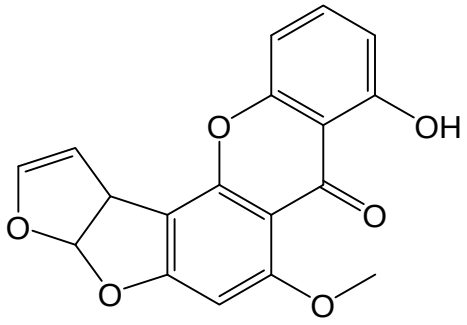
Sterigmatocystin



(3a*R*-*cis*)3*a*,12*c*-dihydro-8-hydroxy-6-methoxy -7*H*-furo [3',2':4,5]furo [2,3-*c*]xanthen-7-one
$C_{18}H_{12}O_6$ MW: 324.28 CAS No.: 10048-13-2

[Summary of sterigmatocystin]

Sterigmatocystin is a carcinogenic mycotoxin produced by *Aspergillus versicolor* etc., and has been discussed as an aflatoxin-related substance as an intermediate of aflatoxin B_1 biosynthesis. Its structure is similar to that of aflatoxin, while its toxicity is much lower than aflatoxin.

Monitoring of feeds and feed materials has hardly been conducted, although there is a report that sterigmatocystin at 16.3 mg/kg was detected in brown rice stored in Japan.

[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 5.1]

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.

50 g sample
- 100 mL Acetonitrile- water (21:4)
- Shake for 60 minutes
- Centrifuge (650x g, 5 minutes)

Multifunctional column (Multisep #226)
- Load the supernatant on the colum
- Discard the first 4 mL of eluate

1 mL eluate
- Add 1 mL of acetic acid (1:100)
- Centrifuge (5,000x g, 5 minutes)

LC-MS/MS

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.

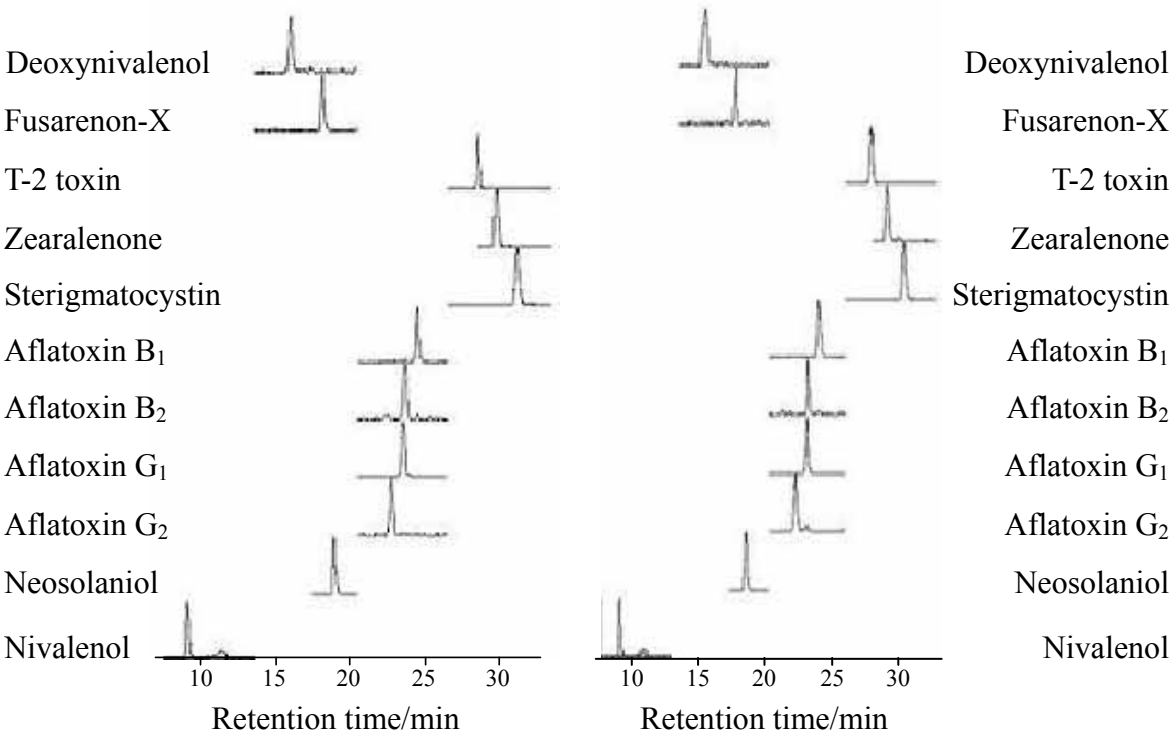


Figure 5.3.1-2 SRM chromatograms of mycotoxin-spiked formula feeds and corn (Left) formula feeds; (Right) corn

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 Liquid chromatography [Feed Analysis Standards, Chapter 5, Section 1 5.2]

A. Reagent preparation

Sterigmatocystin standard solution. Weigh accurately 1 mg of sterigmatocystin [$C_{18}H_{12}O_6$], [1] 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 sterigmatocystin standard stock solution (1 mL of this solution contains 0.2 mg as sterigmatocystin).

Before use, dilute accurately a certain amount of the standard stock solution with acetonitrile/ water (21:4) to prepare several sterigmatocystin standard solutions that contain 0.05-1 μg of sterigmatocystin in 1 mL.

B. Quantification

Extraction. Weigh 25.0 g of an analysis sample, transfer it to a 200-mL stoppered amber Erlenmeyer flask, add 100 mL of acetonitrile/ water (21:4), and extract by shaking for 30 minutes. Transfer the extract to a stoppered amber centrifuge tube, centrifuge at 1,000×g for 5 minutes, to obtain supernatant as a sample solution to be subjected to minicolumn chromatography.

Column treatment. Transfer 9 mL of the sample solution to a test tube, slowly push in a multifunctional column (for aflatoxin/ zearalenone pretreatment), Note 1 and discard the first 4 mL of the eluent that passed packing.

Further push in the column mentioned above[2], and elute 2 mL. Homogenize the eluate, transfer a part of it to a plastic centrifuge tube (volume: 1.5 mL), centrifuge at 5,000×g for 5 minutes, to obtain supernatant to be a sample solution to be subjected to liquid chromatography.

Liquid chromatography. Inject 20 μL each of the sample solution and respective sterigmatocystin standard solutions to a liquid chromatograph to obtain chromatograms.[3]

Example of measurement conditions

- Detector: UV absorptiometer UV absorptiometer (measurement wavelength : 330 nm)
- Column: Octadecylsilyl silica gel column[4] (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm) Note 2
- Eluent: Methanol -water (13:7)
- Flow rate: 1.0 mL/min
- Column oven temperature: 40°C

Calculation. Obtain peak heights from the resulting chromatograms to prepare a calibration curve, and calculate the amount of sterigmatocystin in a sample.

Note 1 MycoSep 226 AflaZon+ (Romer Labs) or equivalents

2 Mightysil RP-18 GP (Kanto Chemical) or equivalents

<<Summary of analysis method>>

In this method, sterigmatocystin in a sample is extracted with aqueous acetonitrile, purified with a multifunctional cleanup (MFC) column, and quantitated by a liquid chromatograph with a UV absorptiometer.

Quantitation procedures from extraction to the middle of column treatment are the same as those in the analysis method in 6 Zearalenone 3.1 in this chapter.

The flow sheet of the analysis method is shown in Figure 5.1.5-1.

25 g sample
- 100 mL Acetonitrile/ water (21:4)
- Shake for 30 minutes
- Centrifuge (1,000x g (2,000 rpm), 5 minutes)

Multifunctional column (MycoSep 226 AflaZon+)
- Transfer 9 mL of the supernatant to a test tube.
- Push in the multifunctional column until the first 4 mL elutes
- Discard the first eluate
- Further push in the multifunctional column until 2 mL elutes
- High speed centrifuge (5,000x g (10,000 rpm), 5 minutes)

LC-UV (330 nm)

Figure 5.1.5-1 Flow sheet of the analysis method for sterigmatocystin

References: Yuji Shirai, Ikumi Kobayashi, Tosiaki Hayakawa: Research Report of Animal Feed, 27, 1 (2002)

History in the Feed Analysis Standards [22] New

<<Analysis method validation>>

- Spike recovery and repeatability

Sample type	Spike concentration (μg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Pig formula feed	200~1,000	3	98.0~100.0	3.3
Cattle formula feed	200~1,000	3	93.9~97.6	2.9
Corn	200~1,000	3	82.7~94.9	4.0
Wheat	200~1,000	3	96.9~97.2	1.8

- Collaborative study

Sample type	Number of laboratories	Spike concentration (μg/kg)	Spike recovery (%)	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Pig formula feed	7	1,000	99.7	3.3	4.2	0.26

- Lower limit of quantification: 100 μg/kg in sample

<<Notes and precautions>>

[1] 50 μg/mL sterigmatocystin standard solution is commercially available from Kanto Chemical etc.
In addition, as for the type of the standard where a specified amount (1 mg) of sterigmatocystin is packaged in an aluminum-sealed vial, the standard stock solution is prepared by injecting accurately 5 mL of acetonitrile with a syringe and dissolving sterigmatocystin by sonication etc. Store this standard stock solution frozen.

[2] For how to use and usage example, see Section 3 2 Simultaneous analysis of aflatoxins by liquid chromatography <<Notes and precautions>> [5] in this chapter.

[3] Examples of chromatograms are shown in Figure 5.1.5-2.

[4] The column to be used only needs to be one that uses packing treated by corresponding endcapping.

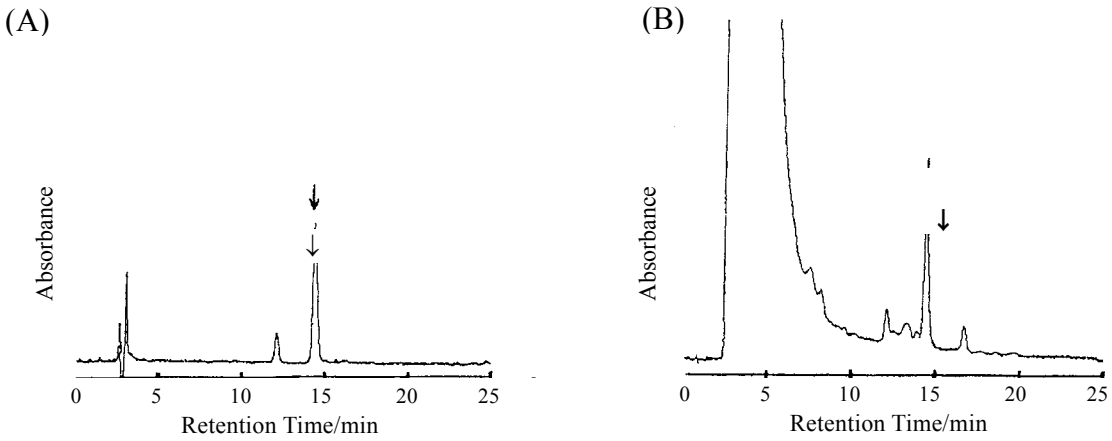


Figure 5.1.5-2 Chromatograms of sterigmatocystin
(Arrows indicate the peak of sterigmatocystin.)
LC conditions are as shown in Example of measurement conditions.
(A) Standard solution (5 ng as sterigmatocystin)
(B) Sample solution of a pig formula feed spiked with 1 mg/kg as sterigmatocystin