Aflatoxin and related compounds

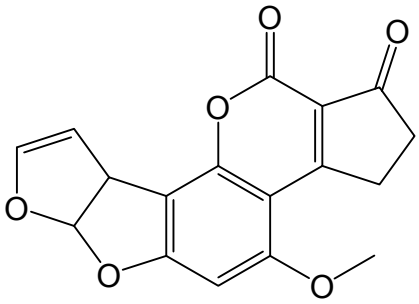
1 Aflatoxin B_1

2 Aflatoxin B_2

3 Aflatoxin G_1

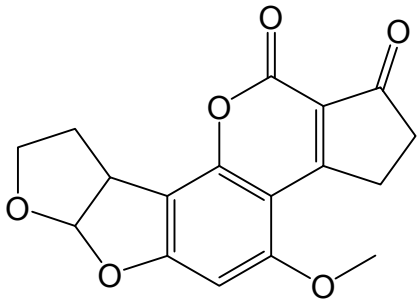
4 Aflatoxin G_2

Aflatoxin B_1



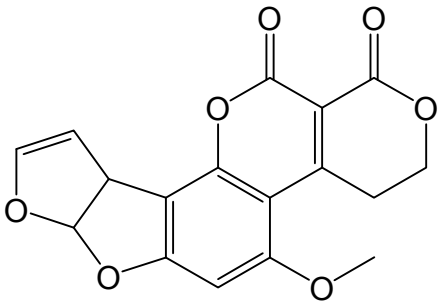
(6aR,9aS)-2,3,6a,9a-tetrahydro-4-methoxycyclo penta [c]furo [3',2':4,5]furo [2,3-h] [1]benzopyran-1,11-dione
$C_{17}H_{12}O_6$ MW: 312.06 CAS No.: 1162-65-8

Aflatoxin B_2



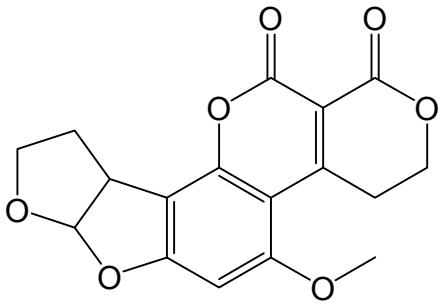
(6aR,9aS)-2,3,6a,8,9,9a-hexahydro-4-methoxy-cyclopenta [c]furo [3',2':4,5]furo [2,3-h] [1]benzopyran-1,11-dione
$C_{17}H_{14}O_6$ MW: 314.08 CAS No.: 7220-81-7

Aflatoxin G_1



(7aR,10aS)-3,4,7a,10a-tetrahydro-5-methoxy-1 H,12H-furo [3',2':4,5]furo [2,3-h]pyrano [3,4-c] [1]benzopyran-1,12-dione
$C_{17}H_{12}O_7$ MW: 328.06 CAS No.: 1165-39-5

Aflatoxin G_2



(7aR,10aS)-3,4,7a,9,10,10a-hexahydro-5-metho xy-1H,12H-furo [3',2':4,5]furo [2,3-h] pyrano [3,4-c] [1]benzopyran-1,12-dione
$C_{17}H_{14}O_7$ MW: 330.07 CAS No.: 7241-98-7

[Summary of aflatoxin]
Aflatoxin is a kind of mycotoxin that was discovered from the mass poisoning of turkeys in Britain in 1960, and has strong carcinogenicity.

The typical mold that produces this aflatoxin is Aspergillus flavus which is related to Aspergillus oryzae. This producer widely distributed in the tropical and subtropical areas, such as Southeast Asia, the United States, and Brazil among others, and grows in agricultural products, especially in peanut and cottonseed, causing aflatoxin contamination.

Ten-odd isomers of aflatoxin have been discovered. However, most of those detected in agricultural products contaminated with molds are B_1, B_2, G_1 and G_2. M_1 is a substance that is detected in the milk of cows which have taken agricultural products contaminated with B_1. Codex designates M_1 allowance in milk as 0.5 ppb.

Toxicity is the highest for B_1, followed by G_1, B_2, and G_2 in this order. In addition, M_1 also shows toxicity at a similar level to B_1.

As for physicochemical properties, aflatoxin is a highly fluorescent substance, and B_1, B_2, M_1 and M_2 emit blue fluorescence, while G_1 and G_2 emit green fluorescence.

In Japan, the Food Sanitation Act prohibits selling foods in which aflatoxin B_1 is detected (Lower limit of detection: 10 μg/kg) by the analysis method in the Notification No. 0728003 (July 28, 2008) from the Inspection and Safety Division, MHLW. Additionally, as for corn, the kit method stipulated in the Notification No. 0713001 (July 13, 2006) from the Inspection and Safety Division, MHLW, can also be used.

<<Standards and specifications in the Act on Safety Assurance and Quality Improvement of Feeds>>

1 Ministerial Ordinance Concerning the Ingredient Specifications for Feed and Feed Additives

(1) Appended Table 1, 1-(3)-B-(c)
Feeds that contain cottonseed meal as a material may not be used in cultured aquatic animals.

(2) Appended Table 1, 2-(1)
(a) The aflatoxin B_1 content in peanut meal may not exceed 1 ppm.
(b) Types of feeds that may contain peanut meal as a material and the percentage should be as shown in the following table:

Feed type	Percentage
Chicken feeds (except for chick feeds and broiler starter)	4 % or less
Pig feeds (except for lactation period)	4 % or less
Dairy cow [Note] feeds	2 % or less
Cattle (except for lactation period and dairy cows) feeds	4 % or less

Note: dairy cows are cows of approximately over 18 months old for milking.

As shown above, peanut meal and cottonseed meal may not used in feeds for cultured aquatic animals considering that aflatoxin is known to sensitively affect trouts in particular (carcinogenicity), that there are not enough information to set standards and specifications as feeds for cultured aquatic animals, and that little amount of peanut meal has been used for feeds for cultured aquatic animals.

2 Guidelines for hazardous substances in feeds

(1) Guideline on aflatoxin B_1 concentration in feeds for calves during lactation, cows, piglets during lactation, chicks, and broiler starters: 0.01 ppm

(2) Guideline in formula feeds other than (1) above: 0.02 ppm

<<Notes for aflatoxin analysis procedure >>

Aflatoxin is soluble in chloroform, methanol, acetone, and benzene, while it is slightly soluble in petroleum ether, hexane, and in water. Also, it is easily degradable by light and thus it needs to be protected from light as much as possible. As for pH, it is easily degradable in alkaline conditions, as well as in strongly acidic conditions, therefore it needs to be analyzed in the stable range of pH 4-6.

Aflatoxin extraction solution after purification is unstable in the sunlight and artificial ultraviolet light, thus a darkroom or a laboratory with shielding is required. When fluorescence densitometry (4 in this section) or screening (5 in this section) is conducted in a laboratory without such facility, an incandescent bulb is preferred to a fluorescent light.

After the end of the experiment, monitor around the laboratory table with a UV lamp; if a blue-violet fluorescent substance, possibly caused by aflatoxin, is found, it is required to wipe off the area using 5% sodium hypochlorite solution.

To purchase aflatoxin standard, a certificate is required that indicates it is used for study purposes in order to prevent the production and use of biotoxin weapons. In addition, import or export procedures may need several months.

For the handling of aflatoxin standard, use protective gloves and a mask etc.

After the end of the experiment, immerse all devices used except the extraction container in 5% sodium hypochlorite solution in a sink for 15-30 minutes, then wash with water.

In case of contact with aflatoxin, immediately immerse in 0.5 % sodium hypochlorite solution for 20-30 seconds, wash with water, and then wash well with soap.

[Methods listed in the Feed Analysis Standards]

1 Simultaneous analysis of mycotoxins by liquid chromatography/ tandem mass spectrometry [Feed Analysis standards, Chapter 5, Section 1 1.1, 2.1, 3.1 and 4.1]

Analyte compounds: Aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, sterigmatocystin, zearalenone, T-2 toxin, deoxynivalenol, nivalenol, neosolaniol and fusarenon-X (11 components)

Scope of application: Feeds

A. Reagent preparation
1) Mycotoxin standard stock solutions. Weigh accurately 1 mg each of aflatoxin B_1 [$C_{17}H_{12}O_6$], aflatoxin B_2 [$C_{17}H_{14}O_6$], aflatoxin G_1 [$C_{17}H_{12}O_7$], aflatoxin G_2 [$C_{17}H_{14}O_7$],

sterigmatocystin [$C_{18}H_{12}O_6$] and zearalenone [$C_{18}H_{22}O_5$]; 5 mg each of T-2 toxin [$C_{24}H_{34}O_9$] and neosolaniol [$C_{19}H_{26}O_8$]; and 10 mg each of deoxynivalenol [$C_{15}H_{20}O_6$], nivalenol [$C_{15}H_{20}O_7$] and fusarenon-X [$C_{17}H_{22}O_8$]. Put each of them in a 50- mL amber volumetric flask, respectively, and dissolve by the addition of acetonitrile. Add the same solvent to each volumetric flask up to the graduation line to prepare the standard stock solutions of mycotoxins (1 mL each of these solutions contains 20 μg respectively as aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, sterigmatocystin and zearalenone; 100 μg respectively as T-2 toxin and neosolaniol; and 200 μg respectively as deoxynivalenol, nivalenol and fusarenon-X.).

2) Mycotoxin mixture standard solution. Transfer 1 mL each of the aflatoxin B_1 and aflatoxin B_2 standard stock solutions, 2 mL of the zearalenone standard stock solution, 3 mL each of the aflatoxin G_1 and aflatoxin G_2 standard stock solutions, 10 mL each of the sterigmatocystin, deoxynivalenol and fusarenon-X standard stock solutions, 20 mL each of the T-2 toxin and neosolaniol standard stock solutions and 30 mL of the nivalenol standard stock solution to a 200- mL amber volumetric flask, add 32 mL of water and mix, and add acetonitrile up to the graduation line to prepare the mycotoxin mixture standard stock solution (1 mL of this solution contains 0.1 μg respectively as aflatoxin B_1 and aflatoxin B_2; 0.2 μg as zearalenone; 0.3 μg respectively as aflatoxin G_1 and aflatoxin G_2; 1 μg as sterigmatocystin; 10 μg respectively as deoxynivalenol, fusarenon-X, T-2 toxin and neosolaniol; and 30 μg as nivalenol.).

Before use, dilute accurately a certain amount of the mycotoxin mixture standard stock solution with acetonitrile- water (21:4) to be a series of dilutions in the range between 10- to 200-fold, then dilute a certain amount of the dilutions with acetic acid (1:100) to be accurately 2-fold to prepare the mycotoxin mixture standard solutions.

B. Quantification

Extraction. Weigh 50 g of an analysis sample, transfer it to a 300- mL stoppered amber Erlenmeyer flask, add 100 mL of acetonitrile- water (21:4), and extract by shaking for 60 minutes. [Note 1] Transfer the extract to a stoppered centrifuge tube, centrifuge at 650×*g* for 5 minutes, to obtain supernatant to be a sample solution to be subjected to column treatment.

Column treatment. Load 10 mL of the sample solution to a multifunctional column (for mycotoxin pretreatment), [Note 2] and discard the first 4 mL of the eluate. [1] Place a 10- mL amber test tube under the column, and collect the following 2 mL of the eluate. Transfer accurately 1 mL of the eluate to another 10- mL amber test tube, and dilute by the addition of accurately 1 mL of acetic acid (1:100). [2] [3] Transfer a certain amount of this solution to a plastic centrifuge tube (capacity: 1.5 mL), centrifuge at 5,000×*g* for 5 minutes, to obtain supernatant to be a sample solution to be subjected to analysis by liquid chromatography- tandem mass spectrometry.

Measurement by liquid chromatography- tandem mass spectrometry. Inject 10 μL each of the sample solution and respective mixture standard solutions to a liquid chromatograph-tandem mass spectrometer to obtain selected reaction monitoring chromatograms.

Example of measurement conditions

(Liquid chromatography part)

Column: Octadecylsilyl silica gel column (4.6 mm in inner diameter, 150 mm in length, particle size 5 μm) [Note 3]

Eluent: 10 mmol/L ammonium acetate solution- acetonitrile (9:1) (1 min retention) →19 min→10 mmol/L ammonium acetate solution-

acetonitrile (1:4) (15 min retention)
Flow rate: 0.2 mL/min
Column oven temperature: 40 °C
(Tandem mass spectrometry part Note 4)
Ionization method: Electrospray ionization (ESI)
Ion source temperature: 120 °C
Desolvation temperature: 300 °C
Capillary voltage: Positive 4.0 kV, negative 1.5 kV
Cone voltage: As shown in the table below
Collision energy: As shown in the table below
Monitor ion: As shown in the table below

Table: Monitor ion conditions for mycotoxins

Name of mycotoxin	Measurement mode	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Aflatoxin B_1	+	313	241	40	35
Aflatoxin B_2	+	315	243	40	35
Aflatoxin G_1	+	329	214	40	35
Aflatoxin G_2	+	331	217	40	35
Sterigmatocystin	+	325	281	40	35
T-2 toxin	+	484	305	20	15
Neosolaniol	+	400	305	15	15
Zearalenone	−	317	175	40	25
Deoxynivalenol	−	355	295	10	10
Nivalenol	−	371	281	10	15
Fusarenon-X	−	353	263	25	15

Calculation. Obtain peak areas from the resulting selected reaction monitoring chromatograms[4] to prepare a calibration curve, and calculate the amount of respective mycotoxins in the sample.

Note 1 When the analysis sample absorbs the extraction solvent and cannot be shaken, use 150 mL of the extraction solvent.
2 MultiSep 226 AflaZon+ (Romer Labs) or equivalents.
3 ZORBAX XDB-C18 (Agilent Technologies) or equivalents.
4 Example conditions for Quattro micro API Mass Analyzer (Waters).

<<Summary of analysis method>>
This is a simultaneous analysis method to extract aflatoxin B_1, B_2, G_1 and G_2, sterigmatocystin, zearalenone, T-2 toxin, neosolaniol, deoxynivalenol, nivalenol and fusarenon-X in feeds with acetonitrile- water (21:4), purify with a multifunctional cleanup (MFC) column, and quantitate by a liquid chromatograph- tandem mass spectrometer.

The accuracy of this method is currently inferior to individual analysis methods of respective mycotoxins by LC or LC-MS (or similar simultaneous analysis methods of mycotoxins); therefore if the analysis result is over the standard value, the result needs to be confirmed by individual analysis methods.

The flow sheet of the analysis method is shown in Figure 5.3.1-1.

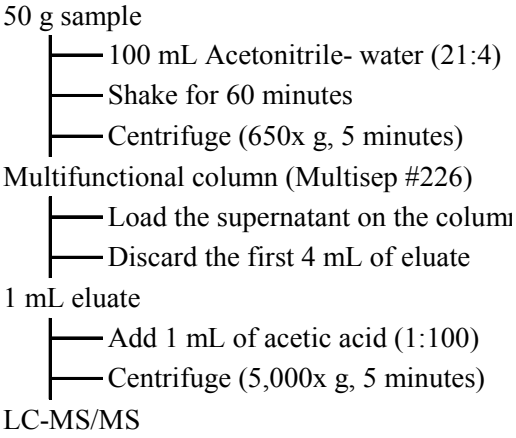


Figure 5.3.1-1 Flow sheet of the simultaneous analysis method for mycotoxins by liquid chromatography- tandem mass spectrometry

References:Rie Fukunaka, Hisaaki Hiraoka: Research Report of Animal Feed, **31**, 2 (2006) History in the Feed Analysis Standards [29] New

<<Analysis method validation>>

- Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (μg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Aflatoxin B_1	Corn	1~4	3	98.6~106.0	6.2
	Cattle formula feed	1~4	3	96.2~99.5	7.8
Aflatoxin B_2	Corn	1~4	3	101.4~105.5	6.4
	Cattle formula feed	1~4	3	94.2~100.8	7.5
Aflatoxin G_1	Corn	3~12	3	98.7~103.0	4.9
	Cattle formula feed	3~12	3	93.4~100.4	7.3
Aflatoxin G_2	Corn	3~12	3	100.3~103.0	5.8
	Cattle formula feed	3~12	3	97.4~101.3	9.1
Sterigmatocystin	Corn	10~40	3	97.5~109.3	15.1
	Cattle formula feed	10~40	3	99.6~101.4	6.2
Zearalenone	Corn	2~8	3	99.8~102.4	14.0
	Cattle formula feed	2~8	3	105.9~109.3	9.8
T-2 toxin	Corn	100~400	3	102.7~103.0	8.6
	Cattle formula feed	100~400	3	100.1~108.1	10.7
Deoxynivalenol	Corn	100~400	3	104.4~106.2	7.7
	Cattle formula feed	100~400	3	96.4~103.9	9.9
Nivalenol	Corn	300~1,200	3	99.6~106.6	11.3
	Cattle formula feed	300~1,200	3	91.8~101.8	12.5
Neosolaniol	Corn	100~400	3	101.8~110.3	13.0
	Cattle formula feed	100~400	3	91.1~92.6	12.4
Fusarenon-X	Corn	100~400	3	97.9~106.2	8.3
	Cattle formula feed	100~400	3	104.6~110.2	12.2

Collaborative study

Name of analyzed component	Sample type	Number of laboratories	Spike concentration (μg/kg)	Spike recovery (%) (measured value (μg/kg))	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Aflatoxin B_1	Corn	6	4	97.1	6.0	23.2	1.05
	Cattle formula feed	6	4	89.7	12.3	36.3	1.65
Aflatoxin B_2	Corn	6	4	100.0	7.9	26.2	1.19
	Cattle formula feed	5	4	99.1	3.5	35.2	1.60
Aflatoxin G_1	Corn	6	12	86.3	6.3	41.4	1.88
	Cattle formula feed	5	12	82.0	5.1	47.1	2.14
Aflatoxin G_2	Corn	6	12	93.8	5.7	28.5	1.30
	Cattle formula feed	6	12	85.3	17.1	37.1	1.69
Sterigmatocystin	Corn	6	40	113.3	7.0	11.6	0.53
	Cattle formula feed	5	40	113.9	7.0	17.4	0.79
Zearalenone	Corn	6	8+Natural contamination	(16.2)	13.0	14.6	0.66
	Cattle formula feed	6	8+Natural contamination	(27.9)	19.0	36.1	1.64
T-2 toxin	Corn	6	400	108.7	2.6	13.8	0.75
	Cattle formula feed	5	400	107.4	3.6	17.9	0.97
Deoxynivalenol	Corn	6	400+Natural contamination	(444.3)	4.5	5.6	0.31
	Cattle formula feed	5	400	112.8	5.2	17.6	0.96
Nivalenol	Corn	5	1,200	86.7	9.9	14.9	0.96
	Cattle formula feed	6	1,200	61.7	27.6	23.9	1.54
Neosolaniol	Corn	5	400	109.6	1.4	13.1	0.71
	Cattle formula feed	6	400	83.3	17.9	30.0	1.63
Fusarenon-X	Corn	5	400	104.4	6.2	11.3	0.62
	Cattle formula feed	4	400	105.6	5.8	5.8	0.32

- Lower limit of quantification: 1 μg/kg for aflatoxin B_1, B_2, G_1 and G_2, sterigmatocystin and zearalenone; 8 μg/kg for T-2 toxin and neosolaniol; 40 μg/kg for deoxynivalenol; 60 μg/kg for nivalenol; and 80 μg/kg for fusarenon-X (*SN* ratio)
- Lower limit of detection: 0.3 μg/kg for aflatoxin B_1, B_2, G_1 and G_2, sterigmatocystin and zearalenone; 2.4 μg/kg for T-2 toxin and neosolaniol; 12 μg/kg for deoxynivalenol; 18 μg/kg for nivalenol; and 24 μg/kg for fusarenon-X (*SN* ratio)

<<Notes and precautions>>

[1] Recovery of sterigmatocystin, zearalenone, T-2 toxin, deoxynivalenol, nivalenol and fusarenon-X is low in the fraction of 0-4 mL eluate.

[2] Ionization of aflatoxin B_1, B_2, G_1 and G_2, T-2 toxin, neosolaniol, deoxynivalenol, nivalenol and fusarenon-X is enhanced by the addition of acetic acid to the solution to be injected.

[3] Ion suppression of sterigmatocystin and zearalenone is prevented by diluting to be 2-fold.

[4] Examples of selected reaction monitoring (SRM) chromatograms are shown in Figure 5.3.1-2.

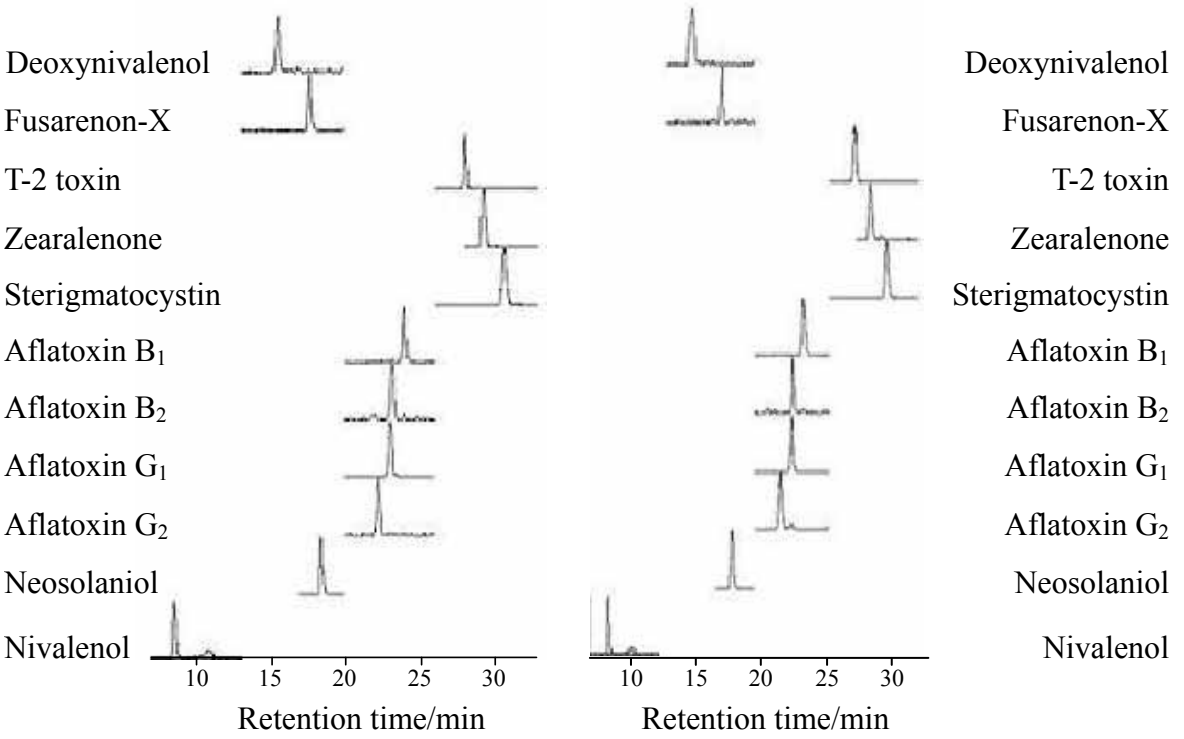


Figure 5.3.1-2 SRM chromatograms of mycotoxin-spiked formula feeds and corn (Left) formula feeds; (Right) corn

Spike concentration: 200 μg/kg for deoxynivalenol, fusarenon-X and T-2 toxin; 4 μg/kg for zearalenone; 20 μg/kg for sterigmatocystin; 2 μg/kg for aflatoxin B_1 and B_2; 6 μg/kg for aflatoxin G_1 and G_2; 200 μg/kg for neosolaniol; and 600 μg/kg for nivalenol.

2 Simultaneous analysis of aflatoxins by liquid chromatography [Feed Analysis standards, Chapter 5, Section 1 1.2, 2.2, 3.2 and 4.2]

Analyte compounds: aflatoxin B_1, B_2, G_1 and G_2 (4 components)

Scope of application: formula feeds and corn [2]

A. Reagent preparation

1) Aflatoxin B_1 standard stock solution. Weigh accurately 1 mg of Aflatoxin B_1 [$C_{17}H_{12}O_6$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the aflatoxin B_1 standard stock solution (1 mL of this solution contains 0.2 mg as aflatoxin B_1.). [3]
2) Aflatoxin B_2 standard stock solution. Weigh accurately 1 mg of Aflatoxin B_2 [$C_{17}H_{14}O_6$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the aflatoxin B_2 standard stock solution (1 mL of this solution contains 0.2 mg as aflatoxin B_2.). [3]
3) Aflatoxin G_1 standard stock solution. Weigh accurately 1 mg of Aflatoxin G_1 [$C_{17}H_{12}O_7$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the aflatoxin G_1 standard stock solution (1 mL of this solution contains 0.2 mg as aflatoxin G_1.). [3]
4) Aflatoxin G_2 standard stock solution. Weigh accurately 1 mg of Aflatoxin G_2 [$C_{17}H_{14}O_7$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the aflatoxin G_2 standard stock solution (1 mL of this solution contains 0.2 mg as aflatoxin G_2.). [3]
5) Aflatoxin mixture standard stock solution. Mix a certain amount of each of the Aflatoxin B_1, B_2, G_1 and G_2 standard stock solutions, dilute accurately with acetonitrile to prepare the aflatoxin mixture standard stock solution that contains 0.5 μg respectively as aflatoxin B_1, B_2, G_1 and G_2 in 1 mL. [4]

B. Quantification

Extraction. Weigh 50 g of an analysis sample,[Note 2] transfer it to a 300- mL stoppered amber Erlenmeyer flask, add 100 mL of acetonitrile- water (9:1), and extract by shaking for 30 minutes. Transfer the extract to a stoppered centrifuge tube, centrifuge at 650×*g* for 5 minutes, to obtain supernatant to be a sample solution to be subjected to column treatment.

Column treatment. Transfer 4.5 mL of the sample solution to a test tube, slowly push in a multifunctional column (for aflatoxin pretreatment), [Note 3] [5] to obtain eluate that passed the packing to be a sample solution to be subjected to derivatization.

Derivatization. Transfer 1 mL of the sample solution accurately to a 50- mL recovery flask,[6] [7] concentrate under vacuum in a water bath at 40°C or less to be almost dried up, and then dry up by nitrogen gas flow. Add accurately 0.1 mL of trifluoroacetic acid to the residue,[8] seal the recovery flask, and then leave at rest for 15 minutes, and further add accurately 0.9 mL of water- acetone (9:1) to the recovery flask and shake.[7] Transfer this solution to a plastic centrifuge tube (capacity: 1.5 mL), centrifuge at 5,000×*g* for 5 minutes, to obtain supernatant to be a sample solution to be subjected to analysis by liquid chromatography.

At the same time, transfer accurately several points within 2-40 μL of the aflatoxin mixture standard stock solution to 50- mL recovery flasks, respectively, and add accurately 0.1 mL of trifluoroacetic acid. Operate in the same way as the sample solution to prepare standard solutions that contain 1-20 ng as aflatoxin B_1, B_2, G_1 and G_2,

respectively, in 1 mL.

Liquid chromatography. Inject 20 μL each of the sample solution and respective standard solutions to a liquid chromatograph to obtain chromatograms.

Example of measurement conditions

Detector: Fluorescence detector (excitation wavelength, 365 nm; emission wavelength, 450 nm)

Column: Octadecylsilyl silica gel column[9] (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm) Note 4

Eluent: Water- methanol (3:2)

Flow rate: 0.8 mL/min

Column oven temperature: 40°C

Calculation. Obtain peak heights or peak areas from the resulting chromatograms[10] to prepare a calibration curve, and calculate the amount of aflatoxin B_1, B_2, G_1 and G_2 in the sample.

Note 1 Conduct the quantification procedure under protection from light.

2 The amount shall be 25.0 g for a sample that is difficult to be shaken.

3 Myco Sep 226 AflaZon+ (Romer Labs) or equivalents.

4 Mightysil RP-18 GP (Kanto Chemical) or equivalents.

<<Summary of analysis method>>

In this method, extract is purified by passing through a multifunctional cleanup (MFC) column and quantitated by a liquid chromatography with a fluorescence detector.

Analysis time can be significantly reduced because the time required for purification is short. In addition, stable peaks are obtained by derivatization of aflatoxin B_1 and aflatoxin G_1 and separation using LC (reversed-phase).

The flow sheet of the analysis method is shown in Figure 5.3.2-1.

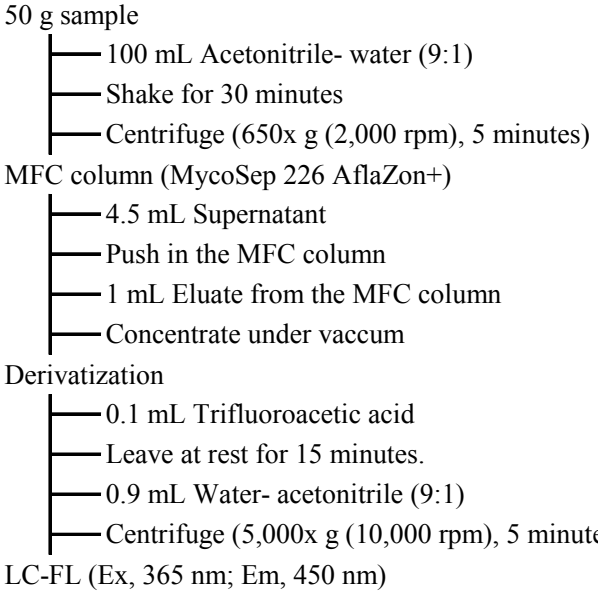


Figure 5.3.2-1 Flow sheet of the simultaneous analysis method for aflatoxins B_1, B_2, G_1, and G_2

References: Yuji Shirai, Yoshihiro Sekiguchi, Masayuki Shimomura, Toshiaki Hayakawa: Research Report of Animal Feed, 24, 10 (1999)

History in the Feed Analysis Standards [21] New, [26] Revision

<<Analysis method validation>>

- Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (μg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Aflatoxin B_1	Chicken formula feed	10~100	3	96.0~103.3	5.2
	Cattle formula feed	10~100	3	96.0~97.3	2.8
	Corn	10~100	3	92.0~100.3	4.0
Aflatoxin B_2	Chicken formula feed	10~100	3	92.0~95.0	4.8
	Cattle formula feed	10~100	3	90.7~100.3	4.7
	Corn	10~100	3	92.7~96.3	5.3
Aflatoxin G_1	Chicken formula feed	10~100	3	95.3~102.7	7.4
	Cattle formula feed	10~100	3	94.0~102.3	4.4
	Corn	10~100	3	95.3~101.0	4.2
Aflatoxin G_2	Chicken formula feed	10~100	3	92.7~96.0	8.0
	Cattle formula feed	10~100	3	91.0~103.7	5.0
	Corn	10~100	3	91.7~97.0	1.7

- Collaborative study

Name of analyzed component	Sample type	Number of laboratories	Spike concentration (μg/kg)	Spike recovery (%)	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Aflatoxin B_1	Chicken formula feed	7	20	98.5	2.9	3.8	0.17
Aflatoxin B_2	Chicken formula feed	7	20	90.6	2.8	7.3	0.33
Aflatoxin G_1	Chicken formula feed	7	20	100.0	2.6	3.0	0.14
Aflatoxin G_2	Chicken formula feed	7	20	93.5	2.6	4.9	0.22

- Lower limit of quantification: 0.5 μg/kg in a sample

<<Notes and precautions>>

[1] For common notes for analysis procedures for aflatoxins, see **<<Notes for aflatoxin analysis procedure>>** (p.6).

[2] This method can be applied to most of other feed materials. However, confirmation by LC-MS is required for feeds with a lot of interfering substances such as silage.

[3] 1-mg standard compounds of Aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 is available from Acros Organics, etc. Recently, solutions in solvents are available from Kanto Chemical, etc., which are safer to be handled.

For standards in the form of an aluminum-sealed vial that contains a predetermined amount (1 mg) of each aflatoxin, prepare each standard stock solution by injecting accurately 5 mL of acetonitrile with a syringe and dissolving aflatoxin by sonication etc. Store this standard stock solution in a cool dark place.

Additionally, the AOACI method (Mary W. Trucksess: Official Mehtod of AOAC International, 17th ed., Chapter 49, 3-5 (2000)) is useful as the preparation method for the standard solution.

[4] Take accurately 25 μL of each aflatoxin standard stock solution with a microsyringe, transfer to a 10- mL amber volumetric flask, mix, and add acetonitrile up to the

graduation line. Additionally, preferably store in a vial sealed with an aluminum seal in a cool dark place.

[5] Usage and an example are shown in Figure 5.3.2-2. This column is a mixture of reversed-phase resin, anion-exchange resin, and cation-exchange resin, and shall be used directly without conditioning.

Also, syringes such as MultiSep 228 AflaPat (Romer Labs), Autoprep MF-A (Showa Denko) can be used. In that case, elute at a flow rate around 1 mL/min, and subject the first 1 mL of eluate to derivatization. Before use, identify the elution flactions using the aflatoxin standard solution because the elution pattern differs between columns used.

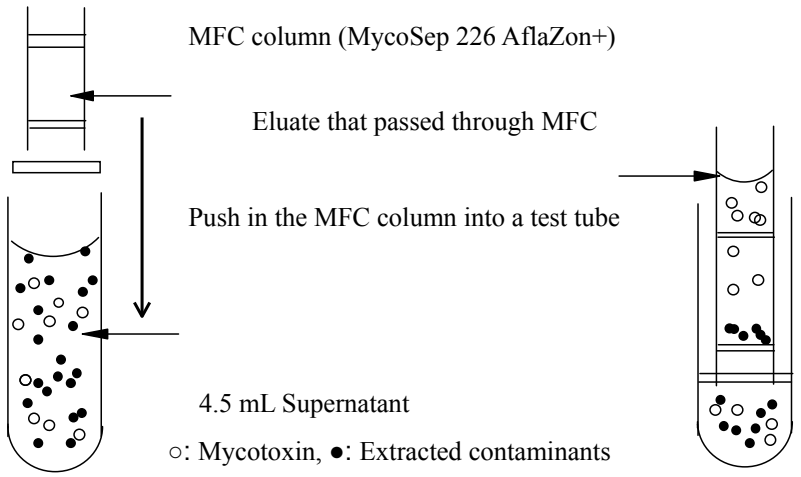


Figure 5.3.2-2 Purification method with a multifunctional cleanup (MFC) column (MycoSep 226 AflaZon+ etc.)

[6] Instead of a recovery flask, the sample can also be accurately transferred to a screw-capped vial or a test tube and evaporated with a centrifugal evaporator etc.

[7] A micropipette (dispenser) can be used.

[8] A micropipette (dispenser) can be used. After derivatization, it is needed to inject to LC after leaving at rest for at least an hour. Anhydrous trifluoroacetic acid had been used previously, however, it was changed to trifluoroacetic acid due to the variance in derivatization and poor reproducibility.

Even using trifluoroacetic acid, there was a difference in performance between the manufacturers. A product from Sigma was comparatively better.

The reaction formula of derivatization is shown in Figure 5.3.2-3.

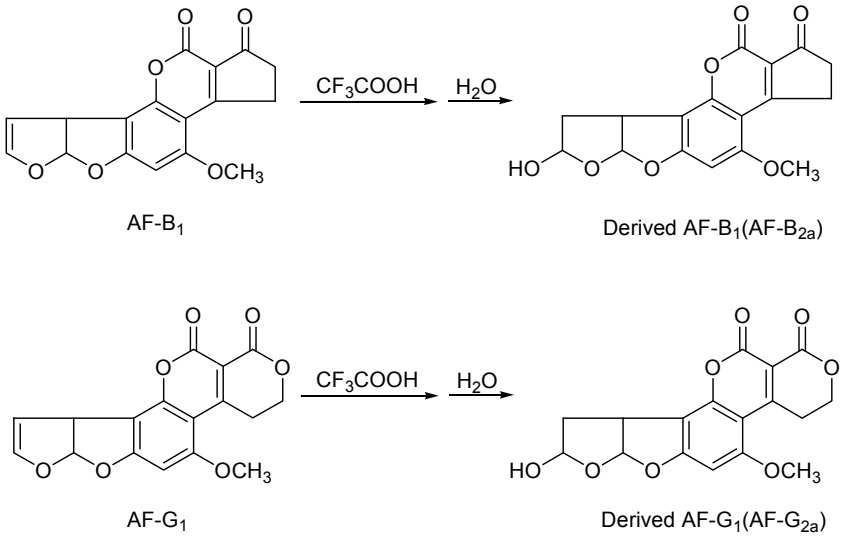


Figure 5.3.2-3 Reaction formula of derivatization

[9] The column to be used only needs to be one that uses packing treated by corresponding endcapping. The column used in the development of this analysis was Mightysil RP-18 GP.

[10] Examples of LC chromatograms of the aflatoxin mixture standard solution and the sample solution of naturally contaminated corn are shown in Figure 5.3.2-4.

In addition, peaks of aflatoxin B_1 and aflatoxin G_1 disappear by changing trifluoroacetic acid to water in derivatization. It can be thus confirmed that these peaks are derived from aflatoxin B_1 and aflatoxin G_1. Examples of LC chromatograms in that case are also shown in Figure 5.3.2-4.

Additionally, care should be taken that multiple peaks may appear by ethoxy- or methoxy-modification of the peak of the aflatoxin B_1 derivative by ethanol or methanol if methanol is used for washing of an microsyringe for LC or if a minute amount of alcohols is remained in the LC instrument or in reagents.

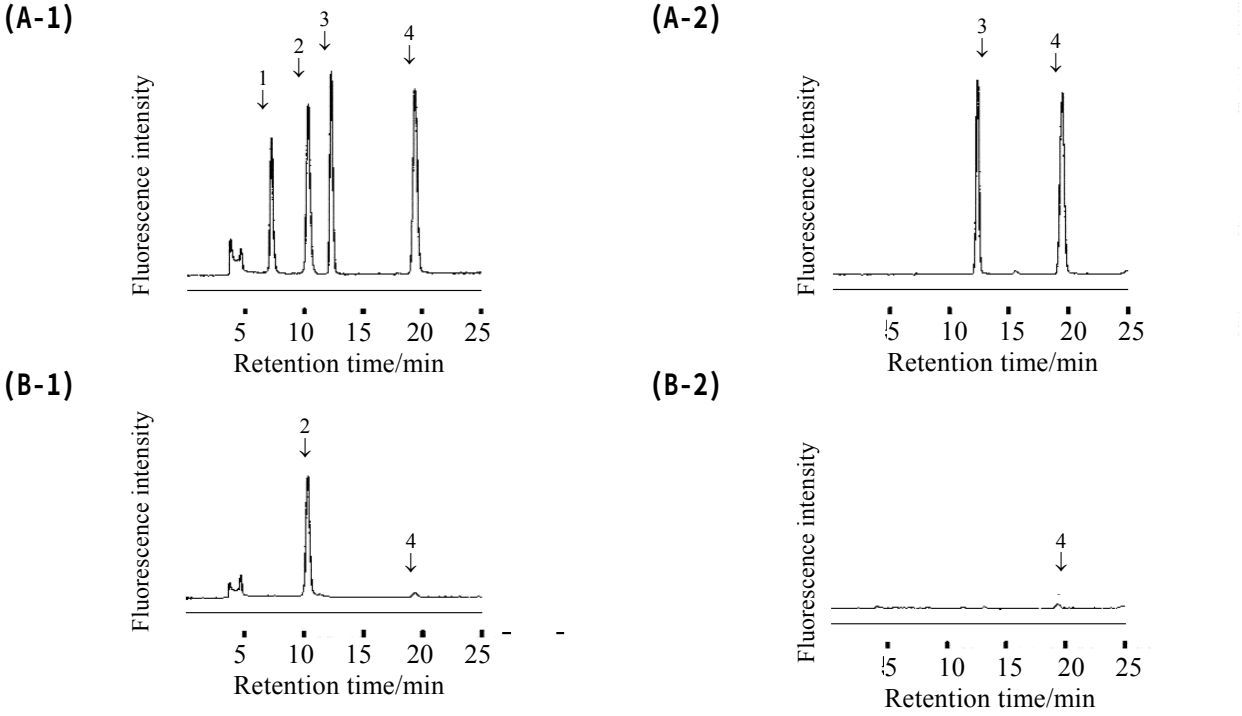


Figure 5.3.2-4 LC chromatograms of the mixture standard solution and a sample solution (quantitation and identification)

For measurement conditions, see the Example of measurement conditions described above.

Peaks 1, 2, 3 and 4 show the locations of peaks of aflatoxin G_1 derivative, aflatoxin B_1 derivative, aflatoxin G_2 and aflatoxin B_2, respectively.

(A-1) Aflatoxin mixture standard solution (0.2 ng as each aflatoxin, with derivatization)

(A-2) Aflatoxin mixture standard solution (0.2 ng as each aflatoxin, without derivatization)

(B-1) Sample solution of corn naturally contaminated with Aflatoxin B_1 and aflatoxin B_2 (with derivatization)

(B-2) Sample solution of corn naturally contaminated with Aflatoxin B_1 and aflatoxin B_2 (without derivatization)

3 Simultaneous analysis of aflatoxins by liquid chromatography - photochemical reactor [Feed Analysis standards, Chapter 5, Section 1 1.3, 2.3, 3.3 and 4.3]

Analyte compounds: Aflatoxin B_1, B_2, G_1 and G_2 (4 components)

Scope of application: Feeds except soybean meal

A. Reagent preparation

1) Aflatoxin B_1 standard stock solution. [1][2] Weigh accurately 1 mg of aflatoxin B_1 [$C_{17}H_{12}O_6$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the aflatoxin B_1 standard stock solution (1 mL of this solution contains 0.2 mg as aflatoxin B_1.).
2) Aflatoxin B_2 standard stock solution. Weigh accurately 1 mg of aflatoxin B_2 [$C_{17}H_{14}O_6$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the aflatoxin B_2 standard stock solution (1 mL of this solution contains 0.2 mg as aflatoxin B_2.).
3) Aflatoxin G_1 standard stock solution. Weigh accurately 1 mg of aflatoxin G_1 [$C_{17}H_{12}O_7$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the aflatoxin G_1 standard stock solution (1 mL of this solution contains 0.2 mg as aflatoxin G_1.).
4) Aflatoxin G_2 standard stock solution. Weigh accurately 1 mg of aflatoxin G_2 [$C_{17}H_{14}O_7$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the aflatoxin G_2 standard stock solution (1 mL of this solution contains 0.2 mg as aflatoxin G_2.).
5) Mixture standard solution. Before use, mix a certain amount of each of the aflatoxin B_1, B_2, G_1 and G_2 standard stock solutions, dilute accurately with acetonitrile- water (9:1), to prepare several mixture standard solutions that contain 2.5-20 ng respectively as aflatoxin B_1, B_2, G_1 and G_2 in 1 mL.

B. Quantification

Extraction. Weigh 50 g of an analysis sample, Note 2 transfer it to a 300- mL stoppered amber Erlenmeyer flask, add 100 mL of acetonitrile- water (9:1), and extract by shaking for 30 minutes. Filter the extract with filter paper (No. 5A), to obtain filtrate to be a sample solution to be subjected to column treatment.

Column treatment. Transfer 6 mL of the sample solution to a test tube, slowly push in a multifunctional column (for aflatoxin pretreatment), Note 3 [3] and discard the first 1 mL of eluate that passed the packing. Further push in the column above similarly to elute aflatoxin B_1, B_2, G_1 and G_2. After homogenizing the eluate, transfer a part of it to a plastic centrifuge tube (capacity: 1.5 mL), centrifuge at 6,000×g for 5 minutes, to obtain supernatant to be a sample solution to be subjected to analysis by liquid chromatography.

Liquid chromatography. Inject 20 μL each of the sample solution and respective mixture standard solutions to a liquid chromatograph to obtain chromatograms.

Example of measurement conditions

Detector: Fluorescence detector (excitation wavelength, 365 nm; emission wavelength, 450 nm)
Column: Octadecylsilyl silica gel column (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm) Note 4
Eluent: Water- methanol- acetonitrile (11:8:1)
Flow rate: 0.7 mL/min

Column oven temperature: 35°C
Photochemical reactor system [Note 5, 6]: A 245-nm low-pressure mercury lamp (15 W) irradiation system [4] with a reaction coil (0.25 mm in inner diameter, 10 min length) installed in a reaction coil holder

Calculation. Obtain peak heights or areas from the resulting chromatograms [5] to prepare a calibration curve, and calculate the amount of aflatoxin B_1, B_2, G_1 and G_2 in the sample.

Note 1 Conduct the quantification procedure under protection from light.
2 The amount shall be 25.0 g for a sample that is difficult to be shaken.
3 MycoSep 226 AflaZon+, MycoSep 228 AflaPat (Romer Labs) or equivalents.
4 Shodex C18M4E (Showa Denko) or equivalents.
5 Use PHRED (AURA industries) or equivalents, and connect between the column and the detector.
6 Peaks of aflatoxin B_1 and aflatoxin G_1 disappear when ultraviolet irradiation by the system is not conducted because fluorescent derivatives of aflatoxin B_1 and G_1 are not produced. This can be used for the identification of detected peaks.

<<Summary of analysis method>>
In this method, extract is purified by a multifunctional cleanup (MFC) column, and aflatoxin B_1 and G_1 are derivatized by post-column derivatization using a photochemical reactor to enhance fluorescence intensity to be quantitated. In addition, peaks of B_1 and G_1 on the chromatogram disappear by turning off the photochemical reactor, and this can be used for the confirmation of detection. Moreover, introduction to LC by the post-column method can reduce analysis time compared to pre-column derivatization.

If there are contaminants that cannot be separated by this method, a method using an immunoaffinity column (Section 1. I.1.7., p.15, in this chapter) is useful.

History in the Feed Analysis Standards [27] New

<<Analysis method validation>>
- Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (μg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Aflatoxin B_1	Chicken formula feed	5~40	3	84.0~96.9	7.1
	Cattle formula feed	5~40	3	79.3~98.3	4.3
	Corn	5~40	3	87.1~99.3	10.3
	Soybean meal	5~40	3	54.9~91.7	21.2
Aflatoxin B_2	Chicken formula feed	5~40	3	79.4~92.0	13.9
	Cattle formula feed	5~40	3	80.9~102.5	4.0
	Corn	5~40	3	89.0~94.0	4.2
	Soybean meal	5~40	3	79.4~96.6	7.9
Aflatoxin G_1	Chicken formula feed	5~40	3	77.2~93.3	7.1
	Cattle formula feed	5~40	3	78.5~89.3	8.1
	Corn	5~40	3	72.5~86.8	9.3
	Soybean meal	5~40	3	84.1~92.1	6.0
Aflatoxin G_2	Chicken formula feed	5~40	3	72.4~100.5	11.2
	Cattle formula feed	5~40	3	88.5~96.5	8.1
	Corn	5~40	3	78.0~94.9	4.5
	Soybean meal	5~40	3	91.5~104.7	3.5

- Collaborative study

Name of analyzed component	Sample type	Number of laboratories	Spike concentration (μg/kg)	Spike recovery (%) (measured value (μg/kg))	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Aflatoxin B_1	Cattle formula feed	7	5	110.8	3.2	11.6	0.53
	Corn	7	Natural contamination	(8.89)	6.7	17.3	0.79
Aflatoxin B_2	Cattle formula feed	7	5	94.6	1.9	9.5	0.43
Aflatoxin G_1	Cattle formula feed	7	5	108.0	2.8	15.9	0.72
Aflatoxin G_2	Cattle formula feed	7	5	87.0	3.7	9.0	0.41

- Lower limit of quantification: 1 μg/kg for aflatoxin B_1 and G_1; 0.5 μg/kg for aflatoxin B_2 and G_2 (*SN* ratio)
- Lower limit of detection: 0.5 μg/kg for aflatoxin B_1 and G_1; 0.2 μg/kg for aflatoxin B_2 and G_2 (*SN* ratio)

<<Notes and precautions>>

[1] For common notes for analysis procedures for aflatoxins, see <<Notes for aflatoxin analysis procedure>> (p.144).

[2] For purchase and preparation of standards, see 2 <<Notes and precautions>> [3] and [4] in this section.

[3] For a multifunctional cleanup (MFC) column, see [5] Usage and an example in 2 <<Notes and precautions>> in this section. Also, syringes such as MultiSep 226 AflaZon+ and

MultiSep 228 AflaPat (Romer Labs) can be used.

[4] Energy of the lamp of a photochemical reactor decreases even if it is not in use, therefore exchange for a newly purchased lamp when the peak intensity of the standard solution decreases. In addition, when the inner diameter or the length of the reaction coil is changed, confirm the peak intensity using the aflatoxin standard solution before use.

The reaction formula of derivatization is shown in Figure 5.3.3-1.

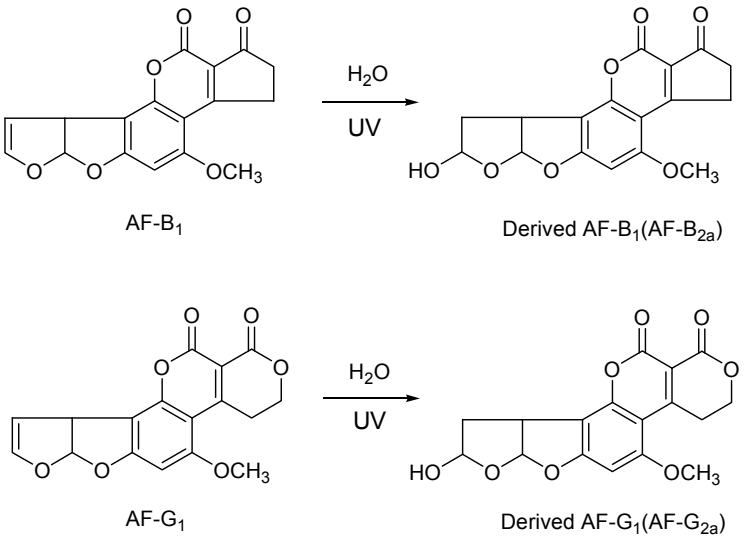
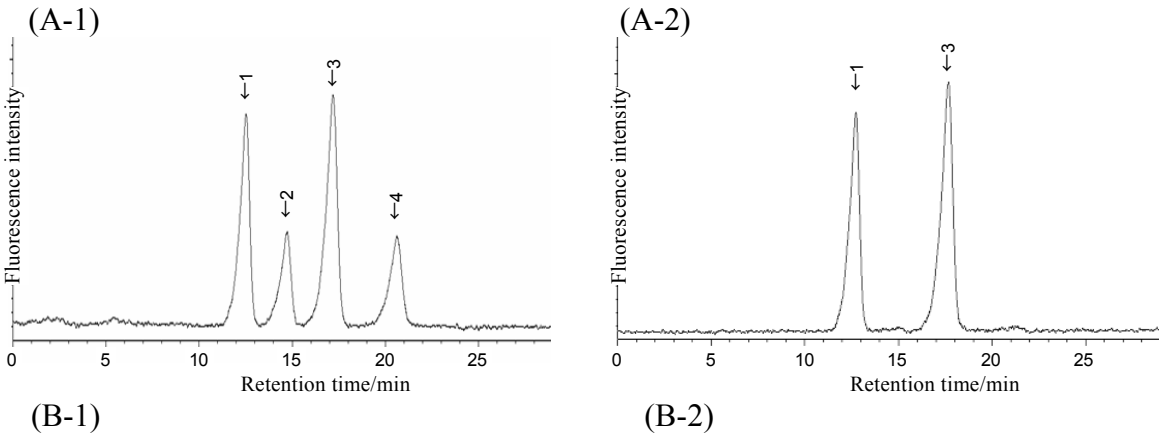


Figure 5.3.3-1 Reaction formula of derivatization

[5] Examples of chromatograms of the aflatoxin mixture standard solution and the sample solution of naturally contaminated corn are shown in Figure 5.3.3-2.

As this is a post-column reaction, elution of aflatoxins by LC is in the order of G_2, G_1, B_2, and B_1, and is different from 2. Simultaneous analysis of aflatoxins by liquid chromatography in this section.

In addition, peaks of aflatoxin B_1 derivative and G_1 derivative disappear by turning off the photochemical reactor. It can be thus confirmed that these peaks are derived from aflatoxin B_1 and aflatoxin G_1. Examples of LC chromatograms in that case are also shown in Figure 5.3.3-2.



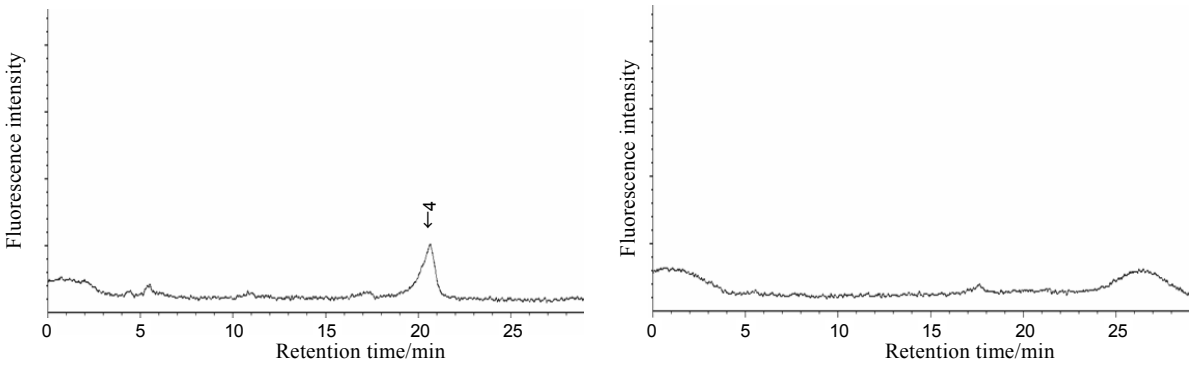


Figure 5.3.3-2 Chromatograms of the mixture standard solution and a sample solution (quantitation and identification)

For measurement conditions, see the Example of measurement conditions.

Peaks 1, 2, 3 and 4 show the locations of peaks of aflatoxin G_2, aflatoxin G_1 derivative, aflatoxin B_2 and aflatoxin B_1 derivative, respectively.

(A-1) Aflatoxin mixture standard solution (0.2 ng as each aflatoxin, with the photochemical reactor on)

(A-2) Aflatoxin mixture standard solution (0.2 ng as each aflatoxin, with the photochemical reactor off)

(B-1) Sample solution of corn naturally contaminated with aflatoxin B_1 (with the photochemical reactor on)

(B-2) Sample solution of corn naturally contaminated with aflatoxin B_1 (with the photochemical reactor off)

4 Fluorescence densitometry

4.1 Peanut meal[1] [Feed Analysis standards, Chapter 5, Section 1 1.4 (1)]

A. Reagent preparation

1) Aflatoxin mixture standard solution. Measure accurately 5 mg each of Aflatoxin B_1 [$C_{17}H_{12}O_6$], aflatoxin B_2 [$C_{17}H_{14}O_6$], aflatoxin G_1 [$C_{17}H_{12}O_7$] and aflatoxin G_2 [$C_{17}H_{14}O_7$], put in a 250-mL amber volumetric flask, respectively, and dissolve by the addition of benzene/acetonitrile (49:1). Add the same solvent to each volumetric flask up to the graduation line to prepare aflatoxin B_1, B_2, G_1 and G_2 standard stock solutions.[2] (1 mL each of these solutions contains 20 μg respectively as aflatoxin B_1, B_2, G_1 and G_2.)

   Before use, mix a certain amount of respective standard stock solutions, dilute accurately with benzene/acetonitrile (49:1) to prepare the aflatoxin mixture standard solution that contains 0.2 μg each of aflatoxin B_1, B_2, G_1 and G_2 in 1 mL.

2) Aflatoxin B_1 standard solution. Before use, dilute accurately a certain amount of the aflatoxin B_1 standard stock solution with benzene/acetonitrile (49:1) to prepare the aflatoxin B_1 standard solution that contains 0.2 μg of aflatoxin B_1 in 1 mL.

B. Quantification

Extraction. Weigh 20.0 g of an analysis sample, transfer it to a 500-mL separatory funnel, moisten by the addition of 10 mL of water, then add 100 mL of chloroform, and extract by shaking for 30 minutes.[3] Filter the extract with filter paper (2 types), to be a sample solution to be subjected to thin-layer chromatography.[4]

Thin-layer chromatography. Designate a line 2 cm distant from the edge of a thin-layer plate Note 1 as the baseline. Spot on this baseline a certain amount of the sample solution (10-20 μL), 10 μL of the aflatoxin mixture standard solution, and several points within 5-20 μL of the aflatoxin B_1 standard solution for the preparation of the calibration curve at intervals of 1.5 cm using microsyringes.[5] Place the thin-layer plate in a developing container, and develop in unsaturated atmosphere until the front of the developing solvent (chloroform -acetone -hexane (20:1:1)) reaches 10 cm or more above the baseline,[6] then remove the thin-layer plate from the container to air-dry.

Measurement. Measure fluorescence intensity of the developed spots of the aflatoxin B_1 standard solution as well as the developed aflatoxin B_1 spot of the sample solution with a spectro-fluorescence densitometer (excitation wavelength 365 nm, fluorescence wavelength 430 nm).

Calculation. Prepare a calibration curve from the fluorescence intensity of the aflatoxin B_1 standard solution, and calculate the amount of aflatoxin B_1 in the sample.

Note 1: Silica gel 70 plate -Wako (20×20 cm) (Wako Pure Chemicals) or equivalents[7]

<<Summary of analysis method>>

This method is a quantitative method mainly based on the AOACI method with modifications. It is included in the Feed Analysis Standards as the analysis method for aflatoxin B_1.

References: "Official Methods of Analysis of the AOAC Internatinoal", 18th Ed. 49.2.08, 49.2.09 (2005)

History in the Feed Analysis Standards [0] New

<<Notes>>

[1] See <<Notes on analytical procedures for aflatoxin analysis>> for notes on analytical procedures that are common in aflatoxin analysis.

[2] The method to standardize the accurate concentration of the aflatoxin standard stock solution is designated in Section 4 of this chapter.

[3] A 500-mL stoppered Erlenmeyer flask can also be used. Degas from time to time during shaking for extraction. Also, when extraction by a blender (or a homogenizer) is done instead of shaking for extraction, conduct the extraction procedure for about 5 minutes.

[4] If water is contained, dehydrate with an adequate amount of sodium sulfate (anhydrous).

[5] The diameter of a spot should be 5 mm or less.
Use a microsyringe with a blunt end of the tip of the needle.
For spotting, as there is a possible error due to the inner volume of the needle, check the drawn volume and the remaining volume before and after spotting to obtain the spot volume as the difference.
Drummond "Micro Caps" (micropipette) is convenient. The diameter of a spot should be 5 mm or less. In addition, take care not to damage the adsorbent of the thin-layer plate by the tip of the needle of the microsyringe.
The aflatoxin standard mixture solution is used to check separation of aflatoxin B_1 and B_2, G_1 or G_2 on a thin-layer plate.

[6] Use a glass or stainless developing container with a lid (approximate height 25 cm, width 15 cm, depth 30 cm) with a small inner container for the developing solvent (height 22 cm, width 5 cm, depth 5 cm). For development, add the developing solvent to the small inner container to the depth of 2 cm and place it in the developing container, and immediately insert a thin-layer plate to develop with light shielding.
Developing temperature should be 22-25°C.
If marked tailing or diffusing of a developed spot is observed, re-analyze using a thin-layer plate prepared separately.

[7] There are Merck silica gel 60TLC plate (5721) etc.

4.2 Cottonseed meal [Feed Analysis standards, Chapter 5, Section 1 1.4(2)]

A. Reagent preparation

1) Aflatoxin mixture standard solution and aflatoxin B_1 standard solution.[1] As described in (1) A.
2) Extraction solvent. Acetone -water -acetic acid (425: 75: 4)
3) Lead acetate solution. Dissolve 200 g of lead acetate (II) trihydrate to a suitable amount of water, add 3 mL of acetic acid, and further add water to be 1 L.
4) Silica gel. Dry silica gel for column chromatography (particle size 74-149 μm (200-100 mesh)) Note 1 at 110°C for 2 hours, add water equivalent to 1 v/w%, mix, and leave at rest overnight.

B. Quantification

Extraction. Weigh 25.0 g of an analysis sample, transfer it to a 500-mL separatory funnel, add 250 mL of extraction solvent, extract by shaking for 30 minutes, and filter with filter paper (2 types).

Transfer 125 mL of the filtrate to a 500-mL recovery flask, add 20 mL of lead acetate solution[2] and 25 mL of water, concentrate under vacuum in the water bath at 50°C to about 125 mL, and then let it cool. Transfer the concentrate to a stoppered 200-mL graduated cylinder using water, add water to be 200 mL, and filter with filter paper (2 types).[3]

Transfer 160 mL of the filtrate to a 250-mL separatory funnel, add 50 mL of chloroform, shake, and then leave at rest. Transfer the chloroform layer (lower layer) to an Erlenmeyer flask. Add 50 mL of chloroform to the remaining liquid and operate similarly, and combine the chloroform layer to the Erlenmeyer flask above.

Dehydrate the chloroform layer with a suitable amount of sodium sulfate (anhydrous),[4] and filter into a 200-mL recovery flask with filter paper (2 types). Wash the Erlenmeyer flask above and sodium sulfate sequentially with a small amount of chloroform, and add the wash through the filter paper above to the filtrate. Concentrate the filtrate under vacuum in the water bath at 50°C or less to 1-2 mL to be a sample solution to be subjected to column treatment.

Column treatment. Suspend 10 g of sodium sulfate (anhydrous), 15 g of silica gel, and 10 g of sodium sulfate (anhydrous) respectively, in diethyl ether -hexane (3:1),[5] and pour into a column (20 mm inner diameter), and elute so that the liquid level reaches to the height of 3 mm from the upper end of packing to prepare a column.

Load the sample solution on the column.[6] Wash the recovery flask that had contained the sample solution three times with 2 mL each of chloroform, add the wash to the column, and elute so that the liquid level reaches to the height of 3 mm from the upper end of packing. Moreover, add 150 mL of diethyl ether -hexane (3:1) to the column and elute similarly.

Place a 500-mL recovery flask under the column. Add 200 mL of chloroform -acetone (4:1) to the column to elute aflatoxin B_1. Concentrate the eluate under vacuum in the water bath at 50°C or less, and further dry up by the flow of nitrogen gas.[7] Add accurately a specified amount[8] of benzene -acetonitrile (49:1) to dissolve the residue to be a sample solution to be subjected to thin-layer chromatography.

Thin-layer chromatography.[9] As described in the section (1) B Thin-layer chromatography.

Measurement.[9] As described in the section (1) B Measurement.

Calculation.[9] As described in the section (1) B Calculation.

Note 1: Wako gel C-200 (Wako Pure Chemicals) or equivalents [10]

<<Summary of analysis method>>

See 4.1 <<Summary of analysis method>> in this section.

<<Notes and precautions>>

[1] The preparation method is the same as 4.1 A 1) and 2) in this section.

[2] This is used as a protein-removing agent. When heavy metal salts (such as Cu^{2+}, Pd^{2+}, Zn^{2+}, Cd^{2+} etc.) are added to an aqueous solution containing protein, these metal ions bind to protein to precipitate aggregated protein, which is removed by centrifugation or filtering.

[3] When filtering is difficult, add 4-5 g of diatomite as filter aid, mix well, filter or centrifuge, and then filter.

[4] Dehydration can be by passing a column of sodium sulfate (anhydrous). If dehydration is insufficient, the next column chromatography will be difficult.

[5] Add about 40 mL of diethyl ether -hexane (3:1), stir with a glass rod to be slurry.
Note, when diethyl ether is used, that peroxide in diethyl ether and water in diethyl ether may produce fluorescence on the thin-layer plate.

[6] Load the whole amount of the vacuum-concentrated sample solution on the column. A funnel with a long stem is convenient.

[7] To store in this state, cover with aluminum foil etc. and keep in a freezer (−18°C).

[8] Usually 2 mL is used.

[9] The procedure is the same as the corresponding section of 4.1 B in this section.

[10] Mallinckrodt Silic ARCC-7 (60-200 mesh), Merck #7734 (0.063-0.2 mm) or their equivalents can also be used.

4.3 Corn [Feed Analysis standards, Chapter 5, Section 1 1.4 (3)]

A. Reagent preparation

1) Aflatoxin mixture standard solution and aflatoxin B_1 standard solution.[1] As described in (1) A.
2) Silica gel.[2] As described in (2) A 4).

B. Quantification

Extraction. Weigh 50 g of an analysis sample, transfer it to a 500-mL separatory funnel, moisten by the addition of 25 mL of water, then add 250 mL of chloroform, and extract by shaking for 30 minutes.

Transfer the extract to an Erlenmeyer flask, dehydrate with a suitable amount of sodium sulfate (anhydrous), and filter with filter paper (2 types). Transfer 50 mL of the filtrate to a 200-mL recovery flask, and concentrate under vacuum in the water bath at 50°C or less to 1-2 mL to be a sample solution to be subjected to column treatment.

Column treatment. Suspend 10 g of sodium sulfate (anhydrous), 15 g of silica gel, and 10 g of sodium sulfate (anhydrous) respectively, in chloroform,[3] and pour into a column (20 mm inner diameter), and elute so that the liquid level reaches to the height of 3 mm from the upper end of packing to prepare a column.

Load the sample solution on the column.[4] Wash the recovery flask that had contained the sample solution three times with 2 mL each of chloroform, add the wash to the column, and elute so that the liquid level reaches to the height of 3 mm from the upper end of packing. Moreover, add 150 mL of hexane and 150 mL of diethyl ether sequentially to the column and elute similarly.

Place a 200-mL recovery flask under the column. Add 150 mL of chloroform -methanol (9:1) 150 mL to the column to elute aflatoxin B_1. Concentrate the eluate under vacuum in the water bath at 50°C, and further dry up by the flow of nitrogen gas.[5]

Add accurately a specified amount[6] of benzene -acetonitrile (49:1) to dissolve the residue to be a sample solution to be subjected to thin-layer chromatography.

Thin-layer chromatography.[7] As described in the section (1) B Thin-layer chromatography.

Measurement.[7] As described in the section (1) B Measurement.

Calculation[7] As described in the section (1) B Calculation.

<<Summary of analysis method>>

See 4.1 <<Summary of analysis method>> in this section.

<<Notes and precautions>>

[1] The preparation method is the same as 4.1 A 1) and 2) in this section.
[2] The preparation method is the same as 4.2 A 4) in this section.
[3] See 4.2 <<Notes and precautions>> [4] in this section.
[4] See 4.2 の<<Notes and precautions>> [5] in this section.
[5] See 4.2 の<<Notes and precautions>> [6] in this section.
[6] See 4.2 の<<Notes and precautions>> [7] in this section.
[7] See the corresponding section of 4.1 B in this section.

4.4 Formula feeds [Feed Analysis standards, Chapter 5, Section 1 1.4 の(4)]

A. Reagent preparation

1) Aflatoxin mixture standard solution and aflatoxin B_1 standard solution[1] As described in (1) A.
2) Lead acetate solution[2] As described in (2) A 3).
3) Silica gel.[2] As described in (2) A 4).
4) Acidic alumina. Acidic alumina for column chromatography (particle size 63-200 μm (230-70 mesh)) Note 1

B. Quantification

Extraction. Weigh 50 g of an analysis sample, transfer it to a 500-mL separatory funnel, moisten the sample by the addition of 70 mL of hydrochloric acid (0.1 mol/L),[3] then add 250 mL of chloroform, and extract by shaking for 30 minutes.

Transfer the chloroform layer[4] (lower layer) to an Erlenmeyer flask, dehydrate with a suitable amount of sodium sulfate (anhydrous), and filter with filter paper (2 types) to be a sample solution to be subjected to purification.

Purification. Transfer 100 mL of sample solution to a 500-mL recovery flask, and concentrate under vacuum in the water bath at 50°C to be nearly dried up. Dissolve the residue by the addition of 20 mL of acetone, 60 mL of water, and 20 mL of lead acetate solution 20 mL, leave at rest for 5 minutes, and filter this solution with filter paper (2 types).

Transfer 50 mL of the filtrate to a 250-mL separatory funnel, add 50 mL of chloroform, shake, and then leave at rest. Transfer the chloroform layer (lower layer) to an Erlenmeyer flask. Add 50 mL of chloroform to the remaining liquid and operate similarly, and combine the chloroform layer to the Erlenmeyer flask above.

Dehydrate the chloroform layer with a suitable amount of sodium sulfate (anhydrous), and filter into a 200-mL recovery flask with filter paper (2 types). Wash the Erlenmeyer flask above and sodium sulfate sequentially with a small amount of chloroform, and filter the wash similarly and combine it with the filtrate. Concentrate the filtrate under vacuum in the water bath at 50°C to 1-2 mL to be a sample solution to be subjected to column treatment.

Column treatment. Suspend 5 g of acidic alumina, 15 g of silica gel and 10 g of sodium sulfate (anhydrous) 10 g respectively in chloroform, and pour into a column (20 mm inner diameter), and elute so that the liquid level reaches to the height of 3 mm from the upper end of packing to prepare a column.

Load the sample solution on the column. Wash the recovery flask that had contained the sample solution three times with 2 mL each of chloroform, add the wash to the column,[5] and elute so that the liquid level reaches to the height of 3 mm from the upper end of packing. Moreover, add 100 mL of benzene -acetic acid (9:1) and 150 mL of diethyl ether -hexane[6] (3:1) sequentially to the column and elute similarly.

Place a 500-mL recovery flask under the column. Add 200 mL of chloroform -acetone (3:1) to the column to elute aflatoxin B_1. Concentrate the eluate under vacuum in the water bath at 50°C to be almost dried up, and further dry up by the flow of nitrogen gas. Then add accurately a specified amount of benzene -acetonitrile (49:1) to dissolve the residue to be a sample solution to be subjected to thin-layer chromatography.

Thin-layer chromatography.[7] As described in the section (1) B Thin-layer chromatography.

Measurement.[7] As described in the section (1) B Measurement.

Calculation.[7] As described in the section (1) B Calculation.

Note 1: Aluminiumoxid 90 aktiv Art. 1078 (Merck) or equivalents

<<Summary of analysis method>>

See 4.1 **<<Summary of analysis method>>** in this section.

<<Notes and precautions>>

[1] The preparation method is the same as 4.1 A 1) and 2) in this section.

[2] The preparation method is the same as sections in 4.2 A in this section.

[3] The sample is moistened with 70 mL of 0.1 mol/L hydrochloric acid in advance to increase the extraction ratio of aflatoxin. Other than 0.1 mol/L hydrochloric acid, 2% potassium dihydrogen phosphate solution, etc. have a similar effect.

[4] Separation between the chloroform layer and the water layer is especially poor in feeds that contain syrup or skim milk. In such a case, the chloroform layer can preferably be taken after centrifugation at about 650×g.

[5] Washing with benzene -acetic acid is effective for the removal of most of interfering substances.

[6] Washing with diethyl ether -hexane washes away excess benzene -acetic acid, and removes blue fluorescence substances other than aflatoxin that was not washed with benzene -acetic acid.

[7] The procedure is the same as the corresponding section of 4.1 B in this section.

[Other analysis methods]

5 Screening

Scope of application: Formula feeds and materials

A. Reagent preparation

1) Extraction solvent: acetone -water (17:3)
2) Precipitation reagent solution. Dissolve 20 g of iron chloride (III) (anhydrous) in water to be 300 mL. To 30 mL of this solution, add 170 mL of sodium hydroxide solution (0.8 w/v%) (prepare before use.).
3) Washing solution. Dissolve 1.12 g of potassium hydroxide and 10 g of potassium chloride in water to be 1 L.
4) Packing for column chromatography.
   i) Magnesium silicate. Synthetic magnesium silicate (particle size 74-149 μm (200-100 mesh))Note 1
   ii) Silica gel. Silica gel for column chromatography (particle size 63-200 μm (230-70 mesh))Note 2
   iii) Neutral alumina. Neutral alumina for column chromatography (particle size 63-200 μm (230-70 mesh))Note 3
   iv) Sodium sulfate (anhydrous)

   Dry these magnesium silicate, silica gel, neutral alumina and sodium sulfate (anhydrous) at 110-120°C for 2 hours before use.

B. Operation

Extraction. Weigh 50 g of an analysis sample, transfer it to a 500-mL separatory funnel, add 250 mL of extraction solvent, extract by shaking for 30 minutes[1], and filter with filter paper (2 types).

Transfer 150 mL of the filtrate to a beaker. Add 3 g of copper carbonate (II) (alkaline) and stir. Further add 200 mL of the precipitation reagent solution,[2] stir, and leave at rest. Transfer the supernatant to a 300-mL centrifuge tube, centrifuge at 2,000 rpm for 5 minutes, and filter the supernatant with filter paper (2 types). Transfer 150 mL of the filtrate to a 500-mL separatory funnel, add 150 mL of sulfuric acid (1:3,000) and 10 mL of chloroform, shake vigorously and then leave at rest. Transfer the chloroform layer (lower layer) to a 200-mL separatory funnel, add 100 mL of the washing solution, shake mildly[3] and then leave at rest.

Transfer the chloroform layer[4] (lower layer) to a beaker, dehydrate by the addition of a suitable amount of sodium sulfate (anhydrous), and the supernatant is subjected to column chromatography as a sample solution.

Column chromatography. Pack the bottom of a column (6 mm inner diameter)[5] with glass wool, then with sodium sulfate (anhydrous) to the height of 8-10 mm, magnesium silicate to 8-10 mm, silica gel to 16-20 mm, neutral alumina to 8-10 mm and sodium sulfate (anhydrous) to 8-10 mm sequentially by the dry method, and pack the top end with glass wool to prepare a column.

Load 2 mL of the sample solution on the column. Elute so that the liquid level reaches to the height of 3 mm from the upper end of packing. Then add 3 mL of chloroform -acetone (9:1) and elute similarly.

Determination. Place the column in the dark and illuminate by ultraviolet light (wavelength 365 nm).[6] If there is aflatoxin, a blue fluorescent band is observed at the upper end of the magnesium silicate layer .[7]

Note 1: Florisil (Floridin) or equivalents
2: Kiselgel 60 Art. 7734 (Merck) or equivalents
3: Aluminiumoxid 90 aktiv neutral Art. 1077 (Merck) or equivalents

<<Summary of analysis method>>

This method determines the presence of absence of aflatoxin contamination in feeds (screening), by extracting aflatoxin in a sample with extraction solvent, purifying it by liquid-liquid partition, developing it with a column, and illuminating the column after development by ultraviolet light (wavelength 365 nm) to confirm the presence or absence of a blue fluorescent band (if there is aflatoxin, a blue fluorescent band is observed at the upper end of the magnesium silicate layer).

This is applicable to formula feeds and feed materials (corn, peanut meal, cottonseed meal etc.) with the aflatoxin concentration (concentration in feed) of 10 μg/kg or more. As liquid chromatography instruments seem to have become sufficiently common, this method was excluded from the Feed Analysis Standards.

References: Romer, T. R.: J. AOAC., 58, 500 (1975)
History in the Feed Analysis Standards [0] New, [31] Abolished

<<Notes and precautions>>

[1] Instead of extraction by a shaker for 30 minutes, extraction by a blender (homogenizer) for 3 minutes will do.

[2] Copper carbonate (II) (alkaline) and iron chloride (III) (anhydrous) are used as precipitation reagents. As a way of cleanup, interfering substances in the sample are precipitated by divalent metal ions (Pb^{2+}, Cu^{2+}, Fe^{2+} etc.) to be removed by filtration.

[3] Note that vigorous shaking will lead to emulsification and difficulty in separation.

[4] When the chloroform layer is suspended, transfer the suspended layer to a 200-mL separatory funnel, add 50 mL of sulfuric acid (1:3,000) to this and mix, and leave at rest to take the chloroform layer. Add to this a suitable amount of sodium sulfate (anhydrous) to dehydrate, and the supernatant is subjected to column chromatography as a sample solution.

[5] A column for vitamin B_1 quantification (See Figure 8.1.11-4 (p.362) in <<Notes and precautions>> in Chapter 8, Section 1 11 Decoquinate 1.2.2 [2]) is convenient.

[6] Such as a UV light (Manaslu light from Manaslu Co. Ltd), a mini cabinet CM type (Irie Shokai Co. Ltd.).
To illuminate an ultraviolet light, make sure to wear clear protective glasses against ultraviolet light.

[7] When the activity of alumina and silica gel is decreased, interfering pigments and fluorescent substances may develop to the magnesium silicate layer and conceal the fluorescent band of aflatoxin. Also, when the activity of magnesium silicate is decreased, the fluorescent band of aflatoxin diffuses to the magnesium silicate layer.
When a blue fluorescent band is diffused to the magnesium silicate layer, it cannot be decided that the fluorescence is caused by aflatoxin.
If necessary, develop 20 ng of aflatoxin B_1 (chloroform solution) in a separately prepared column, and judge by comparison to this column for the location of the fluorescent band and fluorescence color.

6 Quantification of aflatoxin B1 by international standard

ISO 14718 (1998) Animal feeds- Quantification of aflatoxin B_1 in formula feeds
Flow sheet of the analysis method

50.0 g sample
- 20 g celite, 250 mL chloroform, 25 mL water
- Shake for 30 minutes
- Filter

Florisil cartridge (washed with 10 mL of chloroform and degassed in advanc
- Load 50 mL of the filtrate and elute by natural flow.
- Wash with 5 mL of chloroform and 20 mL of methanol.
- Elute Aflatoxin B1 with 50 mL of acetone/ water (49:1)
- Concentrate under vacuum (40-50°C, to about 0.5 mL)
- Dissolve in 1 mL of methanol and add 4 mL of water

C_{18} cartridge (washed with 10 mL of methanol and 10 mL of water in advan
- Load the sample solution
- Wash with 5 mL of methanol/ water (4:1) (twice)
- Elute with 25 mL of methanol/ water (1:4)
- Elute Aflatoxin B_1 with 25 mL of water/ acetone (17:3)

Fill up to be 50 mL

LC-FL

7 Aflatoxin B_1 test in grains, beans, nuts and seeds, and spices [Notification No. 0728003 dated July 28, 2008 from the Inspection and Safety Division, Department of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare]

Scope of application: Grains, beans, nuts and seeds, and spices

When the analysis result is positive by high performance liquid chromatography shown below, identification is conducted by high performance liquid chromatography/ mass spectrometry etc.

A. Multifunctional column method

1 Instruments and devices

A rotary evaporator, a high performance liquid chromatograph (with a fluorescence detector) and a cartridge column for cleanup (multifunctional column packed with a mixture of reversed phase resin, anion exchange resin, and cation exchange resin[1] [2]) are used.

2 Reagents and test solutions

Except those listed below, use those shown in the Standards and Criteria for Food and Food Additives, etc. (Notification No. 370 of Ministry of Health and Welfare, 1959) Part I Food D Monographs ○ Grains, Beans, Fruits, Vegetables, Nuts and Seeds, Tea and Hop, 2 Tests for Ingredient Specifications of Grains, Beans, Fruits, Vegetables, Nuts and Seeds, Tea and Hop (2) Reagents and Test Solutions.

Trifluoroacetic acid (store in a cool and dark place)

Filter paper: Whatman No.5 or equivalents

3 Standards

Preparation of the aflatoxin B_1 standard stock solution.[3] Weigh accurately an amount of aflatoxin B_1 to prepare the final standard solution (2.5 ng/mL) with toluene/ acetonitrile (9:1) in a way like as follows. To a certified 1.0-mg weight of, or accurately weighed 1.0 mg of aflatoxin B_1 in a container, add toluene/ acetonitrile (9:1) or 50 mL of acetonitrile, stir vigorously to be the standard stock solution (20 μg/mL). Seal the standard stock solution, cover with aluminum foil, and store in a refrigerator. Take 0.5 mL of the standard stock solution, and fill up to be 200 mL with toluene/ acetonitrile (9:1) or acetonitrile to be the standard solution I (50 ng/mL). Take 1.0 mL of the standard solution I, and fill up to be 20 mL with toluene/ acetonitrile (9: 1) or acetonitrile to prepare the final standard solution (2.5 ng/mL).

4 Preparation of the test solution and the standard solution.

i Grains, beans and nuts and seeds[4]

Weigh 50 g of a ground and homogenized sample into a blender container or a stoppered Erlenmeyer flask, etc. Add 100 mL of acetonitrile/ water (9:1) to this, blend for 5 minutes or shake for 30 minutes, and filter with filter paper or centrifuge. Inject 5 mL of the filtrate carefully to a multifunctional column[1] [2] and elute at a flow rate of 1.0 mL per minute. Collect about 1 mL of the first part of eluate[5] into a test tube to be a test solution.

Take accurately 0.5 mL of the test solution to a screw-capped vial or a stoppered test tube or centrifuge tube, and remove solvent by nitrogen flow or by using an evaporator. Add 0.1 mL of trifluoroacetic acid to the residue, seal and shake vigorously. Leave at rest at room temperature in a dark place for 15 minutes, and then add 0.9 mL of acetonitrile/ water (1:9) to be a test solution for high performance liquid chromatography.[6]

Separately take 1.0 mL of the final standard solution (accurately measured) to a screw-capped vial or a stoppered test tube or centrifuge tube, dry up by nitrogen flow or by an evaporator under vacuum, then add 0.1 mL of trifluoroacetic acid, respectively, and seal and stir vigorously. Leave at rest at room temperature in a dark place for 15 minutes, and then add 0.9 mL of acetonitrile/ water (1:9) to be the standard solution for high performance liquid chromatography for Grains, beans and nuts and seeds.

ii Spices[7]

Weigh 50 g of a ground and homogenized sample into a blender container or a stoppered recovery flask, etc. Add 400 mL of acetonitrile/ water (9:1) to this, blend for 5 minutes or shake for 30 minutes, and filter with filter paper or centrifuge. Inject 5 mL of the filtrate carefully to a multifunctional column[1] and elute at a flow rate of 1.0 mL per minute. Collect about 1 mL of the first part of eluate[5] into a test tube to be a test solution.

Take accurately 0.5 mL of the test solution to a screw-capped vial or a stoppered test tube or centrifuge tube, and remove solvent by nitrogen flow or by using an evaporator. Add 0.1 mL of trifluoroacetic acid to the residue, seal and shake vigorously. Leave at rest at room temperature in a dark place for 15 minutes, and then add 0.4 mL of acetonitrile/ water (1:9) to be a test solution for high performance liquid chromatography.[6]

On the other hand, take accurately 0.25 mL of the final standard solution to a screw-capped vial or a stoppered test tube or centrifuge tube, dry up by nitrogen flow or by an evaporator under vacuum, then add 0.1 mL of trifluoroacetic acid, and seal

and stir vigorously. Leave at rest at room temperature in a dark place for 15 minutes, and then add 0.4 mL of acetonitrile/ water (1:9) to be the standard solution for high performance liquid chromatography for spices.

5 Operation

a High performance liquid chromatography analysis[8] [9] [10]

Test 20 μL each of the test solution for high performance liquid chromatography and the standard solution for high performance liquid chromatography under the following conditions. Qualify by comparing the retention time of a peak on the chromatogram obtained from the test solution to the peak of the standard.

The result is judged to be positive when the peak height or peak area obtained from the measurement of the test solution for high performance liquid chromatography exceeds the peak height or peak area obtained from the measurement of the standard solution for high performance liquid chromatography.

Operation conditions

Column packing:	Use octadecylsilyl silica gel (particle size 3-5 μm).[11]
Column:	4.6 mm in inner diameter, 150 mm or 250 mm in length
Column temperature:	40°C
Mobile phase:	Use acetonitrile/ methanol/ water (1: 3: 6).
Flow rate:	1.0 mL/min
Detection wavelength:	Measure at the excitation wavelength of 365 nm, fluorescence wavelength of 450 nm.
Injection volume:	20 μL

b Identification by high performance liquid chromatography mass spectrometry

Identification is conducted using 0.4 mL of the remaining test solution obtained in A.4 after using 0.5 mL in the high performance liquid chromatography analysis.

Example of measurement

Operation conditions

Column packing:	Octadecylsilyl silica gel for column chromatography (particle size 5 μm)[11] is used.
Column:	2.0 mm in inner diameter, 150 mm in length
Column temperature:	40°C
Mobile phase:	Acetonitrile/ methanol/ 10 mmol/L ammonium acetate (2: 6: 15) is used.
Flow rate: 0.2 mL/min	
Detection wavelength:	Measurement is conducted at excitation wavelength 365 nm and fluorescence wavelength 450 nm.
Injection volume:	5-10 μL

Mass spectrometer conditions

Ionization mode:	ESI-positive
Monitor ion:	(*m*/*z*) aflatoxin B_1, 313

6 Treatment of aflatoxin B_1

Devices, pretreatment column, samples etc, used in the experiment should be treated after immersing in 1 %(v/v) sodium hypochlorite for 2 hours or more.

B. Immunoaffinity column method[12]

1 Instruments and devices

As described in A.1.
Additionally use an immunoaffinity column as a cleanup column.[13]

2 Reagents and test solutions
As described in A.2, except:
use filter paper Whatman No.4 or equivalents, and glass fiber filter paper Whatman 934AH or equivalents. Phosphate buffered saline (pH 7.4, hereinafter referred to as "PBS") - dissolve 0.20 g KCl, 0.20 g KH_2PO_4, 1.16 g anhydrous Na_2HPO_4 (or 2.92 g $Na_2HPO_4 \cdot 12H_2O$), 8.00 g NaCl in 900 mL of water, then adjust with 0.1 mol/L HCl or NaOH to pH 7.4, and fill up to be 1 L.[14] Polyoxiethylene (20) sorbitan monolaurate (those equivalent to ICI trademark Tween20, hereinafter referred to as "Tween20")

3 Standards
As described in A.3.

4 Preparation of the test solution and the standard solution
i Grains, beans and nuts and seeds[4]
Weigh 50 g of a ground and homogenized sample into a blender container or a stoppered Erlenmeyer flask, etc. Add 100 mL of acetonitrile/ water (9:1)[15] to this, blend for 5 minutes or shake for 30 minutes, and filter with filter paper or centrifuge. Take accurately 5.0 mL of the filtrate, and add PBS[14] to be 50.0 mL. Mix well, and filter with glass fiber filter paper,[16] and take accurately 10.0 mL of the filtrate and inject into an immunoaffinity column.[17] After eluting all of the filtrate, wash the column with 10 mL or more of PBS and 10 mL or more of purified water,[18] and pressurize to sufficiently clear out water in the column.[19] Then elute with 3 mL of acetonitrile,[20] and fill up with acetonitrile to be accurately 5.0 mL, and mix well to be a test solution.
Take accurately 2.5 mL of the test solution to a screw-capped vial or a stoppered test tube or centrifuge tube, and remove solvent by nitrogen flow or by using an evaporator.[21] Add 0.1 mL of trifluoroacetic acid to the residue, seal and shake vigorously. Leave at rest at room temperature in a dark place for 15 minutes, and then add 0.9 mL of acetonitrile/ water (1:9) to be a test solution for high performance liquid chromatography.
Preparation of the final standard solution is as described in A.4 i.
ii Spices
Weigh 50 g of a ground and homogenized sample into a blender container or a stoppered recovery flask, etc. Add 400 mL of acetonitrile/ water (9:1)[15] to this, blend for 5 minutes or shake for 30 minutes, and filter with filter paper or centrifuge. Take accurately 5.0 mL of the filtrate, and add PBS containing 2% Tween20[22] to be 50.0 mL. Mix well, and filter with glass fiber filter paper if necessary,[16] and take accurately 10.0 mL of the filtrate and inject into an immunoaffinity column.[17] After eluting all of the filtrate, wash the column with 10 mL or more of PBS containing 0.01% Tween20 and 10 mL or more of purified water,[23] and pressurize to sufficiently clear out water in the column.[19] Then elute with 3 mL of acetonitrile,[20] and fill up with acetonitrile to be accurately 5.0 mL, and mix well to be the test solution.
Take accurately 2.5 mL of the test solution to a screw-capped vial or a stoppered test tube or centrifuge tube, and remove solvent by nitrogen flow or by using an evaporator.[21] Add 0.1 mL of trifluoroacetic acid to the residue, seal and shake vigorously. Leave at rest at room temperature in a dark place for 15 minutes, and then

add 0.4 mL of acetonitrile/ water (1:9) to be a test solution for high performance liquid chromatography.
Preparation of the final standard solution is as described in A. 4 ii.

5 Operation

a High performance liquid chromatography analysis

As described in A.5 a.

b Identification by high performance liquid chromatography/ mass spectrometry

Identification is conducted using 2.0 mL of the remaining test solution obtained in B.4 after using 2.5 mL in the high performance liquid chromatography analysis.

6 Treatment of aflatoxin B_1

As described in A. 6.

<<**Notes**>> [These Notes are part of the Notification, and the original text is quoted.]

[1] Syringe type multifunctional columns MultiSep #228 (Romer Labs) and Autoprep MF-A (Showa Denko) etc. can be used. Use directly without conditioning. In addition, as the elution pattern is different by the column used, check the elution volume in advance using the standard solution. The multifunctional column to be used should be confirmed in advance that the recovery of aflatoxin B_1 is 90% or over (the method listed in AOAC (Mary W. Trucksess: Official Methods of Analysis of AOAC International (18th Edition) Chapter 49, p.26-27, (2005)) is useful.

[2] Plunger type multifunctional columns MycoSep #226 and MycoSep #228 (Romer Labs) etc. also can be used.

[3] As aflatoxin is a strong carcinogen, its careful handling is required. Accurately weighed and commercially available aflatoxin from Sigma, etc. is convenient. In addition, for the preparation of the standard solution, the method listed in AOAC (Mary W. Trucksess: Official Methods of Analysis of AOAC International (18th Edition) Chapter 49, p.3-5, (2005)) is useful.

[4] Employ the method in ii Spices when 100 mL of acetonitrile/ water (9:1) is insufficient for stirring and extraction of the sample, or for recovery of the filtrate.

[5] In multifunctional column purification, contaminants are retained by the column to be eluted later, and aflatoxin elutes at a constant concentration without being retained by the column and thus purified. Therefore, the first 1.0 mL of the eluate from the multifunctional column has the highest purity.

[6] If necessary, remove insoluble matters by centrifugation etc. to obtain the test solution for high performance liquid chromatography.

[7] "Spices" are applicable to chili pepper, red pepper and nutmeg.

[8] In continuous analyses of spice samples and samples with a lot of contaminants which are strongly retained by the HPLC column, initialize after the elution of aflatoxin B_1 by washing the HPLC column with acetonitrile for 5 minutes to 10 minutes followed by the flow of the mobile phase for 10 minutes.

[9] In addition to fluorescent derivatization by trifluoroacetic acid (TFA method), fluorescent derivatization by a photochemical reactor (PR method, Joshua, H. *et al*.: J. chromatogr A., 654, 247-254, 1993) and the Kobracel method (KC method, Kok, W. T. *et al*.: J. Chromatogr., 367, 231-236, 1986, Papadopoulou-Bouraoui, A. *et al*.: J. AOAC Int., 85, 411-416, 2002) are also applicable. These are simple methods, and PR method measures fluorescent derivatives produced by post-column illumination of ultraviolet

light, while KC method bromo-derivatives produced by post-column electrochemistry. As these are post-column reactions, the elution order of aflatoxins by high performance liquid chromatography changes to the order of G_2, G_1, B_2, and B_1. In PR or KC method, reaction by trifluoroacetic acid is not conducted; therefore for the preparation of the standard solution for high performance liquid chromatography, take accurately 0.5 mL of the test solution to a screw-capped vial or a stoppered test tube or centrifuge tube, and remove solvent by nitrogen flow or by using a evaporator, then add accurately acetonitrile/ water (1: 9) (1.0 mL in "Grains, beans and nuts and seeds," while 0.5 mL in "Spices") to be the test solution for high performance liquid chromatography. Prepare by transferring accurately the final standard solution (1.0 mL in "Grains, beans and nuts and seeds," while 0.25 mL in "Spices") to a screw-capped vial or a stoppered test tube or centrifuge tube, and removing solvent by nitrogen flow or by using a evaporator, then adding accurately acetonitrile/ water (1: 9) (1.0 mL in "Grains, beans and nuts and seeds," while 0.5 mL in "Spices"). Additionally, examples of measurement conditions for PR and KC methods are shown below.

<PR method>

High performance liquid chromatography conditions

Column, Octadecylsilyl silica gel column[11] (4.6 mm in inner diameter, 150 or 250 mm in length, particle size 3-5 μm); Column temperature, 40°C; Mobile phase, methanol/ water (4,6); Flow, 0.7 mL/min; Detection wavelength, excitation wavelength 365 nm, measurement wavelength 450 nm; PR reaction system, 245 nm low-pressure mercury lamp (15 W) illumination system; Reaction coil, 0.25 mm in inner diameter, 15-20 m in length; Injection volume, 20 μL.

<KC Method>

High performance liquid chromatography conditions

Column, Octadecylsilyl silica gel column[11] (4.6 mm in inner diameter, 150 or 250 mm in length, particle size 3-5 μm); Column temperature, 40°C; Mobile phase, methanol/ water (4:6) (add 119 mg potassium bromide and 350 μL of 4 mol/L nitric acid per 1 L); Flow, 1.0 mL/min; Detection wavelength, excitation wavelength 365 nm, measurement wavelength 450 nm; KC reaction system, Kobracel (R-Biopharm Rhône); Current 100 μA; Injection volume 20 μL.

[10] Immunoaffinity is effective when there clearly is a contaminant that cannot be separated and the peak height or peak area obtained from the measurement of the test solution for high performance liquid chromatography exceeds the peak height or peak area obtained from the measurement of the standard solution for high performance liquid chromatography. If the influence of the contaminant etc. is not improved, column length, particle size of packing, or mobile phase can be changed.

[11] Inertsil ODS-3 (GL Sciences), Shodex Silica C18M 4E (Showa Denko) and Cadenza CD-C18 CD006 (Imtakt), etc. can be used.

[12] This method is used only when evaluation is difficult such as positive aflatoxin B_1 is dubious using pretreatment by the multifunctional column method for processed food etc. because of contaminants and interfering substances.

[13] AFLAKING (Horiba, Japan, for total aflatoxin, aflatoxin M_1), AflaStar Fit3 (Romer, Austria, for total aflatoxin), AflaTest WB (VICAM, US, for total aflatoxin), RIDA Aflatoxin column (R-Biopharm AG, Germany, for total aflatoxin), EASI-EXTRACT AFLATOXIN (R-Biopharm Rhône, Britain), and Neocolumn (Neogen, US, for total aflatoxin) etc. can be used. Check the status of gel in the column before use to make sure that there is no crack or bubble inside. If there is a crack or a bubble in the gel, it can be removed by pressurizing with a syringe etc. from the top of the column. The immunoaffinity column to be used should be confirmed in advance that the recovery of

aflatoxin B_1 is 80 % or more (the method described in AOAC (Mary W. Trucksess: Official Methods of Analysis of AOAC International (18th Edition) Chapter 49, p.31 (2005)) etc. are useful.

[14] Phosphate Buffered Saline Tablet (Sigma-Aldrich) etc. can be used. Purified water can be used instead of PBS for some immunoaffinity columns.

[15] When extraction with acetonitrile/ water (9:1) is difficult, extraction solvents that have been already validated can be used, for example, acetonitrile/ water (6:4) and methanol: water (7:3, 8:2). When supernatant is separated into 2 layers, the addition of one volume of methanol to the extract may be effective in some cases.

[16] Dilution of a sample extract with PBS may produce precipitation. Direct injection of this into an immunoaffinity column may cause clogging, so filtration with glass fiber filter paper is required. Moreover, in some samples, precipitation cannot be removed by filtration with Whatman 934AH. In such cases, filtration with Whatman GF/F is effective.

[17] Condition the column as follows: connect a stopcock to the bottom of the immunoaffinity column, connect it to a vacuum manifold etc., drain all of solution in the column, then fill the column with PBS and drain totally. After that, inject about a half amount of the inner amount of the column, close the stopcock, and connect to a reservoir (30.0 mL, an Waters product or a syringe can be used) using a connector. To inject the diluted filtrate of a sample, the elution rate needs to be adjusted to about 1-2 drops/second.

[18] Flow rate adjustment by a stopcock is not needed for washing. Additionally, it is effective to repeat the procedure in which the column is filled with the washing solution with a pipette etc and the whole amount is drained. Also, adjust the amount of the washing solution so that the color of the stained gel in the column is removed as much as possible.

[19] Connect a syringe to a reservoir connector, and connect this to the top of the column. Liquid in the column can be removed by sending air.

[20] Inject 1 mL of acetonitrile into the column, elute by natural flow, and leave at rest for 5 minutes. Further inject 1 mL of acetonitrile into the column and elute. Repeat this procedure again.

[21] Aflatoxin may be adsorbed to the vial during evaporation of the eluate. In such a case, use of a silane-treated vial (from Supelco, etc.; washed with e.g., 20-30 % acetonitrile/ water and dried before use) is preferred.

[22] Precipitate from spices can be solubilized by diluting using PBS containing 2% Tween20. When there is precipitate after dilution, it is needed to filter with glass fiber filter paper.

[23] Washing with PBS containing 0.01% Tween20 is effective for decolorization of the stained gel in the column.

8 ELISA

Imported ELISA kits are available from several distributors. They can be generally classified into qualification kits (Agri-Screen®, Rhône Aflacard B_1, etc.) and quantitation kits.

The Ministry of Health, Labour and Welfare evaluated the test methods using fast assay devices (kits) from the view of test time reduction etc. considering the increasing demand of aflatoxin tests for corn produced in the United States since 2005. As a result, it is approved by the Notification No. 0713001 dated July 13, 2006 from the Inspection and Safety Division, MHLW, that aflatoxin in corn can be analyzed using “Charm ROSAaflatoxin test” from Charm, “RIDAscreen FASTaflatoxin” from R-Biopharm AG,

“Aflacard B1 (2ppb)” from R-Biopharm Rhône, “AgraQuant Afla” from Romer or “AgraStrip Afla” from Romer instead of instrumental analysis.
When an ELISA kit is used, 2 or more replicate analyses must be conducted in order to reduce the variance in analytical value and obtain stable results. In addition, as for quantitation analysis, it is required to prepare the calibration curve using the standards by plates each time conducting analysis.
Table 5.1.1-1 shows major kits currently available in Japan and their summaries.

Table 5.1.1-1 ELISA kits for aflatoxin analysis commercially available in Japan

	Item	Lower limit of detection	Analysis time	Shelf life of the kit	Applicable samples	Notes
Qualification kit	Agri-Screen® for Aflatoxin (for total aflatoxin)	20 ppb	5 minutes	9 months	Corn, cottonseed, milo, peanut, rice, soybean, wheat etc.	Neogen AOACI method 990.32 USDA/GIPSA method (2006-09)
	Aflacard B_1	2 ppb	15 minutes	6-9 months	Grains, nuts, spices	R-Biopharm Rhône MHLW Notification method
	Aflacard Total	4 ppb	15 minutes	6-9 months	Grains, nuts, spices	R-Biopharm Rhône
	RIDASCREEN® Aflatoxin EXPRESS	20 ppb	5 minutes	6-9 months	Grains, nuts, spices	R-Biopharm Rhône
	AgraStrip™ AFL	20 ppb	10 minutes	6-9 months	Grains	Romer Labs MHLW Notification method
	Charm ROSA® Aflatoxin Qualitative	5 ppb	5 minutes	6-9 months	Grains	Charm Sciences
Quantitation kit	Veratox® for Aflatoxin (for total aflatoxin)	2 ppb	5 minutes	9 months	Corn, cottonseed, milo, peanut, rice, soybean, wheat etc.	Neogen USDA/GIPSA method (2005-102)
	Veratox® for Aflatoxin Single Test (AST)	3 ppb	10 minutes	6 months	Corn, cottonseed, milo, peanut, rice, soybean, wheat etc.	Neogen USDA/GIPSA method (2007-101)
	Veratox® for Aflatoxin HS	0.5 ppb	20 minutes	9 months	Corn, cottonseed, milo, peanut, rice, soybean, wheat etc.	Neogen
	RIDASCREEN® FAST Aflatoxin	1.7 ppb	25 minutes	6-9 months	Grains, nuts, spices	R-Biopharm Rhône MHLW Notification method
	RIDASCREEN® Aflatoxin B_1	625 ppt	150 minutes	6-9 months	Grains, nuts, spices	R-Biopharm Rhône
	RIDASCREEN® Aflatoxin Total	1.75 ppb	60 minutes	6-9 months	Grains, nuts, spices	R-Biopharm Rhône
	Charm ROSA® Aflatoxin Quantitative	2 ppb	12 minutes	6-9 months	Grains	Charm Sciences MHLW Notification method
	AgraQuant™ AFL	1 ppb	30 minutes	6-9 months	Grains	Romer Labs MHLW Notification method
	MaxSignal™ Total Aflatoxin ELISA Test Kit	0.5 ppb	90 minutes	6-9 months	Grains, feeds, nuts and seeds, milk	BIOO Scientific
	MaxSignal™ Aflatoxin B_1 ELISA Test Kit	0.5 ppb	90 minutes	6-9 months	Grains, feeds, nuts and seeds	BIOO Scientific
	MaxSignal™ Aflatoxin M_1 ELISA Test Kit	0.5 ppb	90 minutes	6-9 months	Milk	BIOO Scientific