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Application News

No.L500

High Performance Liquid Chromatography

Analysis of Aflatoxin B_1, B_2, G_1 and G_2 in Kakkonto Using Nexera-i and RF-20A$_{XS}$

Aflatoxins are a type of mycotoxins that cause severe and acute toxicity. They are also carcinogenic, and testing for aflatoxins is required for crude drugs produced from natural plants or preparations that contain crude drugs. "Analytical methods for aflatoxins in crude drug and its product" was published in Japanese Pharmacopoeial Forum as a proposed revision for the 17th Edition of the Japanese Pharmacopoeia (as of July 2015). This test method proposal proposes a reference level of ≤10 µg/kg for total aflatoxins (sum of aflatoxin B_1, B_2, G_1 and G_2).
This Application News introduces an example analysis of the complex crude drug Kakkonto based on the proposed revision to the Japanese Pharmacopoeia 17th Edition. The proposed revision explains a method of analyzing aflatoxins using a fluorescent detector after derivatization with trifluoroacetic acid (TFA). This Application News describes an example analysis performed using this method, and another example analysis performed without derivatization but with direct fluorescence detection.

Aflatoxins in food are subject to regulation all over the world, and in Japan, a regulation*[1] and notification test method*[2] for aflatoxins have been published. See previous Application News L351, L422, L428, L430 and L435 for example analyses of aflatoxins in food performed using these test methods.

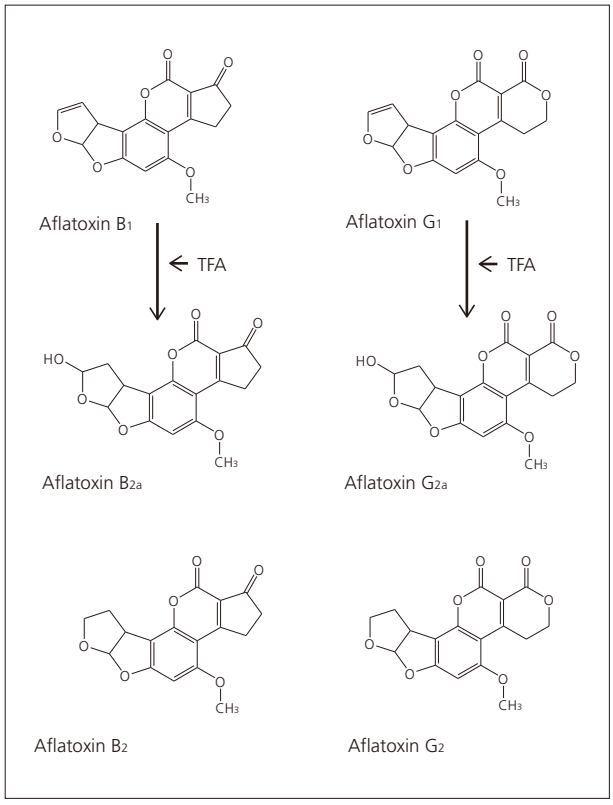

Fig. 1 Structures of Aflatoxin B_1, B_2, G_1 and G_2 and Aflatoxin Structures After TFA Derivatization (B_{2a} and G_{2a})

Analysis with Trifluoroacetic Acid Derivatization

When in a polar solvent, aflatoxins B_1 and G_1 are known to have a lower fluorescence intensity compared to aflatoxins B_2 and G_2. Possible methods of increasing the fluorescence intensity are derivatization with a photochemical reactor, TFA derivatization, and electrochemical derivatization. This Application News uses the TFA derivatization method that appears in the proposed test method. The TFA derivatization reaction is commonly known as the pre-column method, and involves derivatization of the analytical sample before HPLC analysis. The structures of each aflatoxin before and after TFA derivatization are shown in Fig. 1.

Aflatoxin standard solution was added to the complex crude drug Kakkonto prior to analysis. The pretreatment procedure is shown in Fig. 2. This pretreatment was performed based on the proposed revision to the Japanese Pharmacopoeia 17th Edition. An AFLAKING immunoaffinity column (Horiba, Ltd.) was used in a cartridge to remove impurities. Aflatoxin standard solution was added to the crude drug sample so each aflatoxin was present at a concentration of 0.25 µg/kg (total 1 µg/kg). This is equivalent to 1/10th the reference concentration stipulated in the proposed revision to the Japanese Pharmacopoeia 17th Edition.

The example analysis of Kakkonto is shown in Fig. 3, and the analytical conditions are shown in Table 1. An example analysis of the sample with no added aflatoxin standard solution is also shown for comparison. Since an impurity peak was found after aflatoxin B_2, which is the last eluted aflatoxin, a column cleaning process was added to the procedure. See Application News L428 for an example analysis of the standard solution.

Table 1 HPLC Analytical Conditions

System	: Nexera-i
Column	: Shim-pack FC-ODS (150 mm L. × 4.6 mm I.D., 3 µm)
Mobile Phase	: A; Water/methanol/acetonitrile = 6/3/1 (v/v/v)
	: B; Acetonitrile
Time Program	: A Conc. /B Conc. = 100/0 (0.00 - 15.00 min) → 10/90 (16.00 - 23.0 min) → 100/0 (24.00 - 34.00 min)
Flowrate	: 0.80 mL/min
Column Temp.	: 40 °C
Injection Volume	: 20 µL
Detection	: RF-20Axs, Ex. at 365 nm, Em. at 450 nm
Cell Temp.	: 25 °C

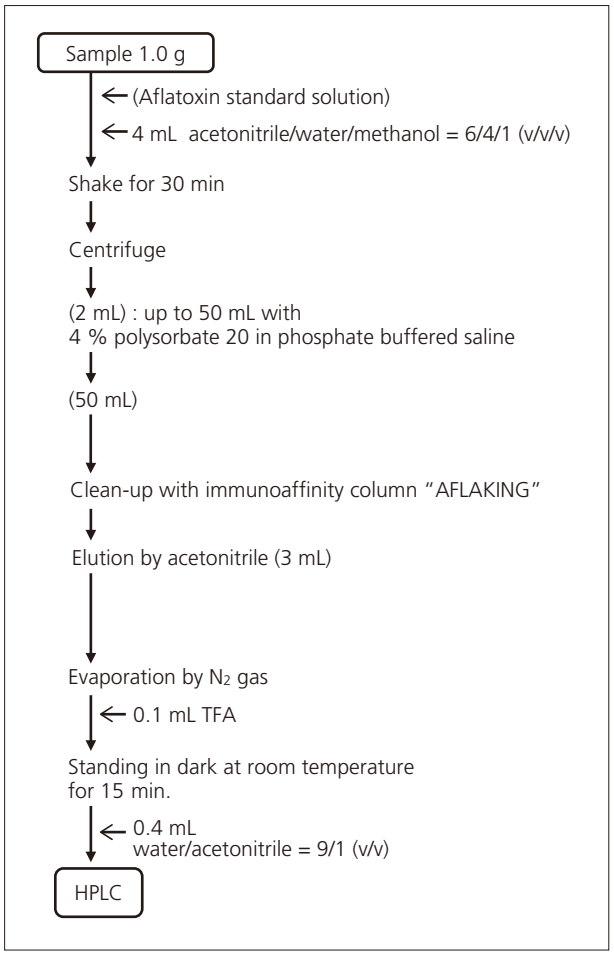

Fig. 2 Pretreatment Procedure

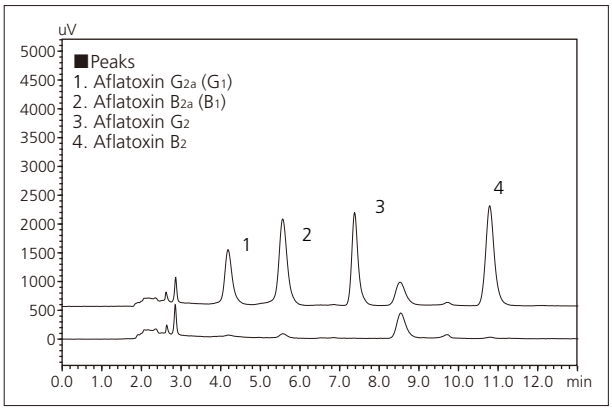

Fig. 3 Chromatogram of Kakkonto After TFA Derivatization —HPLC Analysis (Upper: With Standard Solution, Lower: Without Standard Solution)

Analysis by Direct Detection

Although aflatoxins B_1 and G_1 have a low fluorescence intensity, using the RF-20A$_{XS}$ highly-sensitive fluorescence detector allows for direct detection without derivatization. We performed direct detection using the RF-20A$_{XS}$, and also attempted to shorten the analysis time by using the Shim-pack XR-ODS Ⅱ high-performance column. Fig. 4 shows analysis of the aflatoxin standard solution without TFA derivatization (each aflatoxin at 20 µg/L), and Fig. 5 shows the same analysis performed at low concentrations (each aflatoxin at 0.1 µg/L). Analytical conditions are shown in Table 2. The relative standard deviation (n=6) of the area measured upon analysis of aflatoxin B_1 at 0.1 µg/L was 2.6 %. This result shows that sufficient analytical sensitivity can be obtained by using the RF-20A$_{XS}$ even without performing TFA derivatization. Using the RF-20A$_{XS}$ also shortens the analysis time to approximately 1/3rd of the analysis time with TFA derivatization. Fig. 6 shows the calibration curves for each aflatoxin in the concentration range of 0.1 to 20 µg/L. Good linearity was obtained with all four compounds, with an R^2 of 0.9999 or above.

Table 2 UHPLC Analytical Conditions

System	: Nexera-i
Column	: Shim-pack XR-ODS Ⅱ (100 mm L. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: A; Water : B; Methanol : C; Acetonitrile
Time Program	: A Conc. /B Conc. /C Conc. = 65/30/5 (0.00 - 5.50 min) → 15/5/80 (5.51 - 7.0 min) → 20/80/0 (7.01 - 9.00 min) → 65/30/5 (9.01 - 12.00 min)
Flowrate	: 1.00 mL/min
Column Temp.	: 50 °C
Injection Volume	: 10 µL
Detection	: RF-20A$_{XS}$, Ex. at 365 nm, Em. at 450 nm
Cell Temp.	: 25 °C

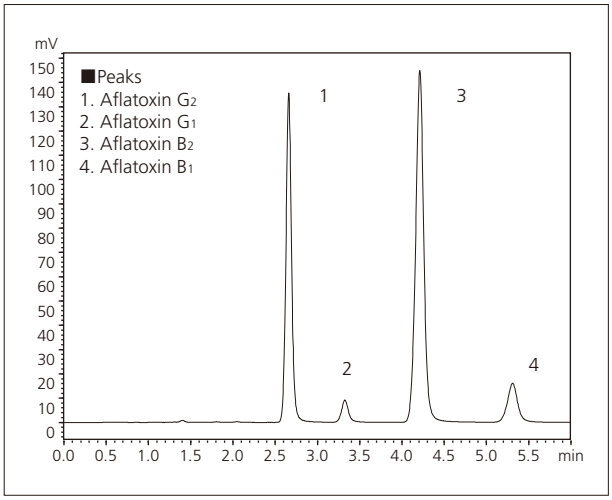

Fig. 4 Chromatogram of Aflatoxin Standard Solution by Direct Detection—UHPLC Analysis (each 20 µg/L, 10 µL)

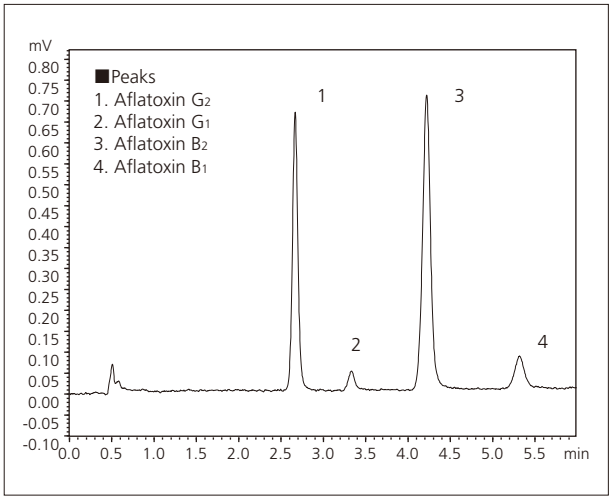

Fig. 5 Chromatogram of Aflatoxin Standard Solution by Direct Detection—UHPLC Analysis (each 0.1 µg/L, 10 µL)

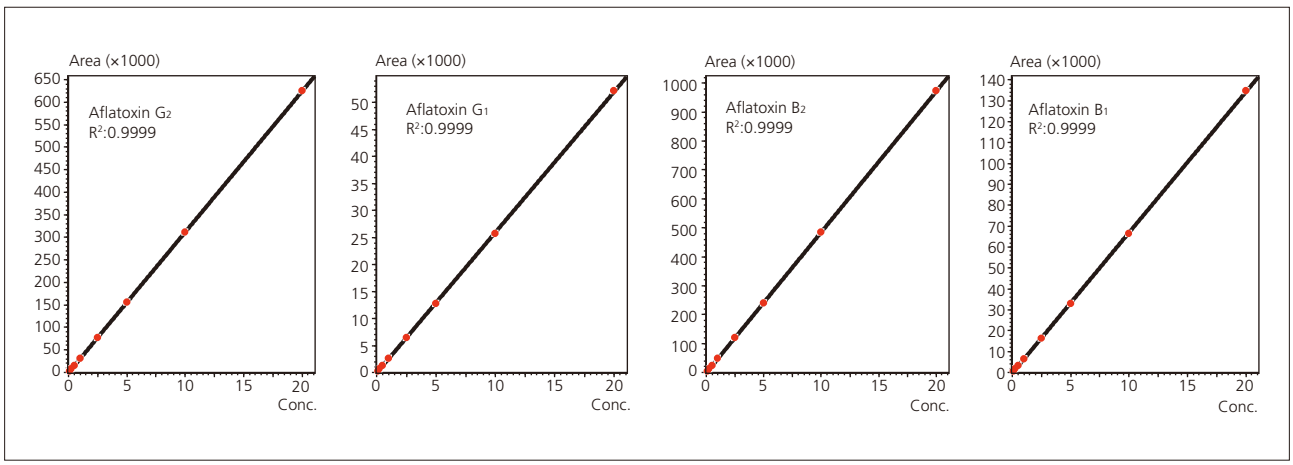

Fig. 6 Aflatoxin Standard Solution Calibration Curves—Direct Detection (each 0.1 to 20 µg/L, 10 µL)

Identical to the analysis with TFA derivatization, aflatoxin standard solution was added to the complex crude drug Kakkonto and analysis performed. The pretreatment procedure is shown in Fig. 7. An AFLAKING immunoaffinity column (Horiba, Ltd.) was also used in a cartridge to remove impurities. The pretreatment procedure up to this purification step is identical to that shown in Fig. 2. Aflatoxin standard solution was added to the crude drug sample so each aflatoxin was present at a concentration of 0.25 µg/kg (total 1 µg/kg). This is equivalent to 1/10th the reference concentration stipulated in the proposed revision to the Japanese Pharmacopoeia 17th Edition.

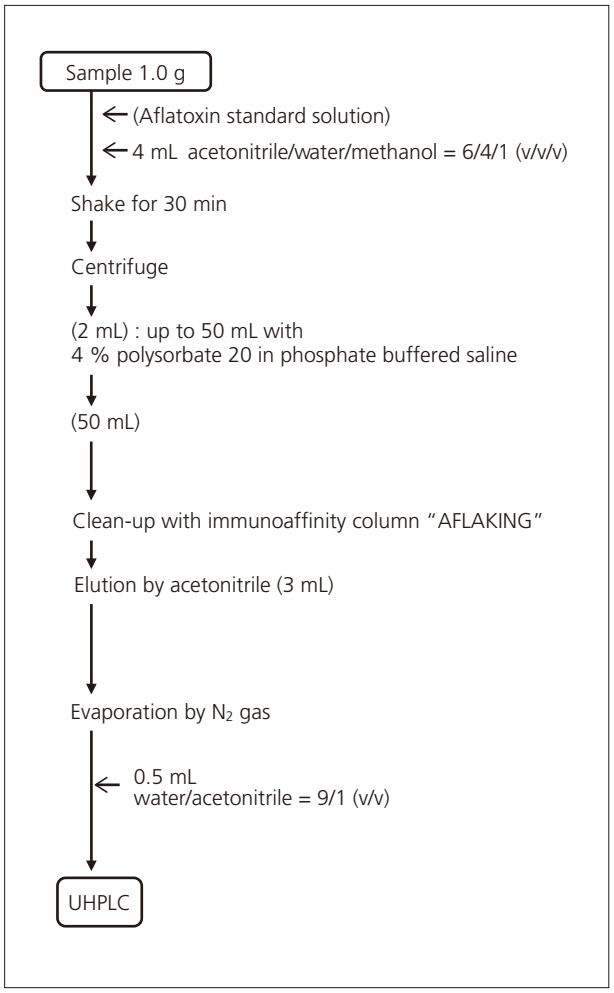

Fig. 7 Pretreatment Procedure

Note: Aflatoxins are degraded by UV light and while in solution will adsorb to glass surfaces. The vials used in analyses were pre-cleaned, low-adsorption brown glass vials.

*1) "Handling of Foods Containing Aflatoxins" (Japanese Ministry of Health, Labour and Welfare, Dept. of Food Safety Notification 0331 No. 5, March 31, 2011)

*2) "Test Method for Total Aflatoxins" (Japanese Ministry of Health, Labour and Welfare, Dept. of Food Safety Notification 0816 No. 2, August 16, 2011)

The example analysis of Kakkonto is shown in Fig. 8, and the analytical conditions are shown in Table 2. Although an impurity peak was eluted between aflatoxin G_1 and B_2 despite use of the immunoaffinity column, the impurity peak was fully separate from the two aflatoxin peaks, and the analysis time was completed in 12 minutes even after adding a cleaning process.

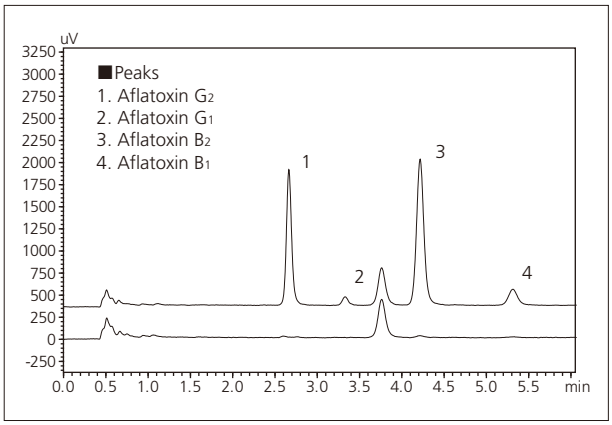

Fig. 8 Chromatogram of Kakkonto by Direct Detection—UHPLC Analysis
(Upper: With Standard Solution, Lower: Without Standard Solution)

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