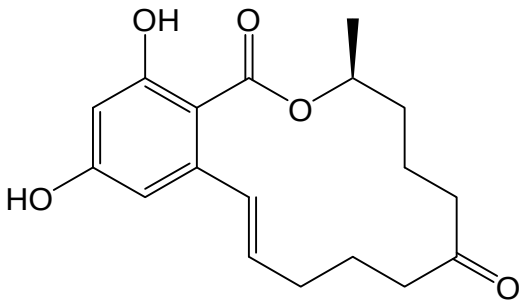
Zearalenone and related compounds

Zearalenone



(4*S*,12*E*)-15,17-dihydroxy-4-methyl-3-oxabicyclo
[12.4.0]octadeca-12,15,17,19-tetraene-2,8-dione
$C_{18}H_{22}O_5$ MW: 318.364 CAS No.: 17924-92-4

[Summary of zearalenone]

Zearalenone is a mycotoxin produced by *Fusarium graminearum* etc., which shows estrogen-like effects and is especially known for causing reproductive disturbance in pigs. Moreover, it also has been attracting attention as one of endocrine disruptors (so-called environmental hormones).

Natural contamination has been reported for corn, milo, barley, wheat, and rye etc. among major feed materials, and the detection ratio was high in milo for feeds that was imported from the United States in 2002, thus the interim acceptable value for zearalenone in feeds has been designated as shown below.

Monitoring results by the Fertilizer and Feed Inspection Services in 2002 showed that zearalenone was detected in about 90 % of milo (0.08-3.32 ppm), and that zearalenone was detected in about 40 % of formula feeds (0.04-1.42 ppm). Additionally, one item of pig formula feed exceeded the interim acceptable value.

<<Standards and specifications in the Act on Safety Assurance and Quality Improvement of Feeds>> [Detection of zearalenone (Notification No. 7269, 13 Seichiku, Feed Division, Livestock Industry Department, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries, Japan dated March 25, 2002)]

The maximum value of zearalenone acceptable to be contained in feed given to livestock: 1.0 ppm

Feeds containing zearalenone over the acceptable value shall be handled as the feed containing harmful substances in Article 23, (formerly Article 2-6), Paragraph 1 in the Act on Safety Assurance and Quality Improvement of Feeds, and their manufacturing, importing or marketing shall be inhibited according to the provisions in the article; or the user of the feed may be inhibited the use of the feed according to the provisions in the said article, and may be ordered disposal etc. according to the provisions in Article 24 (formerly Article 2-7) ("Inhibition on marketing etc. according to the provisions in Article 2-6 in the Act on Safety Assurance and Quality Improvement of Feeds" (Notification No. 971, 14 Seichiku, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries, Japan dated May 22, 2002)).

[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 6.1]

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.

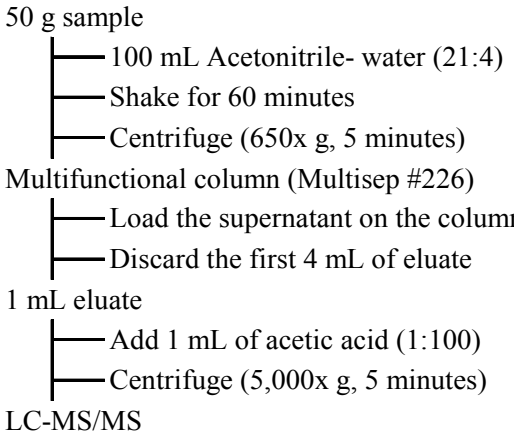


Figure 5.3.1-1 Flow sheet of the simultaneous analysis method for mycotoxins by liquid chromatography- tandem mass spectrometry

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.

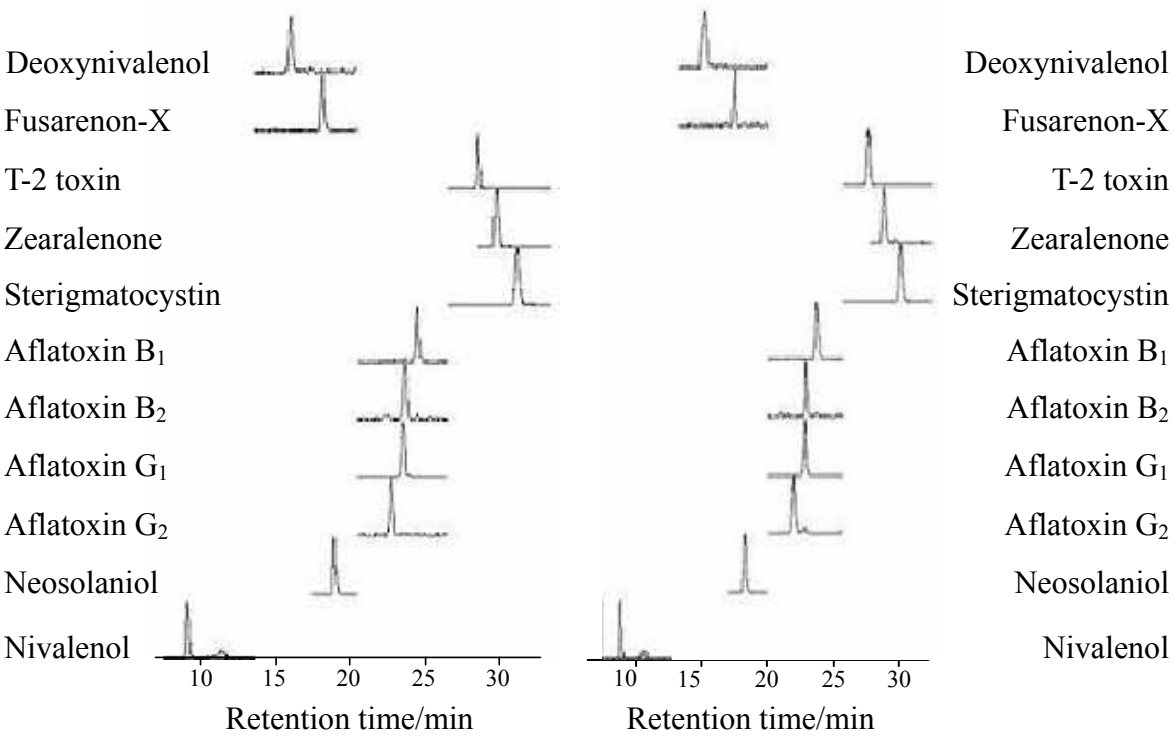


Figure 5.3.1-2 SRM chromatograms of mycotoxin-spiked formula feeds and corn (Left) formula feeds; (Right) corn

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 One-component analysis method by liquid chromatography/ mass spectrometry [Feed Analysis Standards, Chapter 5, Section 1 6.2]

Scope of application: Feeds

A. Reagent preparation

1) Zearalenone standard solution. Weigh accurately 10 mg of zearalenone [$C_{18}H_{22}O_5$], put in a 50-mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the zearalenone standard stock solution (1 mL of this solution contains 0.2 mg as zearalenone.).
   Before use, dilute accurately a certain amount of the standard stock solution with acetonitrile/ water (1:1) to prepare several zearalenone standard solutions that contain 5-1,000 ng of zearalenone in 1 mL.
2) Internal standard solution. Weigh accurately 1 mg of zearalanone [$C_{18}H_{24}O_5$], put in a 20-mL amber volumetric flask, dissolve by the addition of acetonitrile, and add the same solvent up to the graduation line to prepare the internal standard stock solution (1 mL of this solution contains 50 μg as zearalanone.).
   Before use, dilute accurately a certain amount of the internal standard stock solution with acetonitrile/ water (1:1) to prepare the internal standard solution that contains 5 μg of zearalanone in 1 mL.

B. Quantification

Extraction. Weigh 50 g of an analysis sample, transfer it to a 300-mL stoppered Erlenmeyer flask, add 150 mL of acetonitrile/ water (21:4), and extract by shaking for 30 minutes. Transfer the extract to a stoppered centrifuge tube, centrifuge at 1,000×g for 5 minutes, to obtain supernatant as a sample solution to be subjected to column treatment.

Column treatment. Transfer the sample solution to a multifunctional column (for zearalenone pretreatment), [Note 1] discard the first 4 mL of eluent, and collect 3 mL of the following eluent into a 10-mL test tube. Transfer accurately 2 mL of this to a 10-mL test tube, evaporate under vacuum in a water bath at 50°C or less to be almost dried up, and then dry up by nitrogen gas flow.

Dissolve the residue by accurately adding 1 mL of acetonitrile/ water (1:1) and 20 μL of the internal standard solution. Transfer this solution to a plastic centrifuge tube (volume: 1.5 mL), centrifuge at 5,000×*g* for 5 minutes, to obtain supernatant as a sample solution to be subjected to measurement by liquid chromatography/ mass spectrometry.

At the same time, to 1.0 mL each of zearalenone standard solutions, add accurately 20 μL of the internal standard solution to be the standard solutions to be subjected to measurement by liquid chromatography/ mass spectrometry.

Measurement by liquid chromatography/ mass spectrometry. Inject 10 μL each of the sample solution and the standard solutions to a liquid chromatograph/ mass spectrometer, and obtain selected ion monitoring chromatograms.

Example of measurement conditions

Column:	Octadecylsilyl silica gel column (3.0 mm in inner diameter, 250 mm in length, particle size 5 μm) [Note 2]
Eluent:	acetonitrile -methanol -10 mmol/Lammonium acetate solution (4:7:9)
Flow rate:	0.5 mL/min
Column oven temperature: 40°C	
Detector:	Quadrupole mass spectrometer [Note 3]

Ionization method: Atmosphere pressure chemical ionization (APCI) (negative ion mode)
Nebulizer gas: N2 (2.5 L/min)
Dryer gas: N2 (6 L/min)
Interface temperature : 300°C
Heat block temperature : 200°C
CDL temperature : 200°C
Monitor ion: *m/z* 317 (zearalenone), 319 (zearalanone)

Calculation. Obtain peak heights of zearalenone and zearalanone from the resulting selected ion monitoring chromatogram,[1] to prepare a calibration curve by the internal standard method, and calculate the amount of zearalenone in a sample.

Note 1 MultiSep 226 AflaZon+ (Romer Labs) or equivalents
2 ZORBAX Eclipse XDB-C18 (Agilent Technologies) or equivalents
3 Example of conditions for LCMS-2010EV (Shimadzu)

<<Summary of analysis method>>

In this method, zearalenone in feeds is extracted with acetonitrile/ water (21:4), purified with a multifunctional cleanup (MFC) column, spiked with the internal standard, and quantitated by liquid chromatography/ mass spectrometry.

Measurement is conducted with addition of the internal standard because it is feared that recovery may be poor due to ionization suppression in measurement by liquid chromatography/ mass spectrometry.

The flow sheet of the analysis method is shown in Figure 5.1.6-1.

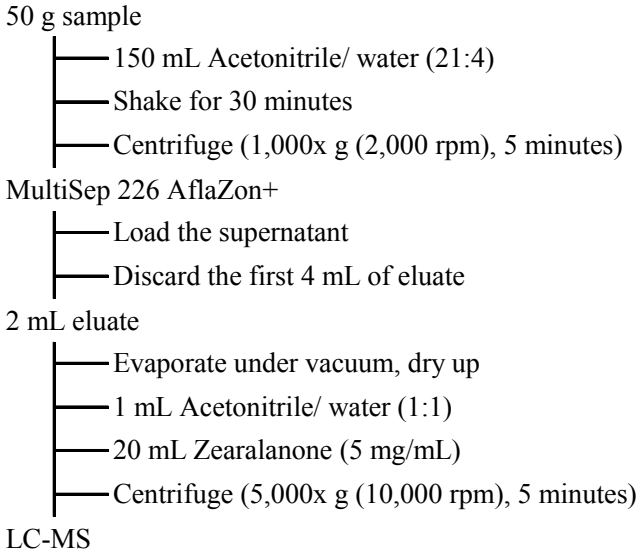


Figure 5.1.6-1 Flow sheet of one-component analysis method for zearalenone by liquid chromatography/ mass spectrometry

References: Kouji Aoyama: Research Report of Animal Feed, 31, 31 (2006)

History in the Feed Analysis Standards [29] New

<<Analysis method validation>>

- Spike recovery and repeatability

Sample type	Spike concentration (μg/kg)	Repeat	Spike recovery (%)	Repeatability RSD RSD (% or less)
Barley	40~1,000	3	98.4~98.7	5.0
Brown rice	40~1,000	3	92.4~94.2	4.2
Formula feed for pigletss in lactatin period	40~1,000	3	106.6~114.5	5.7
Formula feed for beef cattle	40~1,000	3	101.7~106.4	8.7

- Collaborative study

Sample type	Number of laboratories	Spike concentration (μg/kg)	Spike recovery (%) (measured value (μg/kg))	Intra-laboratory repeatability RSDr (%)	Inter-laboratory reproducibility RSDR (%)	HorRat
Brown rice	9	50	112.7	3.9	10.8	0.49
Barley	9	200	112.1	5.3	8.3	0.41
Wheat	9	500	111.7	6.0	10.7	0.60
Formula feed	9	Natural contamination	(38.8)	8.8	13.1	0.60
Milo	9	Natural contamination	(334)	12.0	14.3	0.76

- Lower limit of quantification: 5 μg/kg (107 % spike recovery and 3.3% relative standard deviation)
- Lower limit of detection: 2 μg/kg

<<Notes and precautions>>

[1] An example of selected ion monitoring (SIM) chromatogram is shown in Figure 5.1.6-2.

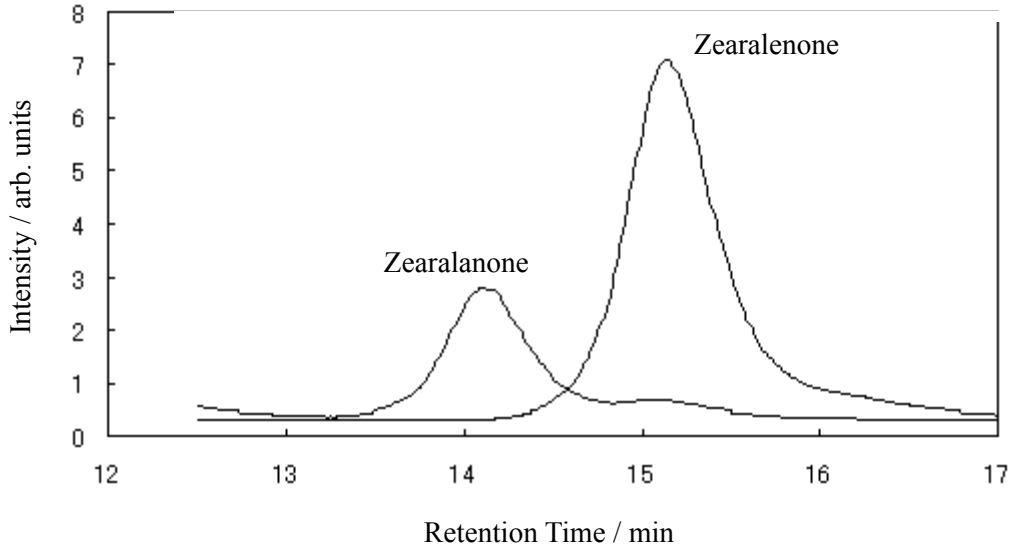


Figure 5.1.6-2 SIM chromatogram of formula feed for beef cattle (spiked with 1,000 μg/kg equivalent).
Measurement conditions were according to Example of measurement conditions.
Column used was Agilent Technologies ZORBAX Eclipse XDB-C18.

3 Liquid chromatography

3.1 Feeds [Feed Analysis Standards, Chapter 5, Section 1 6.3 (1)]

A. Reagent preparation

Zearalenone standard solution. Weigh accurately 1 mg of zearalenone [$C_{18}H_{22}O_5$], 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 zearalenone standard stock solution (1 mL of this solution contains 0.2 mg as zearalenone.).

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

B. Quantification

Extraction. Weigh 50 g of an analysis sample, transfer it to a 300-mL stoppered Erlenmeyer flask, add 150 mL of acetonitrile/ water (21:4), and extract by shaking for 30 minutes. Transfer the extract to a stoppered centrifuge tube, centrifuge at 1,000×g for 5 minutes, to obtain supernatant as a sample solution to be subjected to column treatment.

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 [1] and discard the first 2 mL of the eluent that passed packing.

Further push in the column mentioned above, 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 zearalenone standard solutions to a liquid chromatograph to obtain chromatograms.

Example of measurement conditions

- Detector: Fluorescence detector (excitation wavelength, 278 nm; fluorescence wavelength, 460 nm)
- Column: Octadecylsilyl silica gel column (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm) Note 2 [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[3] to prepare a calibration curve, and calculate the amount of zearalenone 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, zearalenone in a sample is extracted with aqueous acetonitrile, purified with a multifunctional cleanup (MFC) column, and quantitated by a liquid chromatograph with a fluorescence detector.

Quantitation procedures from extraction to the middle of column treatment are the same as those in the analysis method in 5 Sterigmatocystin 2 in this chapter.

The flow sheet of the analysis method is shown in Figure 5.1.6-3.

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 2 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-FL (Ex, 278 nm; Em, 460 nm))

Figure 5.1.6-3 Flow sheet of the analysis method for zearalenone

References: Yuji Shirai, Ikumi Kobayashi, and 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	93.6~101.9	2.6
Cattle formula feed	200~1,000	3	101.4~109.9	1.7
Corn	200~1,000	3	95.2~99.4	1.7
Wheat	200~1,000	3	95.2~99.4	1.7

- Collaborative study

Sample type	Number of laboratories	Spike concentration (μg/kg)	Spike recovery (%)	Intra-laboratory repeatability RSDr (%)	Inter-laboratory reproducibility RSDR (%)	HorRat
Pig formula feed	7	1,000	102.7	3.0	4.2	0.26

- Lower limit of quantification: 50 μg/kg in sample

<<Notes and precautions>>

[1] 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.

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

[3] Examples of chromatograms of the standard solution and the sample solution of a pig formula feed spiked with 1 mg/kg as zearalenone are shown in Figure 5.1.6-4.

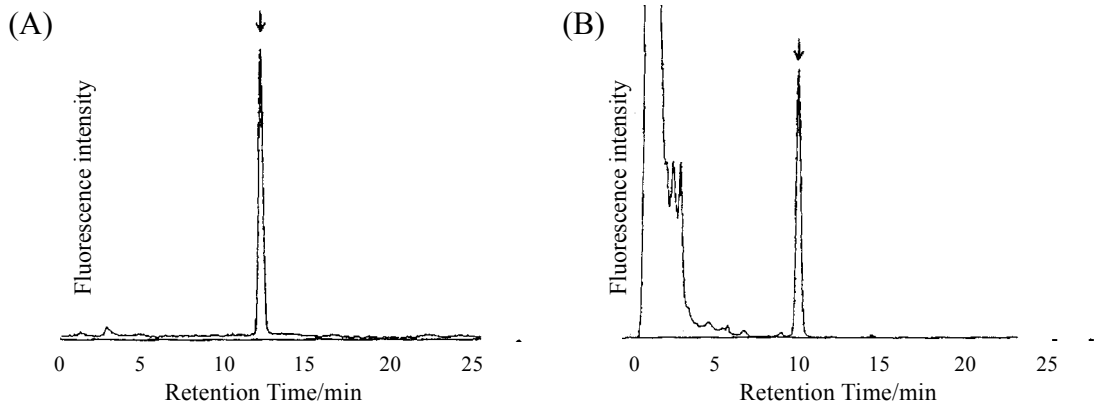


Figure 5.1.6-4 Chromatograms of zearalenone.
(Arrows indicate the peak of zearalenone.)

LC conditions are as shown in Example of measurement conditions.

(A) Standard solution (5 ng as zearalenone)

(B) Sample solution of a pig formula feed spiked with an amount equivalent to 1 mg/kg zearalenone.

3.2 Grains [Feed Analysis Standards, Chapter 5, Section 1 6.3 (2)]

A. Reagent preparation

Zearalenone standard solution. Weigh accurately 10 mg of zearalenone [$C_{18}H_{22}O_5$], put in a 100-mL amber volumetric flask, dissolve by the addition of methanol, and add the same solvent up to the graduation line to prepare the zearalenone standard stock solution (1 mL of this solution contains 0.1 mg as zearalenone.).

Before use, dilute accurately a certain amount of the standard stock solution with methanol to prepare several zearalenone standard solutions that contain 0.5-5 μg of zearalenone in 1 mL.

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 [1] to a stoppered centrifuge tube, and centrifuge at 1,500×*g* for 5 minutes. Transfer the chloroform layer (lower layer) 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 100-mL recovery flask, and concentrate under vacuum in the water bath at 45°C or less to be almost dried up, and further dry up by nitrogen gas flow. Dissolve the residue by the addition of 2 mL of toluene to be a sample solution to be subjected to column treatment.

Column treatment. Wash a silica gel mini column (690 mg) with 10 mL of toluene.

Load the sample solution to the mini column, wash the recovery flask that contained the sample solution twice with 2 mL each of toluene, add the washing to the mini column, and elute by pressurized flow. Note 1 Moreover, add 10 mL of toluene to the mini column, and elute by pressurized flow. Note 1

Place a 50-mL recovery flask under the mini column. Add 12 mL of chloroform 12 mL to the mini column, and pressurize Note 1 to elute zearalenone. Concentrate the eluate under vacuum in the water bath at 45°C or less to be almost dried up, and further dry up by nitrogen gas flow.

Dissolve the residue by accurately adding 2 mL of methanol, filter with membrane filter (pore size 0.5 μm or less), to obtain a sample solution to be subjected to liquid chromatography.

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

Example of measurement conditions

- Detector: Fluorescence detector (excitation wavelength 276 nm; fluorescence wavelength 455 nm) [2]
- Column: Octadecylsilyl silica gel column (4.0 mm in inner diameter, 250 mm in length, particle size 5 μm) Note 2 [3]
- Eluent: Methanol/ water (7:3)
- Flow rate: 1.0 mL/min

Calculation. Obtain peak heights or areas from the resulting chromatograms[4] to prepare a calibration curve, and calculate the amount of zearalenone in a sample.

Note 1 Flow rate shall be about 2 mL/min.

2 Nucleosil $5C_{18}$ (Macherey-Nagel) or equivalents

<<Summary of analysis method>>

In this method, zearalenone in grains is extracted with chloroform, purified with a silica gel mini column, and quantitated by a liquid chromatograph with a fluorescence detector.

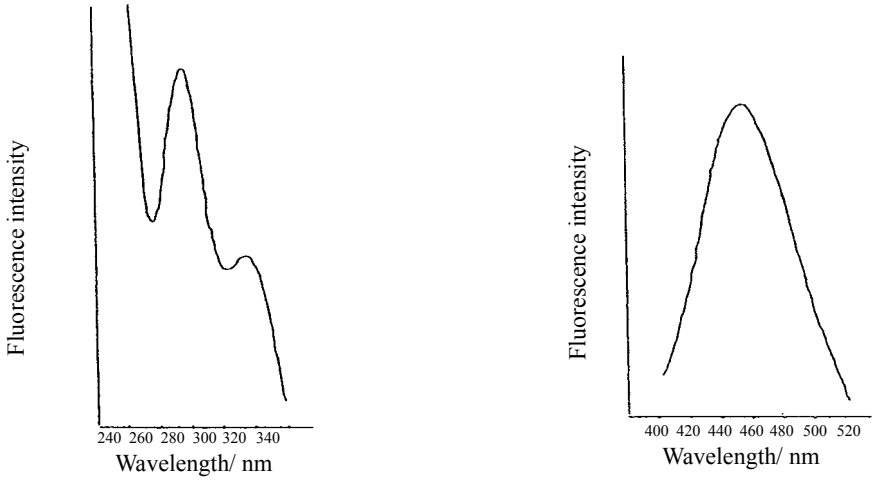
References: Kiyoshi Sugano, Takayuki Ishibashi, and Isao Matsubara: Research Report of Animal Feed, 15, 12 (1990)

History in the Feed Analysis Standards [12] New

<<Notes and precautions>>

[1] If centrifugation is not available, the extract can be filtered with water-repellent filter paper (such as No. 2S, Toyo Roshi) with a layer of a suitable amount of hyflo super-cel (filter aid).

[2] Examples of excitation and fluorescence spectra are shown in Figure 5.1.6-5.

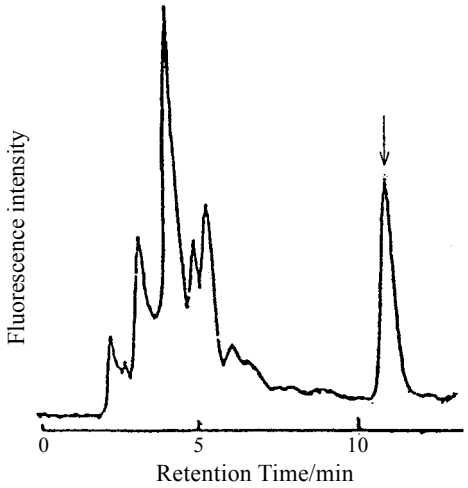


Excitation spectrum (Em: 455 nm) Fluorescence spectrum (Ex: 276 nm)

Figure 5.1.6-5 Excitation and fluorescence spectra of zearalenone (methanol/ water (7:3) solution).

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

[4] An example of chromatogram is shown in Figure 5.1.6-6.



Measurement conditions

Measurement wavelength: Ex. 276 nm, Em. 455 nm

Column: Nucleosil $5C_{18}$

Eluent: Methanol/ water (7:3)

Flow rate: 1.0 mL/min

Figure 5.1.6-6 Chromatogram of corn spiked with an amount equivalent to 100 µg/kg zearalenone
(The arrow indicates the peak of zearalenone.)

3.3 Formula feeds [Feed Analysis Standards, Chapter 5, Section 1 6.3 (3)]

A. Reagent preparation

1) Zearalenone standard solution. Weigh accurately 1 mg of zearalenone [$C_{18}H_{22}O_5$], 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 zearalenone standard stock solution (1 mL of this solution contains 0.2 mg as zearalenone.).
   Before use, dilute accurately a certain amount of the standard stock solution with acetonitrile/ water (21:4) to prepare several zearalenone standard solutions that contain 0.05-1 μg of zearalenone in 1 mL.
2) Citric acid solution. Dissolve 106 g of citric acid monohydrate in water to be 1 L.

B. Quantification

Extraction. Weigh 50 g of an analysis sample, transfer it to a 500-mL separatory funnel, add 25 mL of hydrochloric acid (0.1 mol/L) and 250 mL of chloroform, and extract by shaking for 30 minutes. Transfer the extract[1] to a stoppered centrifuge tube, and centrifuge at 1,500×g for 5 minutes. Transfer the chloroform layer (lower layer) to an Erlenmeyer flask. Dehydrate the chloroform layer with a suitable amount of sodium sulfate (anhydrous), and then filter with water-repellent filter paper to be a sample solution to be subjected to purification.

Purification.[2] Transfer 50 mL of the sample solution to a 300-mL separatory funnel which in advance contains 50 mL of sodium hydroxide solution (0.5 mol/L) and 10 mL of saturated sodium chloride solution, shake vigorously for 1 minute and leave at rest for 30 minutes,[3] and then discard the chloroform layer (lower layer). Add 50 mL of chloroform to the separatory funnel, and operate similarly. Add 50 mL of the citric acid solution and 50 mL of chloroform to the separatory funnel, shake mildly and leave at rest, and filter the chloroform layer into a 300-mL recovery flask with water-repellent filter paper. Add 50 mL of chloroform to the separatory funnel, and operate similarly twice. Concentrate the filtrate under vacuum in the water bath at 50°C or less to be almost dried up, and dry up by nitrogen gas flow.
Dissolve the residue by the accurate addition of 2 mL of methanol, filter with membrane filter (pore size 0.5 μm or less) to be a sample solution to be subjected to liquid chromatography.

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

Example of measurement conditions

- Detector: Fluorescence detector (excitation wavelength : 276 nm, fluorescence wavelength : 455 nm)
- Column: Octadecylsilyl silica gel column (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm) Note 1 [4]
- Eluent: Water/ acetonitrile/ methanol (10:8:5)
- Flow rate: 1.0 mL/min

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

Note 1 Nucleosil $5C_{18}$ (Macherey-Nagel) or equivalents

<<Summary of analysis method>>

In this method, zearalenone in formula feeds is extracted with chloroform, purified by liquid-liquid partition, and quantitated by a liquid chromatograph with a fluorescence detector.

References: Kyoko Ogi and Isao Matsubara: Research Report of Animal Feed, 17, 1(1992)
History in the Feed Analysis Standards[14] New

<<Notes and precautions>>

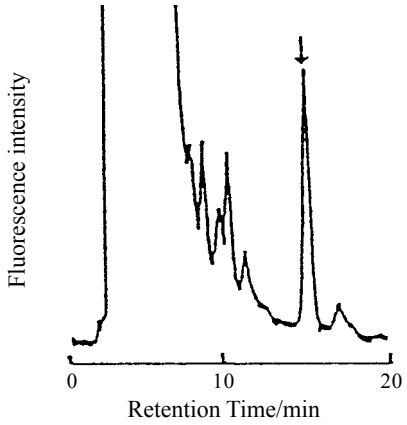
[1] If centrifugation is not available, the extract can be filtered with water-repellent filter paper (such as No. 2S, Toyo Roshi) with a layer of a suitable amount of hyflo super-cel (filter aid).

[2] The purification method utilizes the fact that zearalenone is transferred to the water layer in an alkaline solution, and is re-extracted into the chloroform layer in an acidic solution.

[3] In order to remove interfering substances, it is required to leave at rest for 30 minutes or more after shaking until the alkaline aqueous solution becomes almost transparent. In addition, care should be taken in shaking etc. for operation for re-extraction into a new chloroform layer in an acidic solution because emulsion is likely to be formed.

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

[5] An example of chromatogram is shown in Figure 5.1.6-7.



Measurement conditions
Measurement wavelength: Ex. 276 nm, Em. 455 nm
Column: Nucleosil $5C_{18}$
Eluent: Water/ acetonitrile/ methanol (10:8:5)
Flow rate: 1.0 mL/min

Figure 5.1.6-7 Chromatogram of a formula feed spiked with an amount equivalent to 300 μg/kg zearalenone

[Other analysis methods]

4 ELISA

ELISA kits are available in Japan from several distributors .

Table 5.1.6-1 shows major kits commercially available now in Japan, and their summaries.

Table 5.1.6-1 ELISA kits for zearalenone analysis commercially available in Japan

Item		Lower limit of detection	Analysis time	Shelf life of the kit	Applicable samples	Notes
Quantitation kit	Veratox® for Zearalenone	50 ppb	10 minutes	6 months	Barley, corn, wheat	Neogen
	RIDASCREEN® FAST Zearalenone	50 ppb	25 minutes	6-9 months	Grains, feeds	R-Biopharm Rhône
	RIDASCREEN® Zearalenone	1.25 ppb	150 minutes	6-9 months	Grains, feeds	R-Biopharm Rhône
	AgraQuant® ZON	10 ppb	15 minutes	6-9 months	Grains, feeds	Romer Labs
	Charm ROSA® Zearalenone	50 ppb	15 minutes	6-9 months	Corn	Charm Sciences
	Max Signal™ Zearalenone	1 ppb	60 minutes	6-9 months	Grains, feeds, nuts and seeds, milk	BIOO Scientific