC
Aflatoxin G_1	107	108	101	99
Aflatoxin B_1	106	102	99	98
Aflatoxin G_2	100	100	102	104
Aflatoxin B_2	100	100	101	102

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