SHIMADZU
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Application News

No.L435

High Performance Liquid Chromatography

Quantitation by HPLC and Identification by LC/MS for Total Aflatoxins in Food

Aflatoxins are mycotoxins that are extremely carcinogenic and acutely toxic. In Japan, foods in which the total aflatoxins (sum of B_1, B_2, G_1 and G_2 aflatoxins) are detected at levels exceeding 10 µg/kg are in violation of Article 6, Item 2 of the Food Sanitation Act[1].
Previously, in Application News articles L422, L428 and L430, we introduced examples of simultaneous analysis of these 4 substances (B_1, B_2, G_1 and G_2) using HPLC and UHPLC. In accordance with the test method[2] specified in the notification of August 16, 2011, after pretreatment using an immunoaffinity column, we conducted (1) quantitation using an HPLC system compliant with the test method, (2) identification using a LC/MS system compliant with the test method, and (3) rapid quantitation and identification using the Nexera UHPLC. Here, we present these analyses in addition to the results of the validation.

Total Aflatoxins Test Flow

Fig. 1 shows the complete workflow, including sample preparation, for total aflatoxins according to the specified test method (HPLC or LC/MS), and Fig. 2 shows the structures of those aflatoxins. For processed foods and spices, after extraction with a solution containing an organic solvent, the aflatoxins are purified using an immunoaffinity column (IAC). The fluorescence intensity of aflatoxins B_1 and G_1 is increased by following pretreatment procedure (A) with trifluoroacetic acid (TFA), and quantitative testing is carried out using an HPLC with an RF-20Axs fluorescence detector. If the aflatoxins are detected at a level greater than the reference value, it is re-dissolved in mobile phase without TFA derivatization after drying the purified solution eluted from IAC (pretreatment procedure (B)), and identification of the contained aflatoxins is carried out by LC/MS.
Among the properties of aflatoxins is their susceptibility to degradation when exposed to ultraviolet light, and their tendency in aqueous solution to adsorb to glass surfaces. Therefore, when conducting analysis, it is necessary to use silanized, amber glass vials or polypropylene vials to prevent reduced recoveries. Also, for analysis of actual samples, it is recommended to clean the column after each analysis with mobile phase containing a higher concentration of organic solvent than that used for the analysis.

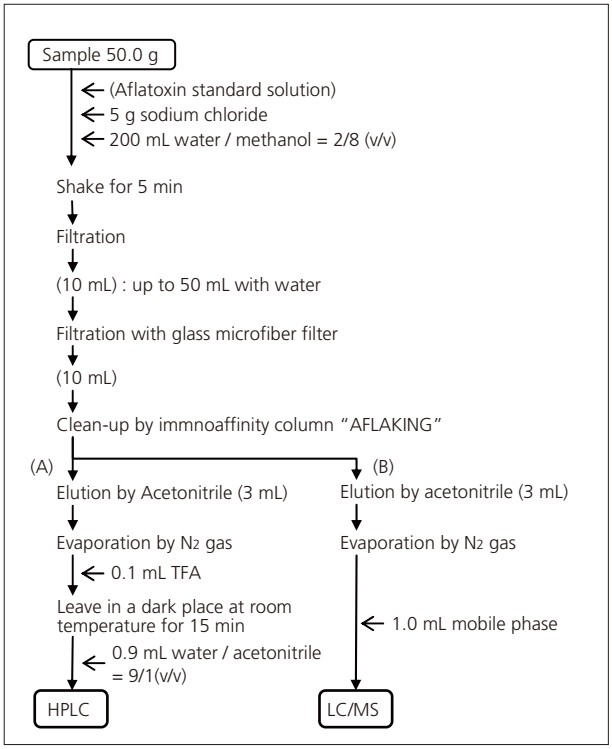

Fig. 1 Total Aflatoxin Test Workflow

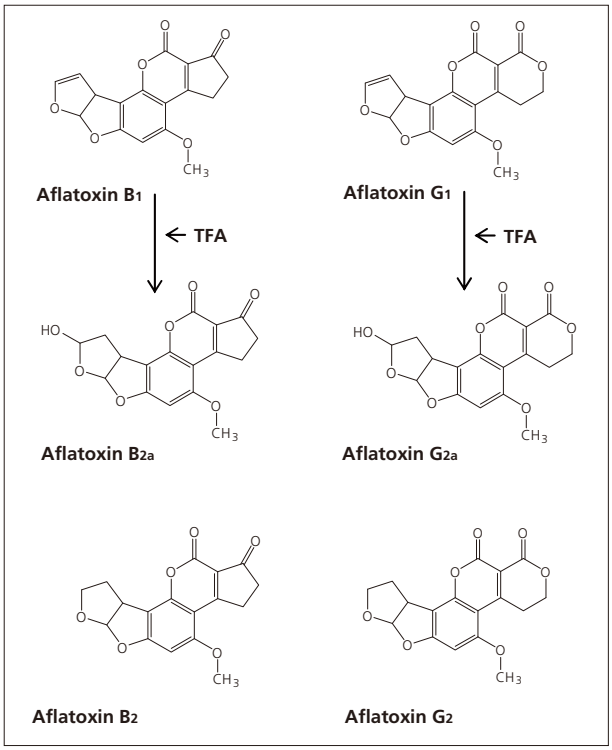

Fig. 2 Structures of Aflatoxins

(1) Quantitation by HPLC –Accuracy and Precision–

Aflatoxins in commercially available roasted peanuts were processed according to procedure (A) in Fig 1, and analysis was conducted by HPLC using the test method to verify the recovery (accuracy) and repeatability (precision). The analytical conditions are shown in Table 1.

The roasted peanuts were spiked with aflatoxin B_1 and G_1 at a concentration of 0.8 µg/kg each, and aflatoxin B_2 and G_2 at a concentration of 0.2 µg/kg each (total aflatoxins: 2 µg/kg). The accuracy and precision with respect to each substance in repeat testing (n = 5) are shown in Table 2. Accuracy of 76–100 % was obtained that satisfied the criteria of the specified reference value in the guideline (70–110 %). In addition, the precision obtained was 5.7–9.6 %, which also met the specified reference value (less than 20 %).

Table 1 Analytical Conditions

Column	: Shim-pack FC-ODS (150 mmL. × 4.6 mmI.D., 3 µm)
Mobile Phase	: Water / Methanol / Acetonitrile = 6/3/1 (v/v/v)
Flow Rate	: 0.8 mL/min
Column Temp.	: 40 °C
Detection	: RF-20Axs, Ex at 365 nm, Em at 450 nm
RF Cell	: Conventional cell
Cell Temp.	: 25 °C
Injection Volume	: 20 µL

Table 2 Accuracy and Precision of Quantitation by HPLC

	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
Recovery (%)	79	90	76	100
%RSD	9.6	8.9	7.3	5.7

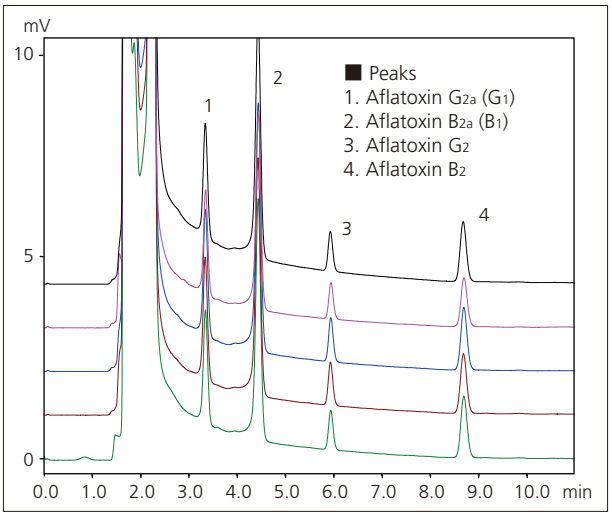

Fig. 3 Chromatograms of Roasted Peanuts Spiked with Aflatoxins (Quantitation by HPLC)

(2) Identification Testing by LC/MS

Commercially available roasted peanuts that were not spiked with the aflatoxin standard solution were subjected to dry processing according to procedure (B) in Fig. 1, and a matrix sample solution (blank) was prepared by dissolving the residue in 1 mL of 10 % acetonitrile solution. The matrix sample solution and aflatoxin standard solution were mixed 1:1, and standard addition matrix samples were prepared at 0.05, 0.25, 0.5, 2.5 and 5 µg/L, respectively (equivalent to 0.2, 1, 2, 10, 20 µg/kg, respectively). Analyses (n = 6) were then conducted using the LCMS-8030 triple stage LC/MS/MS. Fig. 4 shows the MRM* chromatograms obtained from analysis of the 0.5 µg/L standard addition matrix sample, and Table 3 and Table 4 show the analytical conditions. The LCMS-8030 clearly demonstrated sufficient sensitivity to conduct identification testing.

*MRM...Multiple Reaction Monitoring

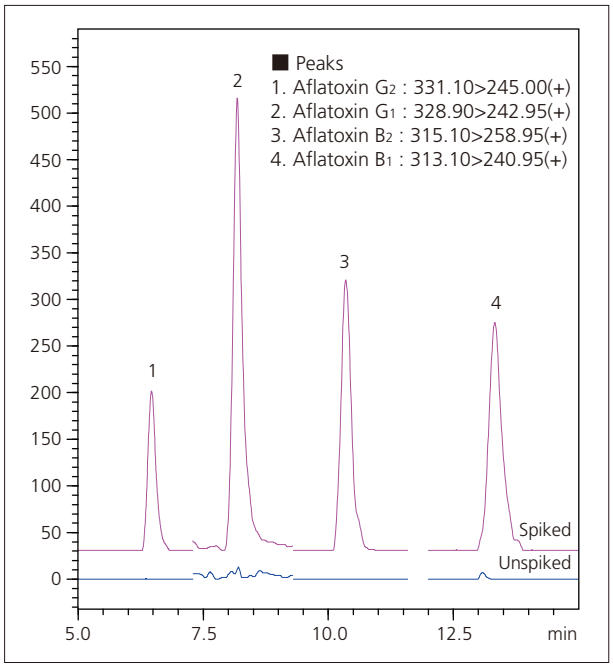

Fig. 4 MRM Chromatograms of Roasted Peanuts Matrix (Upper) Spiked, (Lower) Unspiked

Table 3 Analytical Conditions

[HPLC]	
Column	: Shim-pack FC-ODS (150 mmL. × 2.0 mmI.D., 3 µm)
Mobile Phase	: 10 mmol/L Ammonium acetate water / Methanol = 3/2 (v/v)
Flow Rate	: 0.2 mL/min
Column Temp.	: 40 °C
Injection Volume	: 6 µL
[MS]	
Probe Voltage	: +4.5 kV ESI-positive mode
Nebulizing Gas Flow	: 3 L/min
Drying Gas Flow	: 15 L/min
DL Temp.	: 250 °C
Heat Block Temp.	: 400 °C

Table 4 MRM Mode Parameters

Compound	Transition	Dwell time (ms)	CE (V)	Resolution (Q1, Q3)
Aflatoxin B_1	313.10 > 240.95	200	-40	Unit
Aflatoxin B_2	315.10 > 258.95	200	-33	Unit
Aflatoxin G_1	328.90 > 242.95	200	-30	Unit
Aflatoxin G_2	331.10 > 245.00	200	-32	Unit

In addition, to evaluate the validity of the standard addition method for quantitation of aflatoxins, we checked the linearity of the calibration curves of spiked matrix samples, in addition to the repeatability. Fig. 5 shows the calibration curves, and Table 5 shows the average area and repeatability of the peaks corresponding to 0.25, 0.5 and 5 µg/L. Excellent linearity was obtained over the range of 0.05–5 µg/L, with a coefficient of determination greater than $R^2 = 0.999$, and good repeatability was also confirmed.
As a result, identification testing was confirmed to be possible using the LCMS-8030, and quantitation using calibration curves of matrix samples spiked with standards was also confirmed to be effective.

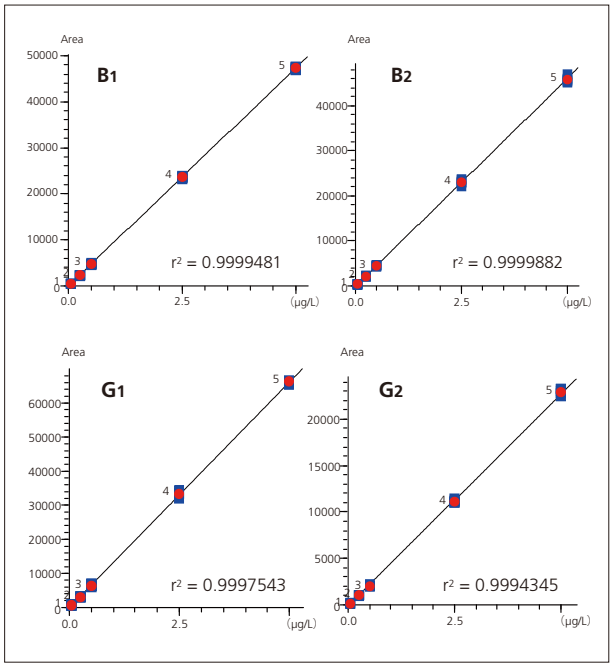

Fig. 5 Calibration Curves of Matrix Samples Spiked with Aflatoxins (Roasted Peanuts, 0.05–5 µg/L)

Table 5 Average and Repeatability of Peak Area (n = 6)

	0.25 µg/L		0.5 µg/L		5 µg/L	
	Area	%RSD	Area	%RSD	Area	%RSD
Aflatoxin B_1	1010	6.37	2033	3.92	22936	1.49
Aflatoxin B_2	3156	1.87	6481	5.09	66012	0.54
Aflatoxin G_1	2209	6.07	4517	3.74	45841	1.45
Aflatoxin G_2	2296	5.96	4749	4.74	47250	0.83

(3) Rapid Quantitation and Identification by Nexera UHPLC with LCMS-8030

Although TFA derivatization is specified in the quantitative test by HPLC according to the official test method, if the obtained value for total aflatoxins exceeds the reference value, follow-up analysis is required. The additional pretreatment is done without TFA derivatization (in Fig. 1 (B) for LC/MS analysis). However, if detection with sufficient sensitivity is achieved through direct detection (without TFA derivatization) as specified in the quantitative test, sample preparation, quantitative testing and identification can all be done using just the procedure of Fig. 1 (B). As a result, such a complicated pretreatment procedure does not need to be repeated. Furthermore, UHPLC what enables the high-throughput analysis brings even greater efficiency of the test for total aflatoxin.

Fig. 6 shows an example of the quantitative testing results obtained using the Shimadzu Nexera UHPLC after conducting pretreatment of aflatoxin-added roasted peanuts according to Fig. 1 (B), and Table 6 shows the analytical conditions. The roasted peanuts were spiked with aflatoxins B_1 and G_1 at 2.4 µg/kg each, and aflatoxins B_2 and G_2 at 0.6 µg/kg each. The accuracy and precision obtained for each substance in repeat analysis (n = 5) are shown in Table 7. Accuracy of 76–80 % and precision of 4.5–6.5 % were obtained, that satisfied the criteria of the specified reference value in the guideline.
In addition to the extremely good sensitivity of this analytical method as shown in Application News L422 and L430, it was confirmed that this method is effective for rapid quantitative testing of aflatoxins.

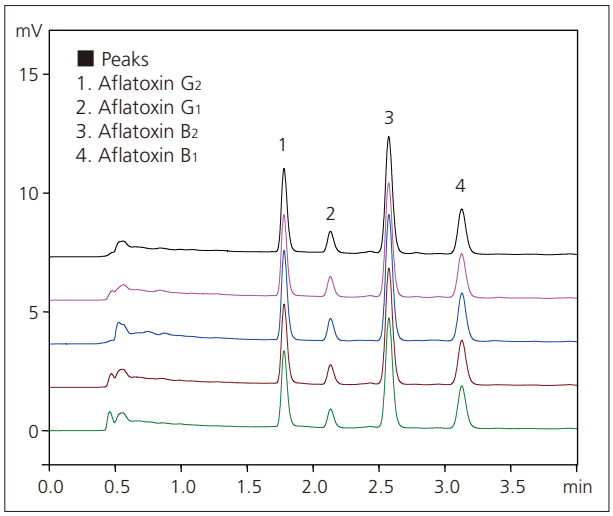

Fig. 6 Chromatograms of Roasted Peanuts Spiked with Aflatoxins (Direct Detection) (10 µL Injected)

Table 6 Analytical Conditions

Column	: Shim-pack XR-ODS Ⅱ (100 mmL. × 3.0 mmI.D., 2.2 µm)
Mobile Phase	: Water / Methanol / Acetonitrile = 6/3/1 (v/v/v)
Flow Rate	: 1.0 mL/min
Column Temp.	: 50 °C
Detection	: RF-20Axs, Ex. at 365 nm, Em. at 450 nm
RF Cell	: Conventional cell
Cell Temp.	: 25 °C
Injection Volume	: 10 µL

Table 7 Accuracy and Precision of Quantitation by UHPLC without Derivatization

	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
Recovery (%)	76	77	77	80
%RSD	4.5	5.1	6.1	6.5

Furthermore, since the LCMS-8030 is an LC/MS/MS that supports UHPLC, when used in combination with the high-speed analysis offered with the above-mentioned Nexera, rapid identification of aflatoxins becomes possible.

Fig. 7 shows the MRM chromatograms generated using high-speed analysis of the same sample of Fig. 4 using the analytical conditions of Table 8 and Table 9. All 4 aflatoxins eluted within 4 minutes, and identification was clearly obtained with sufficient sensitivity even under such high-speed conditions.

Fig. 8 shows the calibration curves generated using the same HPLC analytical conditions (Fig. 5) and matrix samples spiked with aflatoxins. Excellent linearity was obtained over the range of 0.05–25 µg/L, with a coefficient of determination greater than R^2 = 0.999. The peak area repeatability (%RSD, n = 6) obtained for each of the aflatoxin matrix samples at a concentration of 0.25 µg/L was B_1: 14.24 %, B_2: 5.33 %, G_1: 5.45 % and G_2: 18.9 %, demonstrating good repeatability even when using high-speed analysis.

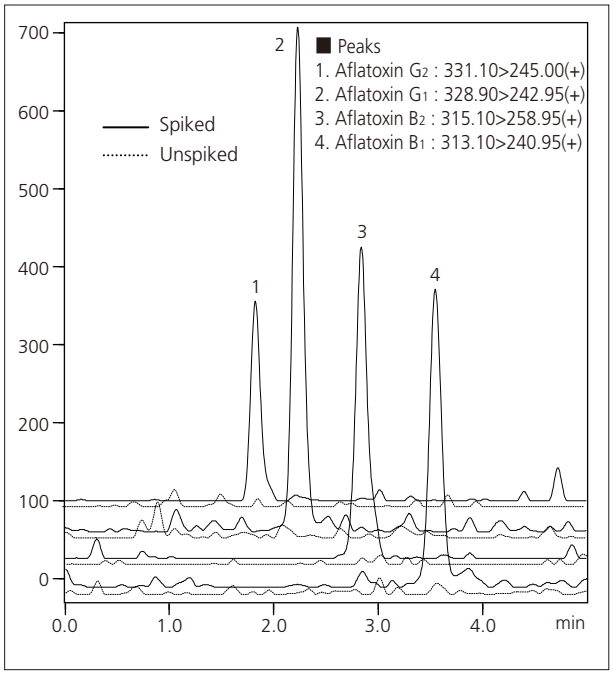

Fig. 7 MRM Chromatograms of Roasted Peanuts (Upper, Solid Line) Spiked, (Lower, Broken Line) Unspiked

Table 8 Analytical Conditions

[HPLC]	
Column	: Shim-pack XR-ODS Ⅱ (100 mmL. × 2.0 mmI.D., 2.2 µm)
Mobile Phase	: 10 mmol/L Ammonium acetate water / Methanol = 3/2 (v/v)
Flow Rate	: 0.45 mL/min
Column Temp.	: 50 °C
Injection Volume	: 6 µL
[MS]	
Probe Voltage	: +4.5 kV ESI-Positive mode
Nebulizing Gas Flow	: 3 L/min
Drying Gas Flow	: 15 L/min
DL Temp.	: 250 °C
Heat Block Temp.	: 400 °C

Table 9 MRM Mode Parameters

Compound	Transition	Dwell time (ms)	CE (V)	Resolution (Q1, Q3)
Aflatoxin B_1	313.10 > 240.95	100	-40	Unit
Aflatoxin B_2	315.10 > 258.95	100	-33	Unit
Aflatoxin G_1	328.90 > 242.95	100	-30	Unit
Aflatoxin G_2	331.10 > 245.00	100	-32	Unit

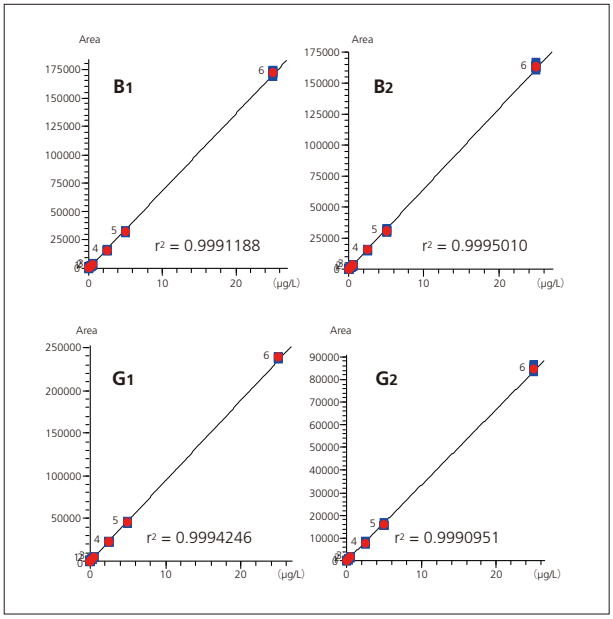

Fig. 8 Calibration Curves of Matrix Samples Spiked with Aflatoxins (Roasted Peanuts, 0.05–25 µg/L)

[References]
1) Handling of Foods Containing Aflatoxins (Japanese Ministry of Health, Labour and Welfare, Dept. of Food Safety Issue 0331 No. 5, March 31, 2011)
2) Test Method for Total Aflatoxins (Dept. of Food Safety Issue 0816 No. 1, August 16, 2011)

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