Deoxynivalenol (Vomitoxin)

Nivalenol

[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 13.1 and 14.1]

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.

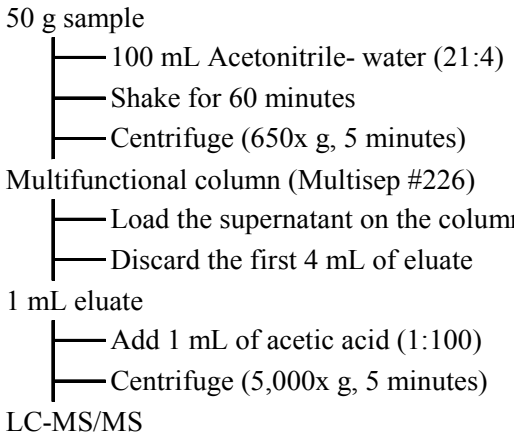


Figure 5.3.1-1 Flow sheet of the simultaneous analysis method for mycotoxins by liquid chromatography- tandem mass spectrometry

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.

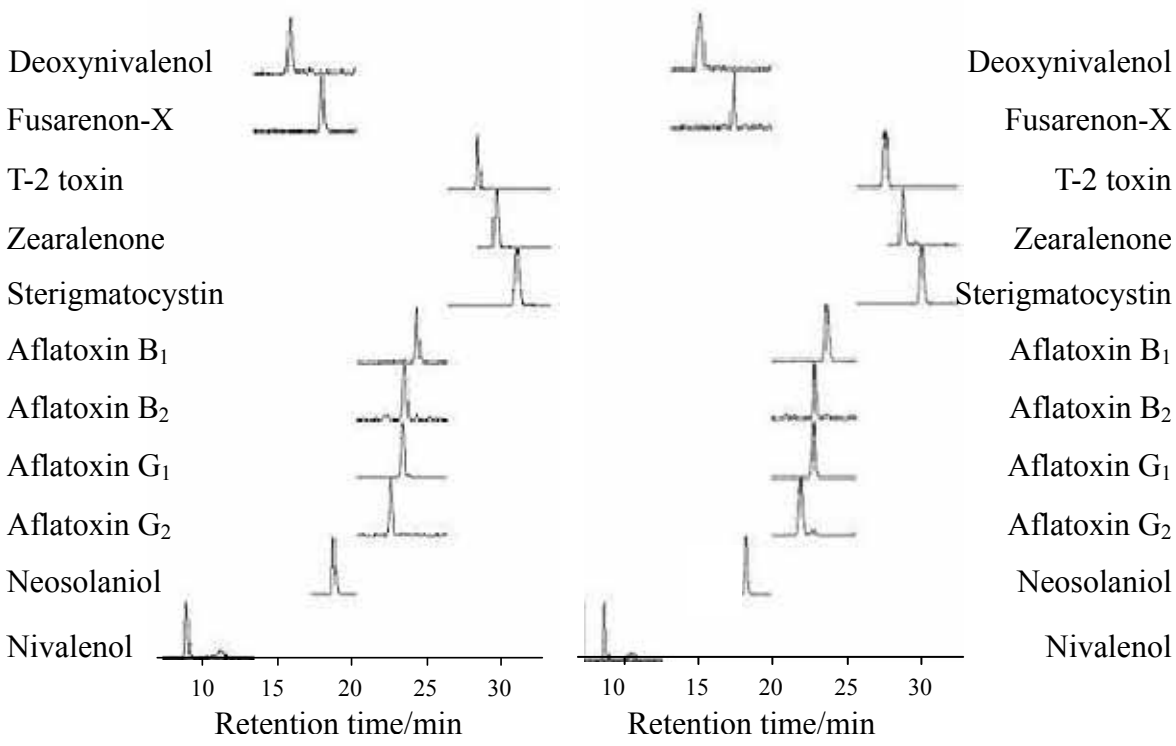


Figure 5.3.1-2 SRM chromatograms of mycotoxin-spiked formula feeds and corn (Left) formula feeds; (Right) corn

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 liquid chromatography/ mass spectrometry [Feed Analysis Standards, Chapter 5, Section 1 13.2 and 14.2]

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 richothecene 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) → 15min→ 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.

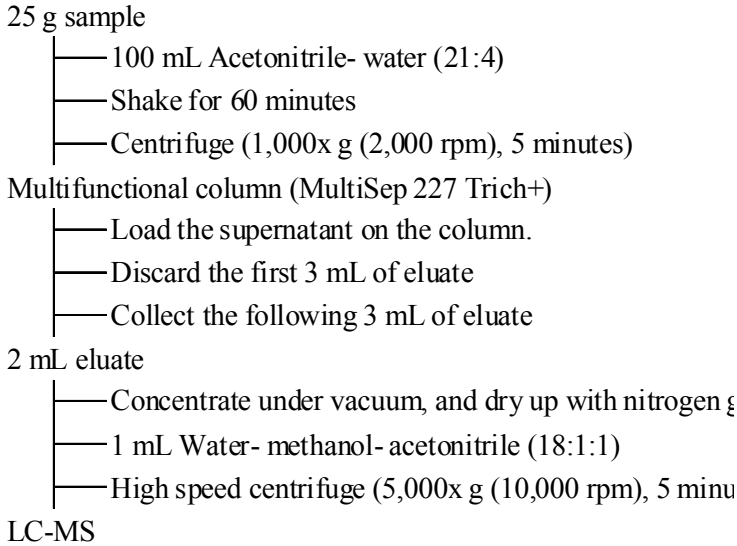


Figure 5.3.4-1 Flow sheet of the simultaneous analysis method for trichothecene mycotoxins by LC-MS

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>>

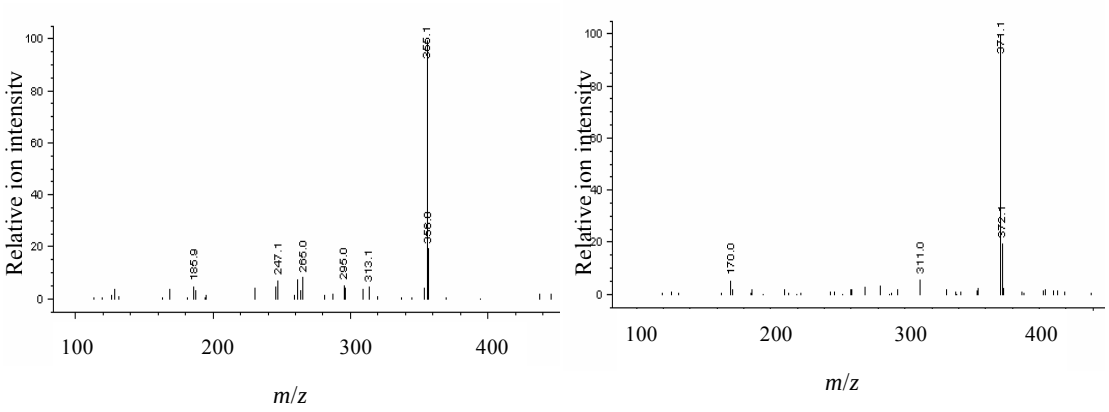
[1] Nivalenol recovery is low in the fraction of 0-3 mL eluate. In addition, ontaminants that interfere the quantitation of mycotoxins may be eluted in the fraction of eluate over 6 mL.

[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

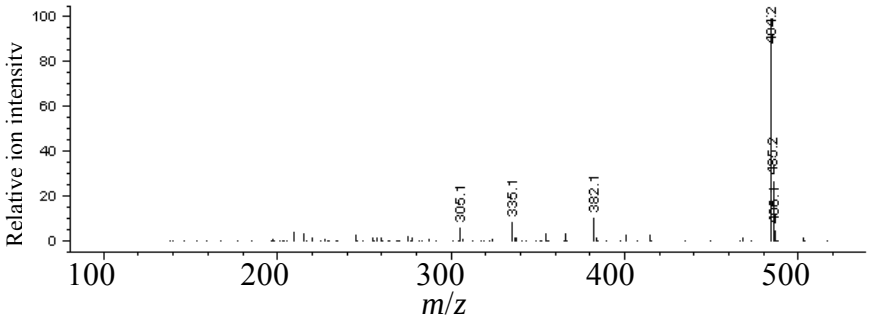


Figure 5.3.4-3 Mass spectrum of T-2 toxin

[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.

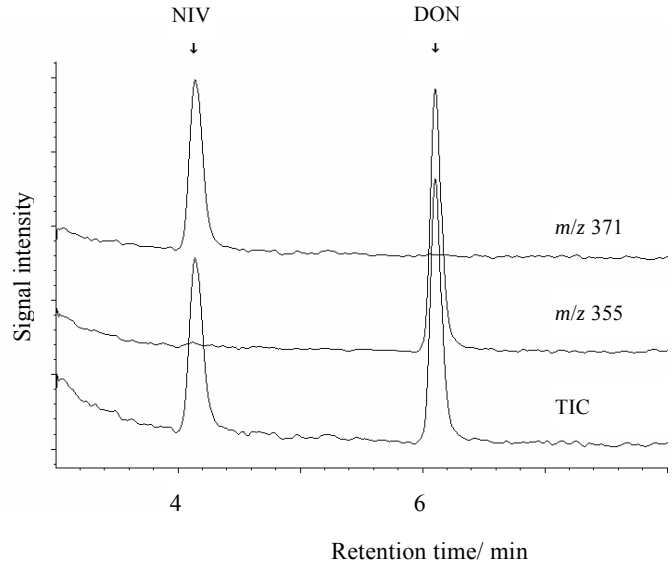


Figure 5.3.4-4 SIM chromatogram and TIC of a pig formula feed spiked with an amount equivalent to 100 µg/kg as DON and NIV

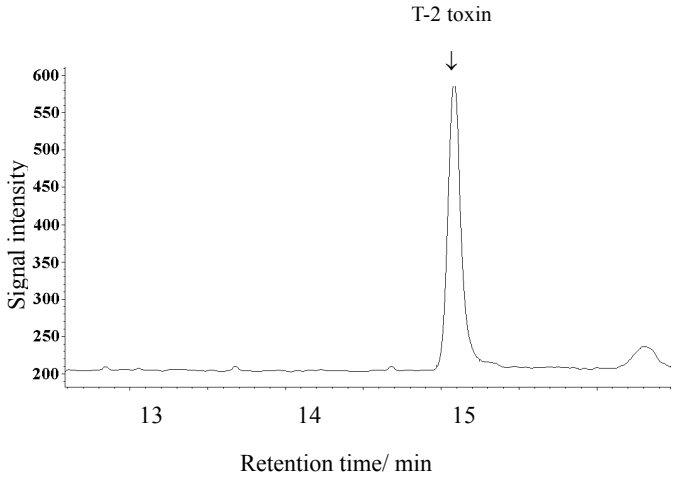


Figure 5.3.4-5 SIM chromatogram of a cattle formula feed spiked with an amount equivalent to 10 µg/kg as T-2 toxin

3 Simultaneous analysis of trichothecene mycotoxin by gas chromatography [Feed Analysis Standards, Chapter 5, Section 1 13.3 and 14.3]

Analyte compounds: 3-Acetyldeoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol, nivalenol and fusarenon-X (5 components)

Scope of application: Feeds

A. Reagent preparation

1) 3-Acetyldeoxynivalenol standard stock solution. Put 1 mg of 3-acetyldeoxynivalenol [$C_{17}H_{22}O_7$][1] in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and further add the same solvent up to the graduation line to prepare the 3-acetyldeoxynivalenol standard stock solution (1 mL of this solution contains 0.2 mg as 3-acetyldeoxynivalenol.).

2) 15-Acetyldeoxynivalenol standard stock solution. Put 1 mg of 15-acetyldeoxynivalenol [$C_{17}H_{22}O_7$][1] in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and further add the same solvent up to the graduation line to prepare the 15-acetyldeoxynivalenol standard stock solution (1 mL of this solution contains 0.2 mg as 15-acetyldeoxynivalenol.).

3) Deoxynivalenol standard stock solution. Put 1 mg of deoxynivalenol [$C_{15}H_{20}O_6$][1] in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and further add the same solvent up to the graduation line to prepare the deoxynivalenol standard stock solution (1 mL of this solution contains 0.2 mg as deoxynivalenol.).

4) Nivalenol standard stock solution. Put 1 mg of nivalenol [$C_{15}H_{20}O_7$][1] in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and further add the same solvent up to the graduation line to prepare the nivalenol standard stock solution (1 mL of this solution contains 0.2 mg as nivalenol.).

5) Fusarenon-X standard stock solution. Put 1 mg of fusarenon-X [$C_{17}H_{22}O_8$][1] 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 fusarenon-X.).

6) Mixture standard stock solution. Mix a certain amount of each of the 3-acetyldeoxynivalenol standard stock solution, 15-acetyldeoxynivalenol standard stock solution, deoxynivalenol standard stock solution, nivalenol standard stock solution and fusarenon-X standard stock solution, and dilute accurately with acetonitrile to prepare mixture standard stock solution that contains 10 μg as each

mycotoxin in 1 mL.

7) Derivatization reagent. [Note 1] *N*-Trimethylsilylimidasol[2]- *N*,*O*-bis (trimethylsilyl) acetamide[2]- trimethylchlorosilane [2] (3:3:2) (prepare before use.)

B. Quantification

xtraction. Weigh 25.0 g of an analysis sample, transfer it to a 200- mL stoppered Erlenmeyer flask, add 100 mL of acetonitrile- water (21:4), and extract by shaking for 60 minutes. [Note 2]. Transfer the extract to a 10- mL 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 the sample solution to a multifunctional column (for trichothecene mycotoxins pretreatment), [Note 3] and discard the first 3 mL of eluate. [3] Transfer accurately 2 mL of the following 3 mL of eluate [4] to a 50 - mL recovery flask to be a sample solution to be subjected to derivatization.

Derivatization. Concentrate the sample solution under vacuum in the water bath at 50°C or less to be almost dried up, and then dry up by nitrogen gas flow. [5] Add 0.1 mL of the derivatization reagent to the residue, seal the recovery flask that contained the sample solution, and leave at rest at room temperature for 15 minutes. Dissolve the residue by the addition of accurately 1 mL of 2,2,4-trimethylpentane, and further add 1 mL of water, and shake for 5 minutes. Transfer the whole amount of this solution to a 10- mL or smaller test tube, shake, and then leave at rest, to obtain the 2,2,4-trimethylpentane layer (upper layer) to be a sample solution to be subjected to gas chromatography.

Derivatization of standard stock solution. Transfer accurately 1 mL of the mycotoxin mixture standard stock solution to a 50- mL recovery flask, and dry up by nitrogen gas flow. Add 0.1 mL of the derivatization reagent to the residue, seal the recovery flask, and leave at rest at room temperature for 15 minutes. Dissolve the residue by the addition of accurately 5 mL of 2,2,4-trimethylpentane, [Note 4] and further add 1 mL of water, and shake for 5 minutes. Transfer the whole amount of this solution to a 10- mL or smaller test tube, shake, and then leave at rest. Dilute the 2,2,4-trimethylpentane layer (upper layer) accurately with the same solvent to prepare several standard solutions that contain 0.01-1 μg respectively as respective mycotoxins in 1 mL to be subjected to gas chromatography.

Gas chromatography. Inject 1 μL each of the sample solution and respective standard solutions to a gas chromatograph, [Note 5] to obtain chromatograms.

Example of measurement conditions

Detector: Electron capture detector

Column [Note 6]: Fused silica capillary column (35 % diphenyl- 65 % dimethylpolisiloxane coating, 0.25 mm in inner diameter, 30 m in length, 0.25 μm in membrane thickness)

Carrier gas: He (1.5 mL/min)

Make-up gas: N_2 (40 mL/min)

Sample introduction: Splitless (60 s)

Injector temperature: 250 °C

Column oven temperature: 80 °C (retained 1 minute) → elevation by 20 °C/min → 180 °C → elevation by 5 °C/min → 300 °C (retained 10 minutes)

Detector temperature: 300 °C

Calculation. Obtain peak heights from the resulting chromatograms [6] to prepare a calibration curve, and calculate the amounts of mycotoxins in the sample.

Note 1 Use the reagent that can sufficiently derivatize mycotoxins to be quantitated.

2 In the case of samples like bran that tends to be pasty, weigh 25.0 g of a sample, transfer it to a 300- mL stoppered Erlenmeyer flask, add 150 mL of acetonitrile- water (21:4), and extract by shaking for 60 minutes.

3 Autoprep MF-T 1500 (Showa Denko), MultiSep 227 Trich+ (Romer Labs) or equivalents.

4 Use reagents for residual pesticide analysis or equivalents.

5 Use a insert treated with silane for the sample injector. Make sure that this insert does not affect the quantitation value.

6 Make sure that the peaks can be sufficiently separated from contaminant peaks.

<<Summary of analysis method>>

This is a simultaneous analysis method to extract trichothecene mycotoxins (Group B, 5 components) in feeds with acetonitrile- water (21:4), purify with a multifunctional cleanup (MFC) column, derivatize, and then quantitate by a gas chromatograph.

The flow sheet of the analysis method is shown in Figure 5.3.5-1.

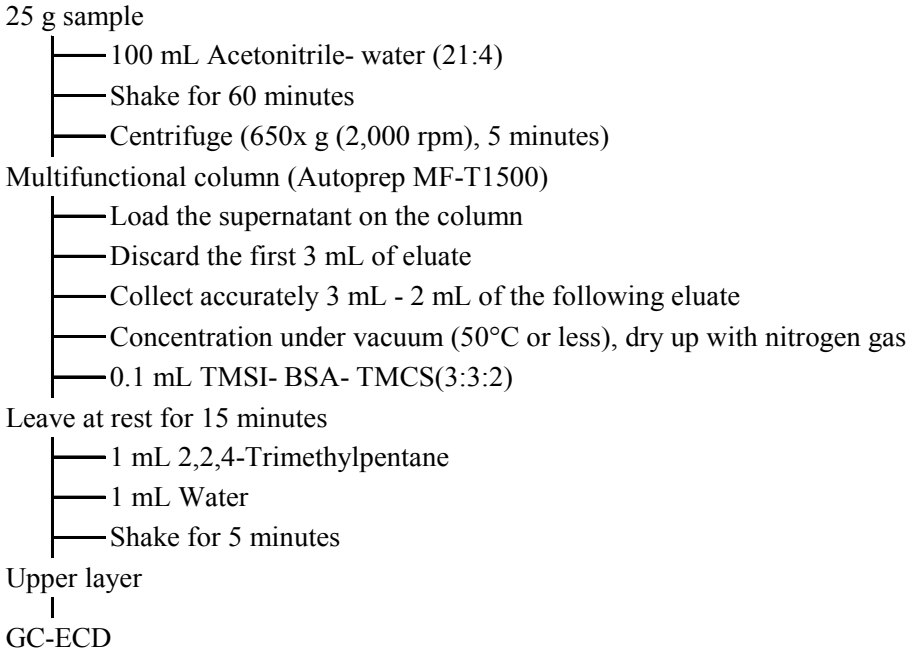


Figure 5.3.5-1 Flow sheet of the simultaneous analysis method for trichothecene mycotoxins (type B) in feeds

References: Yuji Shirai: Research Report of Animal Feed, **28**, 7 (2003)

History in the Feed Analysis Standards [26] 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)
Deoxynivalenol	Chicken formula feed	100~1,000	3	90.8~99.4	10.6
	Pig formula feed	100~1,000	3	93.2~96.8	11.4
	Milo	100~1,000	3	94.2~99.6	2.2
	Barley	100~1,000	3	92.8~98.7	3.6
Nivalenol	Chicken formula feed	100~1,000	3	95.3~105.2	4.0
	Pig formula feed	100~1,000	3	93.5~99.7	8.1
	Milo	100~1,000	3	96.1~96.3	0.7
	Barley	100~1,000	3	85.8~92.4	4.3
3-Acetyldeoxynivalenol	Chicken formula feed	100~1,000	3	95.0~96.5	4.2
	Pig formula feed	100~1,000	3	96.6~99.2	7.6
	Milo	100~1,000	3	93.2~95.7	6.0
	Barley	100~1,000	3	92.3~99.1	3.2
15-acetyldeoxynivalenol	Chicken formula feed	100~1,000	3	98.6~103.7	6.7
	Pig formula feed	100~1,000	3	97.9~98.3	6.2
	Milo	100~1,000	3	92.8~94.7	3.6
	Barley	100~1,000	3	94.2~97.1	3.2
Fusarenon-X	Chicken formula feed	100~1,000	3	94.6~98.1	4.5
	Pig formula feed	100~1,000	3	96.1~99.8	5.3
	Milo	100~1,000	3	92.6~97.0	2.4
	Barley	100~1,000	3	91.4~101.0	2.5

- 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
Deoxynivalenol	Milo	8	400	105.2	4.1	6.2	0.34
	Pig formula feed	8	Natural contamination	(503)	4.7	10.3	0.58
Nivalenol	Milo	8	400	95.4	4.5	6.1	0.33
	Pig formula feed	8	Natural contamination	(56.7)	8.4	14.7	0.67
3-Acetyldeoxynivalenol	Milo	8	400	107.3	5.9	6.6	0.36
15-acetyldeoxynivalenol	Milo	8	400	105.4	5.1	7.3	0.40
	Pig formula feed		Natural contamination	(89.3)	8.4	17.3	0.79
Fusarenon-X	Milo	8	400	106.1	5.4	6.1	0.33

- Lower limit of quantification: 10 μg/kg in a sample for each mycotoxin

<<Notes and precautions>>

[1] Standards are commercially available from Sigma-Aldrich, etc. Also, Mycotoxin Mixture 2 (B-trichothecene) (mixture solution of 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol and nivalenol) is commercially available from Kanto Chemical.

[2] Commercially available from GL Sciences, Tokyo Chemical Industry, and Sigma-Aldrich, etc.

[3] Recovery of nivalenol is low in the fraction of 0-3 mL eluate.

[4] Contaminants that interfere the quantitation of mycotoxins may be eluted in the fraction of eluate over 7 mL.

[5] If water remains, it turns cloudy by the addition of derivatization.

[6] An example of chromatograms is shown in Figure 5.3.5-2.

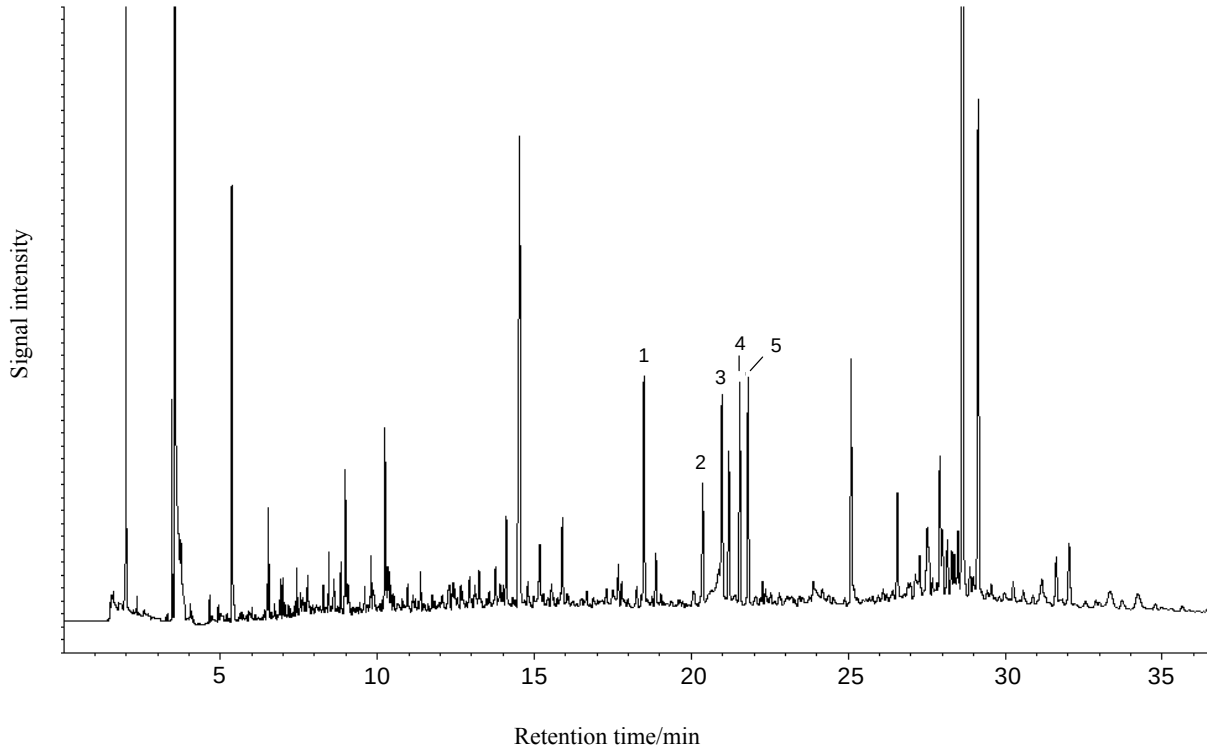


Figure 5.3.5-2 Chromatogram of a pig formula feed spiked with an amount equivalent to 100 µg/kg as respective mycotoxins

Peak name

1 Deoxynivalenol	4 3-Acetyldeoxynivalenol
2 Nivalenol	5 15-Acetyldeoxynivalenol
3 Fusarenon-X	

4 Simultaneous analysis of deoxynivalenol and nivalenol by liquid chromatography [Feed Analysis Standards, Chapter 5, Section 1 13.4 and 14.4]

Analyte compounds: Deoxynivalenol and nivalenol (2 components)

Scope of application: Grains (except barley for nivalenol) and their byproducts

A. Reagent preparation

Mixture standard solution. Weigh accurately 1 mg each of deoxynivalenol [$C_{15}H_{20}O_6$] and nivalenol [$C_{15}H_{20}O_7$], respectively put in a 5- mL amber volumetric flask, dissolve by the addition of acetonitrile, and further add the same solvent to each volumetric flask up to the graduation line to prepare the deoxynivalenol standard stock solution and the nivalenol standard stock solution (1 mL of these solutions contain 0.2 mg as deoxynivalenol and nivalenol, respectively.).

Before use, mix a certain amount of each of the deoxynivalenol and nivalenol standard stock solutions, dilute accurately with acetonitrile, to prepare a solution that contains 25 μg each of deoxynivalenol and nivalenol, respectively, in 1 mL. Further accurately dilute a certain amount of this solution with water- methanol- acetonitrile (18:1:1), to prepare several mixture standard solutions that contain 0.2-2 μg respectively as deoxynivalenol and nivalenol in 1 mL.

.

B. Quantification

Extraction. Weigh 25.0 g of an analysis sample, transfer it to a 200- mL stoppered Erlenmeyer flask, add 100 mL of acetonitrile- water (21:4), and extract by shaking for 60 minutes. [Note 1][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. 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.

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 (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.

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: UV absorptiometer (measurement wavelength: 220 nm)

Column: Octadecylsilyl silica gel column (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm) Note 3 [2]

Eluent: Water- methanol- acetonitrile (18:1:1)

Flow rate: 1 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 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 Shodex silica C18M 4E (Showa Denko) or equivalents.

<<Summary of analysis method>>

This is a simultaneous analysis method to extract deoxynivalenol and nivalenol in feeds with aqueous acetonitrile, purify with a multifunctional cleanup (MFC) column, and quantitate by a liquid chromatograph with a UV absorptiometer.

The flow sheet of the analysis method is shown in Figure 5.3.6-1.

25 g sample
- 100 mL Acetonitrile- water (21:4)
- Shake for 60 minutes
- Centrifuge (650x g (2,000 rpm), 5 minutes)

Multifunctional column (MultiSep 227 Trich+)
- Load the supernatant on the column.
- Discard the first 3 mL of eluate
- Collect accurately 3 mL of the following eluate

2 mL eluate
- Concentrate under vacuum (50°C) and dry up
- 1 mL Water- methanol- acetonitrile (18:1:1)
- High speed centrifuge (5,000x g (10,000 rpm), 5 minutes

LC-UV (220 nm)

Figure 5.3.6-1 Flow sheet of the simultaneous analysis method for deoxynivalenol and nivalenol

References: Yuji Shirai, Yuzo Ono, Kyoko Akimoto: Research Report of Animal Feed, **26**, 1 (2001)

Takayuki Ishibashi, Chie Watabe, Kaori Iwatsuki, Eiichi Ishiguro: Research Report of Animal Feed, 30, 69 (2005)

History in the Feed Analysis Standards [23] New, [28] Partial 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)
Deoxynivalenol	Corn	200~1,000	3	99.7~101.9	11.2
	Milo	200~1,000	3	96.1~96.1	3.8
	Wheat	200~1,000	3	98.2~108.2	4.2
	Barley	200~1,000	3	97.7~97.9	11.0
Nivalenol	Corn	200~1,000	3	88.9~98.3	4.0
	Milo	200~1,000	3	78.2~93.4	2.1
	Wheat	200~1,000	3	78.6~86.5	4.7
	Barley	200~1,000	3	78.2~92.3	3.2

- Collaborative study

Name of analyzed component	Sample type	Number of laboratories	Spike concentration (μg/kg)	Spike recovery (%)	Intra-laboratory repeatability RSDr (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Deoxynivalenol	Corn	7	1,000	102.8	3.4	4.2	0.26
Nivalenol	Corn	7	1,000	84.4	3.1	3.7	0.23

- Lower limit of quantification: 100 μg/kg for respective mycotoxins (Spike

recovery and relative standard deviation)

<<Notes and precautions>>

[1] Extraction time is set as 60 minutes because 30 minutes may not be sufficient in some cases. Additionally, when extraction by shaking is difficult for samples such as bran because of the insufficient amount of the extraction solution, increase the extraction solution as appropriate.

[2] The column to be used only needs to be one that uses packing treated by corresponding endcapping. On the other hand, peaks that interfere the quantitation of deoxynivalenol and nivalenol may appear in some columns; therefore check the presence or absence of interfering peaks before use.
The column used in the development of this analysis was Shodex silica C18M 4E.

[3] Examples of HPLC chromatograms of the mixture standard solution and a sample solution of corn spiked with 1 mg/kg as respective mycotoxins are shown in Figure 5.3.6-2.
In some cases, peaks may appear after 30 minutes, and they may affect the chromatogram of the next sample solution injected. Therefore, it is needed to set rather a long measurement time (60 minutes) after sample injection. Additionally, analysis can also be conducted using 0.2 mmol/L ammonium acetate solution- methanol- acetonitrile (18:1:1) as the eluent and for the sample solution when the baseline around DON is not stable. (In fact, 10 mmol/L ammonium acetate was adjusted to around pH 5, and water- 10 mmol/L ammonium acetate solution (pH 5)- methanol- acetonitrile (17:1:1:1) was used.)

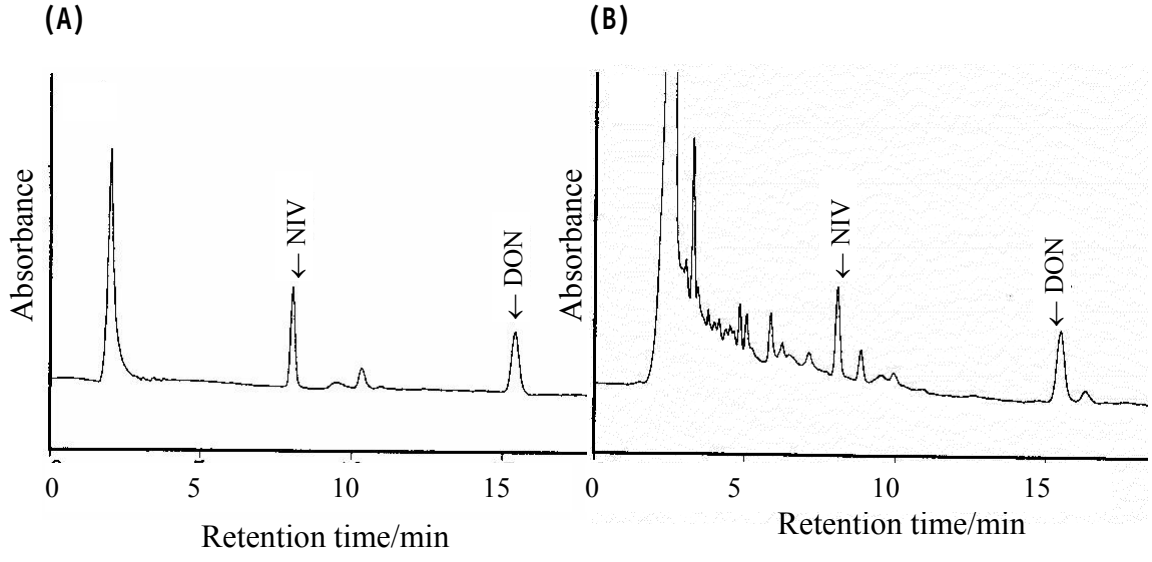


Figure 5.3.6-2 Chromatograms of DON and NIV

Measurement conditions

Eluent: Water- 10 mmol/L ammonium acetate solution (pH 5)- methanol- acetonitrile (17:1:1:1)

Other conditions are according to the Example of measurement conditions.

(A) Mixture standard solution (10 ng as respective mycotoxins)

(B) Sample solution of corn spiked with 1 mg/kg as respective mycotoxins

[Other analysis methods]

5 ELISA

ELISA kits are available in Japan from several distributors .

Table 5.1.13-1 shows major kits commercially available now in Japan, and their summaries.

Table 5.1.13-1 ELISA kits for deoxynivalenol commercially available in Japan

Item		Lower limit of detection	Analysis time	Shelf life of the kit	Applicable samples	Notes
Qualification kit	Agri-Screen® for DON	0.2 ppm	10 minutes	6 months	Grains	Neogen USDA/GIPSA method (2007-005)
	RIDASCREEN® DON FAST	0.22 ppm	5 minutes	6-9 months	Grains	R-Biopharm Rhône FGIS, AOAC RI, GIPSA approved
Quantitation kit	Veratox® for DON 5/5	0.1 ppm	10 minutes	6 months	Grains, bran, flour etc.	Neogen USDA/GIPSA approved
	RIDASCREEN® FAST DON	0.2 ppm	25 minutes	6-9 months	Grains	R-Biopharm Rhône FGIS, AOAC RI, GIPSA approved
	AgraQuant® DON	0.035 ppm	20 minutes	6-9 months	Grains	Romer Labs
	Charm ROSA DON Quantitative	0.25 ppm	20 minutes	6-9 months	Grains	Charm Science USDA/GIPSA method (2007-104)