## 3-Acetyldeoxynivalenol

## 15-Acetyldeoxynivalenol

### [Methods listed in the Feed Analysis Standards]

1 Simultaneous analysis of trichothecene mycotoxin by gas chromatography [Feed Analysis Standards, Chapter 5, Section 1 11.1 and 12.1]

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. <sup>Note 1</sup> *N*-Trimethylsilylimidasol<sup>[2]</sup>- *N*,*O*-bis (trimethylsilyl) acetamide<sup>[2]</sup>- trimethylchlorosilane <sup>[2]</sup> (3:3:2) (prepare before use.)

#### **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. <sup>Note 2</sup>. Transfer the extract to a 10- mL centrifuge tube, centrifuge at  $650 \times g$  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), <sup>Note 3</sup> and discard the first 3 mL of eluate.
  <sup>[3]</sup> Transfer accurately 2 mL of the following 3 mL of eluate <sup>[4]</sup> 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. <sup>[5]</sup> 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, <sup>Note 4</sup> 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  $\mu$ g respectively as respective mycotoxins in 1 mL to be subjected to gas chromatography.

Gas chromatography. Inject 1  $\mu$ L each of the sample solution and respective standard solutions to a gas chromatograph, <sup>Note 5</sup> to obtain chromatograms.

Example of measurement conditions

Detector: Electron capture detector

Column <sup>Note 6</sup>: 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<sub>2</sub> (40 mL/min)

Sample introduction: Splitless (60 s)

Injector temperature: 250 °C

Column oven temperature: 80 °C (retained 1 minute)  $\rightarrow$  elevation by

20 °C/min  $\rightarrow$  180 °C  $\rightarrow$  elevation by 5 °C/min  $\rightarrow$  300 °C (retained 10 minutes)

Detector temperature: 300 °C

Calculation. Obtain peak heights from the resulting chromatograms <sup>[6]</sup> 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.					

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

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<<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.

25 g sample -100 mL Acetonitrile- water (21:4) -Shake for 60 minutes -Centrifuge (650x g (2,000 rpm), 5 minutes) Multifunctional column (Autoprep MF-T1500) -Load the supernatant on the column -Discard the first 3 mL of eluate -Collect accurately 3 mL - 2 mL of the following eluate -Concentration under vacuum (50°C or less), dry up with nitrogen gas -0.1 mL TMSI- BSA- TMCS(3:3:2)Leave at rest for 15 minutes -1 mL 2,2,4-Trimethylpentane -1 mL Water —Shake for 5 minutes Upper layer GC-ECD

# 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>>

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         94.2-99.6         2.2           Barley         100-1,000         3         94.2-99.6         2.2           Milo         Chicken formula feed         100-1,000         3         95.3-105.2         4.0           Pig formula feed         100-1,000         3         95.3-105.2         4.0         7           Milo         100-1,000         3         95.0-96.5         4.2         7           Barley         100-1,000         3         95.0-96.5         4.2         7           Pig formula feed         100-1,000         3         92.8-94.7         3.6           Milo         100-1,000         3         94.6-98.1         4.5           Pig formula feed         100-1,000         3         94.6-98.1         4.5           Pig formula feed         100-1,000         3         94.6-98.1         4.5           Pig formula feed         100-1,000	Name of spiked component	Sample type		Spike concentration (µg/kg)	Repeat	eat Spike recover		Repeatability RSD (% or less)		
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         95.3-105.2         4.0           Milo         100-1,000         3         96.1-96.3         0.7           Barley         100-1,000         3         95.0-96.5         4.2           Pig formula feed         100-1,000         3         95.0-96.5         4.2           Pig formula feed         100-1,000         3         95.0-96.5         4.2           Pig formula feed         100-1,000         3         92.8-94.7         6.0           Milo         100-1,000         3         94.6-98.1         4.5           Pig formula feed         100-1,000         3         94.6-98.1         4.5           Pig formula feed         100-1,000         3         94.6-98.1         4.5           Pig formula feed         100-1,000         3         94.6-98.1         4.5	Deoxynivalenol	Chicken formula feed		100~1,000	3	90.8~99.4			10.6	
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         95,0-96,5         4.2           Pig formula feed         100-1,000         3         95,0-96,5         4.2           Milo         100-1,000         3         95,0-96,5         4.2           Pig formula feed         100-1,000         3         96,6-599,2         7.6           Milo         100-1,000         3         98,6-103,7         6.7           Pig formula feed         100-1,000         3         94,6-98,1         4.5           Milo         100-1,000         3         94,6-98,1         4.5           Pig formula feed         100-1,000         3         94,6-98,1         4.5           Pig formula feed         100-1,000         3         94,6-98,1         4.5           Pig formula feed         100-1,000         3         94,6-98,1         4.5		Pig formula f	feed	100~1,000	3		93.2~96.8		11.4	
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         95,0-96,5         4,2           Pig formula feed         100-1,000         3         95,0-96,5         4,2           Pig formula feed         100-1,000         3         93,2-95,7         6,0           Barley         100-1,000         3         93,2-95,7         6,0           Barley         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         94,6-98,1         4,5           Pig formula feed         100-1,000         3         91,4-101,0         2,5		Milo		100~1,000	3	3 94.2~99		5 2.2		
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         93.5-99.7         8.1           Jardey         100-1,000         3         95.5-99.7         8.1           Arcetyldeoxynivalenol         Chicken formula feed         100-1,000         3         95.0-96.5         4.2           Pig formula feed         100-1,000         3         95.0-99.2         7.6         Milo           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         92.8-94.7         3.6           Barley         100-1,000         3         94.2-97.1         3.2         3.6           Fusarenon-X         Chicken formula feed         100-1,000         3         94.6-98.1         4.5           Pig formula feed         100-1,000         3         92.6-97.0         2.4         Barley           Name of analyzed component         Sample type         Number of laboratories <t< td=""><td></td><td colspan="2">Barley</td><td>100~1,000</td><td>3</td><td colspan="2">92.8~98.7</td><td>3.6</td><td></td></t<>		Barley		100~1,000	3	92.8~98.7		3.6		
Pig formula feed Milo         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         95.0-96.5         4.2           Pig 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         98.6-103.7         6.7           Pig formula feed         100-1,000         3         92.3-99.1         3.2           15-acetyldeoxynivalenol         Chicken formula feed         100-1,000         3         94.2-97.1         3.2           Milo         100-1,000         3         94.2-97.1         3.2         5.3           Fusarenon-X         Chicken formula feed         100-1,000         3         94.2-97.1         3.2           Pig formula feed         100-1,000         3         92.6-97.0         2.4         3.4           Barley         100-1,000         3         91.4-101.0         2.5         5           Vame of analyzed component	Nivalenol	Chicken formula feed		100~1,000	3	95.3~105.2		4.0		
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         95,0-96,5         4.2           Milo         100-1,000         3         93,2-95,7         6.0           Barley         100-1,000         3         93,2-95,7         6.0           Barley         100-1,000         3         98,6-103,7         6.7           Pig formula feed         100-1,000         3         92,8-94,7         3.6           Barley         100-1,000         3         92,8-94,7         3.6           Barley         100-1,000         3         94,6-98,1         4.5           Fusarenon-X         Chicken formula feed         100-1,000         3         94,6-98,1         4.5           Milo         100-1,000         3         94,6-98,1         4.5         5.3           Milo         100-1,000         3         92,6-97,0         2.4         Barley         100-1,000         3         91,4-101,0         2.5		Pig formula feed		100~1,000	3	93.5~99.7		8.1		
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$\begin{array}{c c c c c c c c c c c c c c c c c c c $	15-acetyldeoxynivaleno	L Chicken formula feed		100~1,000	3	98.6~103.7			6.7	
Milo       100 <th< td=""><td></td><td colspan="2">Dig formula feed</td><td>100~1,000</td><td>3</td><td colspan="3">97.9~98.3</td><td>6.2</td><td></td></th<>		Dig formula feed		100~1,000	3	97.9~98.3			6.2	
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       92.6-97.0       2.4         Milo       Sample type       Number of laboratories       Spike concentration (µg/kg)       Intra-laboratory repeatability RSD <sub>R</sub> (%)       Hork         Deoxynivalenol       Milo       8       400       105.2       4.1       6.2       0         Nivalenol       Milo       8       400       95.4       4.5       6.1       0         Nivalenol       Milo       8       400       107.3       5.9       6.6       0         15-acetyldeoxynivalenol       Milo       8       400       105.4       5.1       7.3       0         15-acety		Milo		100~1,000	3	97.9~98.5			3.6	
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Name of analyzed componentSample typeNumber of laboratoriesconcentration (µg/kg)repeatability (measured value (µg/kg))reproducibility RSDr (%)HorRDeoxynivalenolMilo8400105.24.16.20Pig formula feed8Natural contamination(503)4.710.30NivalenolMilo840095.44.56.10Pig formula feed8Natural contamination(56.7)8.414.703-AcetyldeoxynivalenolMilo8400107.35.96.6015-acetyldeoxynivalenolMilo8400105.45.17.30Fusarenon-XMilo8400106.15.46.10	Nous of another d		Noushanad	Spike	Spike re	ecovery (%)	Intra-labora	tory	Inter-laboratory	
Deoxynivalenol         Milo         8         400         105.2         4.1         6.2         0           Pig formula feed         8         Natural contamination         (503)         4.7         10.3         0           Nivalenol         Milo         8         400         95.4         4.5         6.1         0           Pig formula feed         8         Matural contamination         (56.7)         8.4         14.7         0           3-Acetyldeoxynivalenol         Milo         8         400         107.3         5.9         6.6         0           15-acetyldeoxynivalenol         Milo         8         400         105.4         5.1         7.3         0           Pig formula feed         Natural contamination         (89.3)         8.4         17.3         0           Fusarenon-X         Milo         8         400         106.1         5.4         6.1         0	component	Sample type	laboratories	concentration (µg/kg)	(meas (µ	sured value g/kg))	repeatability RSD <sub>r</sub> (%)		reproducibility RSD <sub>R</sub> (%)	HorRat
Pig formula feed         8         Natural contamination         (503)         4.7         10.3         0           Nivalenol         Milo         8         400         95.4         4.5         6.1         0           Pig formula feed         8         Matural contamination         (56.7)         8.4         14.7         0           3-Acetyldeoxynivalenol         Milo         8         400         107.3         5.9         6.6         0           15-acetyldeoxynivalenol         Milo         8         400         105.4         5.1         7.3         0           Pig formula feed         Natural contamination         (89.3)         8.4         17.3         0           Fusarenon-X         Milo         8         400         106.1         5.4         6.1         0	Deoxynivalenol	Milo	8	400		105.2		4.1	6.2	0.34
Nivalenol         Milo         8         400         95.4         4.5         6.1         0           Pig formula feed         8         Natural contamination         (56.7)         8.4         14.7         0           3-Acetyldeoxynivalenol         Milo         8         400         107.3         5.9         6.6         0           15-acetyldeoxynivalenol         Milo         8         400         105.4         5.1         7.3         0           Pig formula feed         Natural contamination         (89.3)         8.4         17.3         0           Fusarenon-X         Milo         8         400         106.1         5.4         6.1         0		Pig formula feed	8	Natural contamination		(503)	I	4.7	10.3	0.58
Pig formula feed         8         Natural contamination         (56.7)         8.4         14.7         0           3-Acetyldeoxynivalenol         Milo         8         400         107.3         5.9         6.6         0           15-acetyldeoxynivalenol         Milo         8         400         105.4         5.1         7.3         0           Pig formula feed         Natural contamination         (89.3)         8.4         17.3         0           Fusarenon-X         Milo         8         400         106.1         5.4         6.1         0	Nivalenol	Milo	8	400		95.4		4.5	6.1	0.33
3-Acetyldeoxynivalenol         Milo         8         400         107.3         5.9         6.6         0           15-acetyldeoxynivalenol         Milo         8         400         105.4         5.1         7.3         0           Pig formula feed         Natural contamination         (89.3)         8.4         17.3         0           Fusarenon-X         Milo         8         400         106.1         5.4         6.1         0		Pig formula feed	8	Natural contamination		(56.7)	1	8.4	14.7	0.67
15-acetyldeoxynivalenol         Milo         8         400         105.4         5.1         7.3         0           Pig formula feed         Natural contamination         (89.3)         8.4         17.3         0           Fusarenon-X         Milo         8         400         106.1         5.4         6.1         0	3-Acetyldeoxynivalenol	Milo	8	400		107.3 5.9		5.9	6.6	0.36
Pig formula feed         Natural contamination         (89.3)         8.4         17.3         0           Fusarenon-X         Milo         8         400         106.1         5.4         6.1         0	15-acetyldeoxynivalenol	Milo	8	400		105.4		5.1	7.3	0.40
Fusarenon-X Milo 8 400 106.1 5.4 6.1 0		Pig formula feed		Natural contamination		(89.3)	1	8.4	17.3	0.79
	Fusarenon-X	Milo	8	400		106.1		5.4	6.1	0.33

#### Spike recovery and repeatability

• Lower limit of quantification:  $10 \mu g/kg$  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.



Retention time/min

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

Peak name

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