T-2 toxin

[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 8.1]

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.

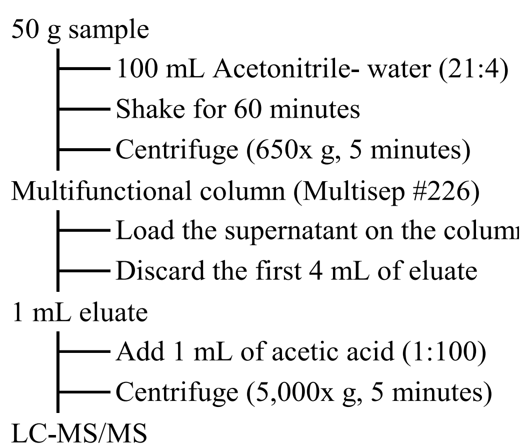


Figure 5.3.1-1 Flow sheet of the simultaneous analysis method for mycotoxins by liquid chromatography- tandem mass spectrometry

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.

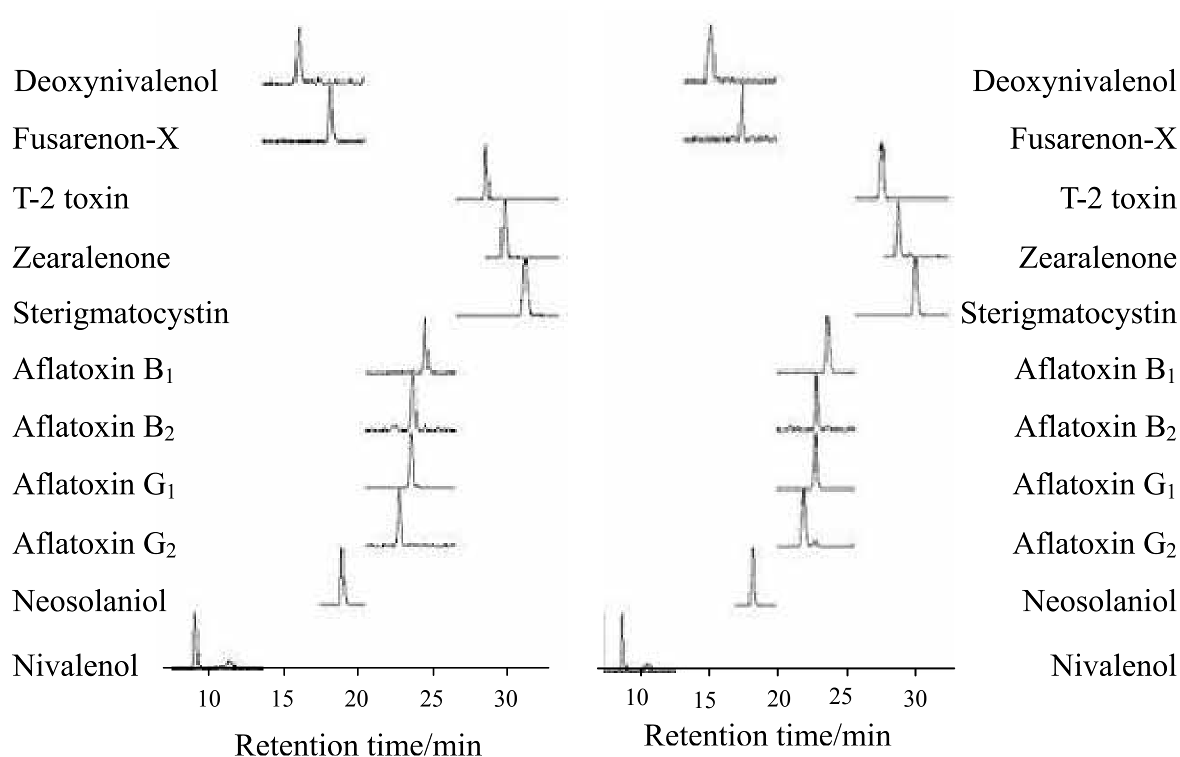


Figure 5.3.1-2 SRM chromatograms of mycotoxin-spiked formula feeds and corn (Left) formula feeds; (Right) corn

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 trichothecene mycotoxin by a liquid chromatograph/ mass spectrometer [Feed Analysis Standards, Chapter 5, Section 1 8.2]

Analyte compounds T-2 toxin, deoxynivalenol and nivalenol (3 components)

Scope of application: Feeds

A. Reagent preparation

1) T-2 toxin standard stock solution. Weigh accurately 1 mg of T-2 toxin [$C_{24}H_{34}O_9$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and further add the same solvent up to the graduation line (1 mL of this solution contains 0.2 mg as T-2 toxin.). Moreover, dilute accurately a certain amount of this solution with acetonitrile to prepare the T-2 toxin standard stock solution that contains 25 µg in 1 mL.
2) Deoxynivalenol standard stock solution. Weigh accurately 1 mg of deoxynivalenol [$C_{15}H_{20}O_6$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and further add the same solvent up to the graduation line (1 mL of this solution contains 0.2 mg as deoxynivalenol.). Moreover, dilute accurately a certain amount of this solution with acetonitrile to prepare the deoxynivalenol standard stock solution that contains 25 µg as deoxynivalenol in 1 mL.
3) Nivalenol standard stock solution. Weigh accurately 1 mg of nivalenol [$C_{15}H_{20}O_7$], put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and further add the same solvent up to the graduation line (1 mL of this solution contains 0.2 mg as nivalenol.). Moreover, dilute accurately a certain amount of this solution with acetonitrile to prepare the nivalenol standard stock solution that contains 25 µg as nivalenol in 1 mL.
4) Mixture standard solution. Before use, mix a certain amount of each of the T-2 toxin, deoxynivalenol and nivalenol standard stock solutions, dilute accurately with water- methanol- acetonitrile (18:1:1), to prepare several mixture standard solutions that contain 0.01-1 µg respectively as T-2 toxin, deoxynivalenol and nivalenol 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 60 minutes. Note 1 Transfer the extract to a stoppered centrifuge tube, centrifuge at 1,000×*g* for 5 minutes, to obtain supernatant to be a sample solution to be subjected to column treatment.

Column treatment. Transfer the sample solution to a multifunctional column (for trichothecene mycotoxins pretreatment), Note 2 discard the first 3 mL of eluate, and collect the following 3 mL of eluate in a 10- mL test tube. [1] Transfer accurately 2 mL of the eluate to a 50 - mL recovery flask, concentrate under vacuum in the 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 water- methanol- acetonitrile (18:1:1), transfer this solution to a plastic centrifuge tube [2] (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- mass spectrometry.

Measurement by liquid chromatography- mass spectrometry. Inject 5 µL each of the sample solution and respective standard solutions to a liquid chromatograph- mass spectrometer to 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 3 [3]

Eluent: 10 mmol/L ammonium acetate solution- methanol (4:1) → 15 min→ methanol (5 minutes retention) [4]

Flow rate: 0.5 mL/min

Column oven temperature: 40 °C

Detector: Quadrupole mass spectrometer Note 4

Ionization method: Atmospheric pressure photo ionization (APPI) or atmospheric pressure chemical ionization (APCI) (positive ion mode, T-2 toxin; negative ion mode, deoxynivalenol and nivalenol)

Fragmentor voltage: 100 V

Nebulizer pressure: N_2 (380 kPa)

Dryer gas: N_2 (7.0 L/min, 350 °C)

Vaporizer temperature: 300 °C

Capillary voltage: 1,500 V

Monitor ion [5]: *m/z* 484 (T-2 toxin), 355 (deoxynivalenol), 371 (nivalenol)

Calculation. Obtain peak areas from the resulting selected ion monitoring chromatograms [6] to prepare a calibration curve, and calculate the amounts of T-2 toxin, deoxynivalenol and nivalenol in the sample.

Note 1 When the analysis sample absorbs the extraction solvent and cannot be shaken, use a 300- mL stoppered Erlenmeyer flask and 150 mL of the extraction solvent.

2 MultiSep 227 Trich+ (Romer Labs) or equivalents.

3 ZORBAX Eclipse XDB-C18 (Agilent Technologies) or equivalents.

4 Example conditions for Agilent 1100 MSD SL (Agilent Technologies).

<<Summary of analysis method>>

This is a simultaneous analysis method to extract trichothecene mycotoxins, specifically T-2 toxin, deoxynivalenol and nivalenol, in feeds with aqueous acetonitrile, purify with a multifunctional cleanup (MFC) column MultiSep 227 Trich+, and quantitate by a liquid chromatograph- mass spectrometer.

The flow sheet of the analysis method is shown in Figure 5.3.4-1.

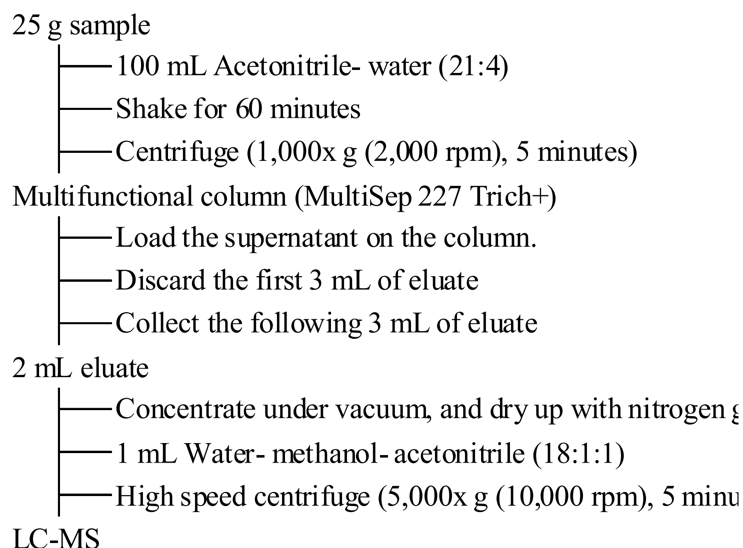


Figure 5.3.4-1 Flow sheet of the simultaneous analysis method for trichothecene mycotoxins by LC-MS

References: Yuzo Ono, Kazutoshi Mizuno, Eiichi Ishiguro: Research Report of Animal Feed, **28**, 20 (2003)

Takayuki Ishibashi, Yuzo Ono: Research Report of Animal Feed, **29**, 1 (2004)
History in the Feed Analysis Standards [26] New (deoxynivalenol and nivalenol), [27] Partial revision (T-2 toxin added)

<<Analysis method validation>>

- Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (µg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Deoxynivalenol	Chicken formula feed	100~1,000	3	104.3~111.0	4.2
	Pig formula feed	100~1,000	3	115.2~115.9	5.1
	Milo	100~1,000	3	100.8~108.4	7.7
	Barley	100~1,000	3	105.6~110.6	8.5
Nivalenol	Chicken formula feed	100~1,000	3	84.9~87.4	9.0
	Pig formula feed	100~1,000	3	86.5~90.6	7.6
	Milo	100~1,000	3	83.7~92.5	3.8
	Barley	100~1,000	3	83.7~85.8	14.4
T-2 toxin	Chick feed	10~1,000	3	96.3~119.7	10.5
	Cattle formula feed	10~1,000	3	104.9~108.7	3.3
	Corn	10~1,000	3	90.0~107.3	14.7
	Wheat	10~1,000	3	102.8~106.7	3.3

- 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
Deoxynivalenol	Chicken formula feed	5	500	113.8	1.6	4.7	0.26
	Chicken formula feed	5	Natural contamination	(211)	5.1	19.1	0.94
Nivalenol	Chicken formula feed	5	500	82.6	1.5	5.8	0.33
T-2 toxin	Broiler finisher	6	200	89.4	3.0	12.9	0.63

- Lower limit of quantification: 10 µg/kg for deoxynivalenol and nivalenol; 5 µg/kg for T-2 toxin (spike recovery and relative standard deviation)

<<Notes and precautions>>

[1] Nivalenol recovery is low in the fraction of 0-3 mL eluate. In addition, contaminants that interfere the quantitation of mycotoxins may be eluted in the fraction of eluate over 6 mL.

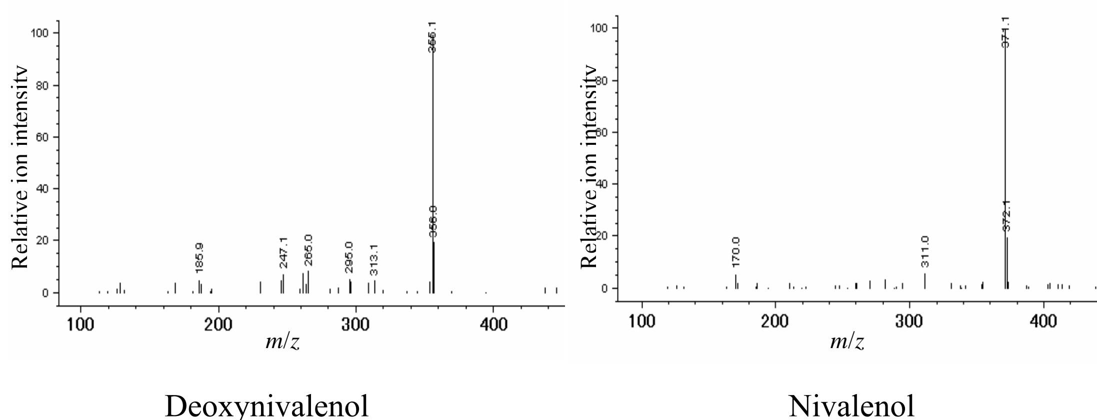
[2] Make sure that there is no background ion that interferes the quantitation of mycotoxins.

[3] 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

ZORBAX Eclipse XDB-C18.

[4] By linear gradient. Additionally, when analysis is conducted according to the Example of measurement conditions, a specific peak that is different from the target component appears at around 16 minutes (about 1 minute after switching the eluent to methanol) in every sample solution, thus washing time with methanol for 5 minutes is included.

[5] Respective acetate adduct ions of deoxynivalenol and nivalenol $[M+CH_3CO_2]^-$ are used as the monitor ion.. The ammonium adduct ion $[M+NH_4]^+$ is used for T-2 toxin. The mass spectra of deoxynivalenol and nivalenol are shown in Figure 5.3.4-2, and the mass spectrum of T-2 toxin is shown in Figure 5.3.4-3.



Deoxynivalenol Nivalenol

Figure 5.3.4-2 Mass spectra of deoxynivalenol and nivalenol

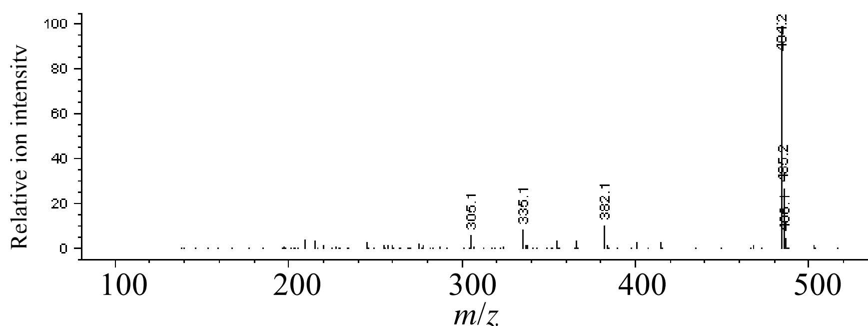


Figure 5.3.4-3 Mass spectrum of T-2 toxin

[6] Examples of selected ion monitoring (SIM) chromatograms and their total, that is, total ion chromatograms (TIC) are shown in Figure 5.3.4-4 and Figure 5.3.4-5.

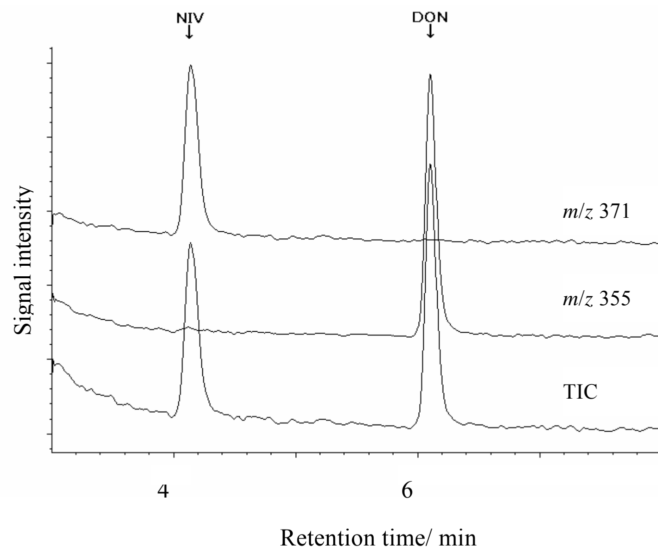


Figure 5.3.4-4 SIM chromatogram and TIC of a pig formula feed spiked with an amount equivalent to 100 µg/kg as DON and NIV

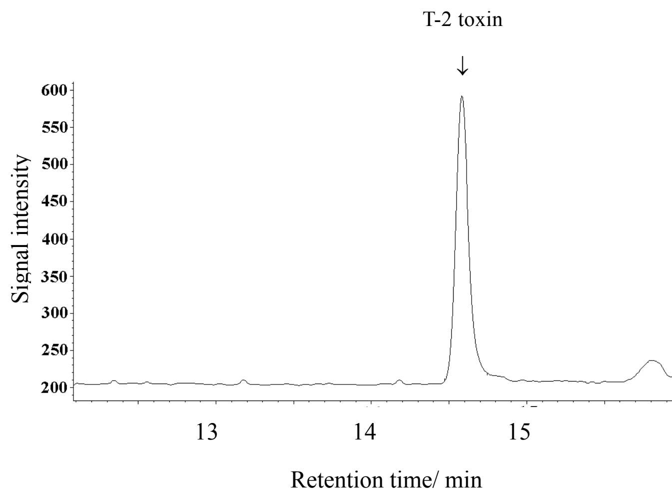


Figure 5.3.4-5 SIM chromatogram of a cattle formula feed spiked with an amount equivalent to 10 µg/kg as T-2 toxin

3 Gas chromatography Note 1 [Feed Analysis Standards, Chapter 5, Section 1 8.3]

A. Reagent preparation

1) T-2 toxin standard solution. Weigh accurately 10 mg of T-2 toxin [$C_{24}H_{34}O_9$], [1] put in a 100-mL amber volumetric flask, dissolve by the addition of chloroform, and add the same solvent up to the graduation line to prepare the T-2 toxin standard stock solution (1 mL of this solution contains 0.1 mg as T-2 toxin.).
   Before use, dilute accurately a certain amount of the standard stock solution with chloroform to prepare the T-2 toxin standard solution that contains 10 µg of T-2 toxin in 1 mL.
2) Internal standard solution. Weigh 0.25 g of methoxychlor[2] and put in a 250-mL amber volumetric flask, dissolve by the addition of *n*-pentane, and add the same solvent up to the graduation line to prepare the internal standard stock solution.
   Before use, dilute a certain amount of the internal standard stock solution with *n*-pentane to prepare the internal standard solution that contains 0.1 µg of methoxychlor in 1 mL.
3) Washing solution. Dissolve 1.12 g of potassium hydroxide and 10 g of potassium chloride in water to be 1 L.
4) Silica gel. Dry silica gel for column chromatography (particle size 74-149 µm (200-100 mesh)) Note 2 at 120°C for 2 hours.
5) Diatomite. Wash diatomite Note 3 sequentially with warm water and methanol, and then air-dry.

B. Quantification

Extraction. Weigh 10.0-50.0 g of an analysis sample, [3] transfer it to a 500-mL separatory funnel, add 250 mL of methanol/ water (1:1), extract by shaking for 30 minutes, and filter with filter paper (2 types).

Transfer 60 mL of the extract to a 500-mL beaker, add 240 mL of ammonium sulfate solution (30 w/v%) [4] and stir. Further add 25 g of diatomite[5] and stir, and filter with filter paper (2 types) to be a sample solution to be subjected to purification.

Purification. Transfer 200 mL of the sample solution to the 300-mL separatory funnel A. Add 10 mL of chloroform,[6] shake vigorously for 1 minute and leave at rest,[7] and transfer the chloroform layer (lower layer) to the 300-mL separatory funnel

B[8] that contains 100 mL of the washing solution in advance. Add 10 mL of chloroform to the separatory funnel A, and treat similarly, and then transfer the chloroform layer to the separatory funnel B,[9] shake mildly[10] and then leave at rest. Pass the chloroform layer through a funnel that contains 25 g of sodium sulfate (anhydrous) in advance for dehydration,[11] to obtain a sample solution to be subjected to column treatment.

Column treatment. Suspend 1 g of sodium sulfate (anhydrous), 2 g of silica gel, and 1 g of sodium sulfate (anhydrous) respectively in chloroform and sequentially pour into a column (10 mm in inner diameter),[12] wash the column by sequentially loading 50 mL of diethyl ether and 20 mL of chloroform, and elute so that the liquid level reaches to the height of 3 mm from the upper end of packing to prepare a column.

Transfer 5 mL of the sample solution to a 50-mL recovery flask, add 20 mL of hexane, mix, and load on the column. Wash the recovery flask with a small amount of hexane three times, and add the washing to the column. Elute so that the liquid level reaches to the height of 3 mm from the upper end of packing, and then sequentially load 20 mL of benzene and 30 mL of benzene/ acetone (19:1) and elute similarly.

Place a 50-mL recovery flask under the column. Add 30 mL of diethyl ether 30 mL to the column to elute T-2 toxin.[13] 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.

Add accurately 2 mL of benzene to dissolve the residue, and transfer to a 10-mL screw-capped amber test tube[14] to be a sample solution to be subjected to derivatization.

At the same time, transfer several amounts between 50-400 µL of the T-2 toxin standard solution to 10-mL screw-capped amber test tubes, respectively, dry up by nitrogen gas flow, and add accurately 2 mL of benzene to dissolve the residue to be the standard solutions to be subjected to derivatization.

Derivatization.[15] Add 1 g of sodium sulfate (anhydrous) to the sample solution, shake, and add 100 µL of *N*-heptafluorobutyrylimidasole.[16] Seal the test tube and shake for 1 minute, further add 2 mL of sodium bicarbonate solution (5 w/v%), seal again and shake for 2 minutes, and then leave at rest. Take accurately a certain amount of the benzene layer (upper layer), dilute accurately 5-fold with the internal standard solution[17] to be a sample solution to be subjected to gas

chromatography.[18]

At the same time, operate using respective standard solutions in the same way as the sample solution, to be the derivatized T-2 toxin standard solutions to be subjected to gas chromatography.

Gas chromatography. Inject a certain amount each of the sample solution and respective derivatized T-2 toxin standard solutions to a gas chromatograph[19] to obtain chromatograms.

Example of measurement conditions

Detector: Electron capture detector

Column: Glass, 3 mm in inner diameter, 1.1 m in length[20]

Column packing: Polydimethysiloxane Note4 (3 %)/ diatomite for gas chromatography (particle size 125-149 µm (120-100 mesh)) Note 5

Carrier gas: N_2 (30 mL/min)

Column oven temperature: 220°C

Injector temperature: 325°C[21]

Detector temperature: 325°C

Calculation. Obtain peak heights or areas of derivatized T-2 toxin and methoxychlor from the resulting chromatograms[22] to prepare a calibration curve[23] by the internal standard method, and calculate the amount of T-2 toxin in the sample..

Note 1 Solvents to be used are reagents for residual agrochemicals or equivalents.

2 Wako gel C-200 (Wako Pure Chemicals) or equivalents

3 Hyflo Supercel (Celite Corporation) or equivalents

4 Silicone SE-30 (GE Toshiba Silicones) or equivalents

5 Gaschrom Q (Applied Science Labs, distributed by Shinwa Chemical Industries) or equivalents

When a column is newly prepared, after column aging, set column oven temperature at 150-180°C, and inject 200 µL of a silanization reagent (Silyl-8 (Pierce Chemical) or equivalents) to the column, react for 1 hour, and then connect the column to the detector to stabilize the baseline.

On and after the second use, inject 100 µL of the silanization reagent and operate similarly before use.

<<Summary of analysis method>>

In this method, T-2 toxin is extracted with methanol/ water (1:1), and purified of protein compounds etc. with ammonium sulfate solution, selectively concentrated with chloroform, and acidic substances are removed with potassium hydroxide solution and the remaining interfering substances are removed with a silica gel column. Then the derivatization reagent (HFBI) is added to the purified sample solution, and resulting ester is diluted with the internal standard solution (methoxychlor) and quantitated by a gas chromatograph with a ^{63}NiElectron capture detector.

References: T. R. Romer, T. M. Boling, J. L. MacDonald: J. AOAC., 61, 801 (1978)
Hisao Tanaka and Kazuhiro Ishizaki: Research Report of Animal Feed, 13, 1 (1988)

History in the Feed Analysis Standards [10] New

<<Notes and precautions>>

[1] 100 µg/mL T-2 toxin standard solution is commercially available from Kanto Chemical, etc.

[2] Commercially available from Wako Pure Chemicals, Kanto Chemical, Hayashi Pure Chemical, GL Sciences, etc.

[3] The sample amount is preferably 10 g for glutenous samples such as wheat.

[4] There is data showing that T-2 toxin recovery reduced extremely when the ammonium sulfate concentration was as high as about 40 w/v%. Therefore, it is needed to prepare a 30 w/v% solution as accurate as possible.

[5] Celite 545 or hyflo super-cel is used. Their specific gravity (apparent) is about 0.25, thus a glassful can be added using a 100-mL beaker.

[6] Add accurately using a transfer pipette. An autoburette etc. is convenient for a large number of samples.

[7] Take sufficient time for wheat, barley, etc.

[8] A little of remaining emulsion layer does not affect the result.

[9] After totally removing chloroform “drops” adhered to the wall, take all including the emulsion layer. Some of the mother liquid (remaining solution) may be mixed as well.

[10] Vigorous shaking leads to emulsification and separation becomes difficult. Hold the funnel upside down, and mild shaking (supporting at the top end as the fulcrum) is sufficient.

[11] Collect the dehydrated solution into a stoppered glass container. Small-volume

Erlenmeyer flasks can be used, however, in that case the 5 mL needs to be separated into a round-bottom flask immediately and accurately.

[12] One with a reservoir of 30-40 mL at the top is convenient.

[13] Adjust the flow rate to about 1 mL/min (1 drop/s).

[14] A preferred cap of the test tube is lined with Teflon. However, it also may adsorb T-2 toxin to some degree, therefore care should be taken that the liquid content does not touch the cap as much as possible.

[15] The derivative thus produced is not very stable; gas chromatography should be completed within the day.

[16] Commercially available from GL Sciences. Preferably stored frozen.

[17] Preferably, take 2 mL of the internal standard solution to a 4-mL screw-capped vial, and add 500 μL of the benzene layer and mix. See [14] for precautions for mixing.

[18] A large peak may appear between the internal standard peak and the T-2 toxin peak in a chromatogram of the solution several dozen minutes after dilution (often completely separated from both of the peaks and does not affect quantitation). Therefore, it is preferred to dilute the sample one by one immediately before injection into a gas chromatograph. Additionally, the volume of *n*-pentane, which is the solvent of the internal standard solution, is rather highly temperature-dependent, thus care should be taken such as to minimize the change in room temperature (change in solution temperature) during gas chromatography operation. (Similar attention is needed for other organic solvents.)

[19] Suitable injection volume is around 5 μL for handling, however, the volume should be changed (in the range about 2-10 μL) depending on the predicted T-2 toxin content to obtain optimum chromatogram.

[20] When a capillary column is used,

Example of measurement conditions

Detector: Electron capture detector (ECD)

Column: Agilent Technologies DB-5 (0.25 mm in inner diameter, 30 m in length, 0.25 μm in membrane thickness)

Carrier gas: He (1.6 mL/min, Initial Flow)

Make-up gas: N_2 (60 mL/min)

Sample introduction: Splitless

Injector temperature: 250°C

Column oven temperature: Initial temperature 70°C (retained 2 min) →

elevation by 20°C/min → 140°C → elevation by 5°C/min→300°C

Detector temperature: 320°C

[21] In a model in which the heater of the sample injector is combined with that for the column oven, both can be the same temperature (325°C) and quantitate with similar accuracy etc.

[22] An example of chromatograms is shown in Figure 5.1.8-1.

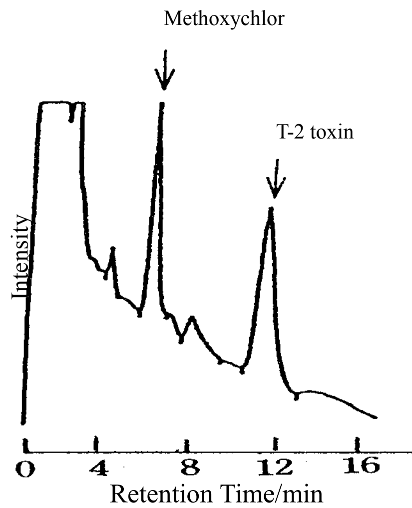


Figure 5.1.8-1 Chromatogram of corn contaminated with T-2 toxin

[23] As for the linearity of the calibration curve, “peak height ratio” exceeds “area ratio,” and the effect of the gap in the peak starting point or end point judged by the autointegrator seems to be slightly smaller in the former than in the latter.

[Other analysis methods]

4 ELISA

ELISA kits are available in Japan from several distributors .

Table 5.1.8-1 shows major kits commercially available now in Japan, and their summaries.

Table 5.1.8-1 ELISA kits for T-2 toxin analysis commercially available in Japan

Item		Lower limit of detection	Analysis time	Shelf life of the kit	Applicable samples	Notes
Quantitation kit	Veratox® for T-2 Toxin	7.5 ppb	10 minutes	6 months	Corn	Neogen
	RIDASCREEN® FAST T-2 Toxin	50 ppb	25 minutes	6-9 months	Grains	R-Biopharm Rhône AOAC RI approved
	RIDASCREEN® T-2 Toxin	3.5 ppb	90 minutes	6-9 months	Grains	R-Biopharm Rhône
	Max Signal T-2toxin	10 ppb	60 minutes	6-9 months	Grains, feeds, nuts and seeds, milk	BIOO Scientific