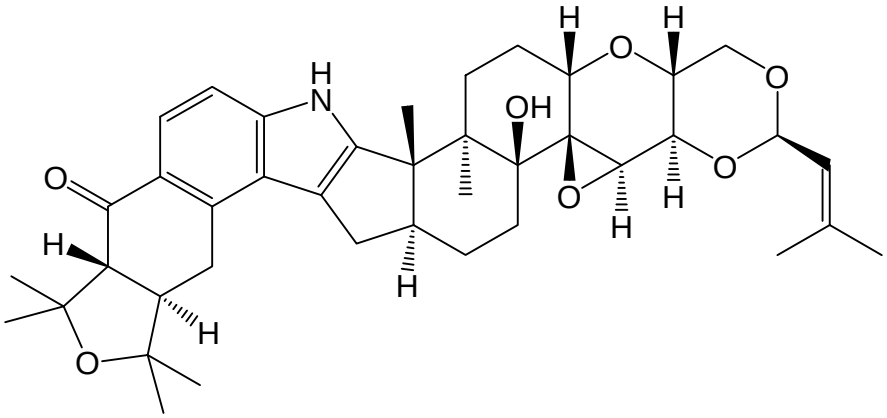
Lolitrem B



$C_{42}H_{55}NO_7$ MW: 685.89 CAS No.: 81771-19-9

[Summary of lolitrem]
Lolitrem B is a biologically active substance produced by *Neotyphodium lolli*, symbiotic endophyte in the body of the grass such as perennial ryegrass, and causes livestock poisoning including ryegrass staggers. In ryegrass staggers, up-and-down motion of the head, difficulty in walking, and tetany-like symptom, etc. are observed, and the risk value of lolitrem B for livestock is believed to be 1,800~2,000 μg/kg (however, Japanese Black cattle are more sensitive.). In Japan, there have been livestock poisoning accidents caused by lolitrem B contained in perennial ryegrass imported from the United States since 1997.

[Methods listed in the Feed Analysis Standards]

1 Liquid chromatography [Note 1] [Feed Analysis Standards, Chapter 5, Section 2 2.1]

Scope of application: hay

A. Reagent preparation

Lolitrem B standard solution. Weigh accurately 1.3 μg of lolitrem B [$C_{42}H_{55}NO_7$], [Note 2] [1] dissolve by the addition of accurately 1 mL of dichloromethane- acetonitrile (4:1), to prepare the lolitrem B standard stock solution that contains 1.3 μg as lolitrem B in 1 mL.

Before use, dilute accurately a certain amount of the standard stock solution with dichloromethane- acetonitrile (4:1) to prepare several lolitrem B standard solutions that contain 2-100 ng respectively as lolitrem B in 1 mL. [2]

B. Quantification

Extraction. Weigh accurately 5 g of an analysis sample, transfer it to a 200-mL stoppered amber Erlenmeyer flask, add 100 mL of ethyl acetate- ethanol (2:1), extract for 2 hours by intermittently shaking for a few seconds, [Note 3] and then filter the extract with filter paper (No. 5 A). [Note 4] Transfer accurately 5 mL of the

filtrate to a 25-mL recovery flask, concentrate under vacuum in a water bath at 40°C or less to be almost dried up, and then dry up by nitrogen gas flow.

Dissolve the residue by the addition of accurately 5 mL of hexane- ethyl acetate (9:1), then filter with membrane filter (pore size 0.5 μm or less),[3] to be a sample solution to be subjected to column treatment.

Column treatment. Wash a silica gel minicolumn (690 mg) with 2 mL of hexane-ethyl acetate (9:1).

Load accurately 2 mL of the sample solution on the minicolumn, elute until the liquid level reaches the upper end of packing, then add 5 mL of hexane- ethyl acetate (9:1) 5 mL and elute similarly.

Place a 25-mL recovery flask under the minicolumn, and add 6 mL of hexane-ethyl acetate (7:3) to elute lolitrem B. Concentrate the eluate under vacuum in a water bath at 40°C or less to be almost dried up, and then dry up by nitrogen gas flow.

Dissolve the residue by the addition of accurately 2 mL of dichloromethane-acetonitrile (4:1), to be a sample solution to be subjected to liquid chromatography.

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

Example of measurement conditions

Detector:	Fluorescence detector (excitation wavelength, 268 nm; emission wavelength, 440 nm)
Column:	Silica gel column (4.6 mm in inner diameter, 250 mm in length, particle size 5 μm)Note 5 [4][5]
Eluent:	Dichloromethane- acetonitrile- water (200:50:1)[6]
Flow rate:	0.5 mL/min

Calculation. Obtain peak heights or areas from the resulting chromatograms[7] to prepare a calibration curve, and calculate the amounts of lolitrem B in the sample.

Note 1 Conduct the quantification procedure under protection from light.

2 Manufactured by New Zealand Pastoral Agriculture Research Institute (Distributed by Wako Pure Chemicals)

3 Mix by shaking for a few seconds 3-4 times per hour.

4 As appropriate, transfer the extract to a 50-mL stoppered amber centrifuge tube, centrifuge at 1,500×g for 5 minutes, and filter supernatant.

5 Use a column with packing of pore size of 7 nm (ZORBAX SIL (Agilent Technologies) or equivalents).

<<Summary of analysis method>>

In this method, lolitrem B is extracted with ethyl acetate- ethanol (2:1), and the sample solution is purified with a silica gel cartridge column, and analyzed by a chromatograph with a fluorescence detector.

The flow sheet of the analysis method is shown in Figure 5.2.2-1.

5 g Analysis sample
- 100 mL Ethyl acetate- ethanol (2:1)
- Extract for 2 hours (sometimes shake vigorously)
- Filter with filter paper (No. 5A)

5 mL Filtrate
- Concentrate under vacuum (40°C or less), and dry up by nitrogen gas
- 5 mL hexane- ethyl acetate (9:1).
- Filter with membrane filter (pore size 0.5 µm or less)

Sep-Pak Plus Silica cartridge (wash with 2 mL hexane- ethyl acetate (9:1) in advance)
- Load 2 mL sample solution.
- Wash with 5 mL hexane- ethyl acetate (9:1).
- Elute with 6 mL hexane- ethyl acetate (7:3)
- Concentrate under vacuum (40°C or less), and dry up by nitrogen gas
- 2 mL Dichloromethane- acetonitrile (4:1)

LC-FL (Ex: 286 nm, Em: 440 nm)

Figure 5.2.2-1 Flow sheet of the analysis method for lolitrem B

References: Yuzo Ono, Kiyoshi Someya, Akira Furukawa, and Kiyoshi Sugano: Research Report of Animal Feed, **25**, 12 (2000)

History in the Feed Analysis Standards [22] New

<<Analysis method validation>>

- Spike recovery and repeatability

Sample type	Spike concentration (µg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Ryegrass	102~1,825	3	94.6~110.9	11.9

- Collaborative study

Sample type	Number of laboratories	Spike concentration (µg/kg)	Spike recovery (%)	Intra-laboratory repeatability RSD_r (%)	Inter-laboratory reproducibility RSD_R (%)	HorRat
Ryegrass	6	255	99.0	2.8	7.5	0.38

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

<<Notes and precautions>>

[1] The standard is commercially available from Wako Pure Chemicals.

[2] As weighing of the standard is technically difficult because of the extremely small amount (about 1 µg), prepare the standard stock solution by adding a certain amount of dichloromethane- acetonitrile (4:1) to the vial of the standard to dissolve. Store the standard stock solution in a freezer.
In addition, the concentration is possibly different between lots; therefore prepare the standard solution by diluting the standard stock solution to contain 2-100 ng

respectively as lolitrem B in 1 mL.

[3] When it is difficult to pass through membrane filter, conduct this procedure after centrifugation.

[4] Store the column after washing with dichloromethane and then replacing with hexane.

[5] The column to be used only needs to meet these specifiPositive.

[6] Prepare the eluate by mixing acetonitrile and water first and then adding dichloromethane.

[7] Chromatograms of the lolitrem B standard solution and a sample solution are shown in Figure 5.2.2-2.

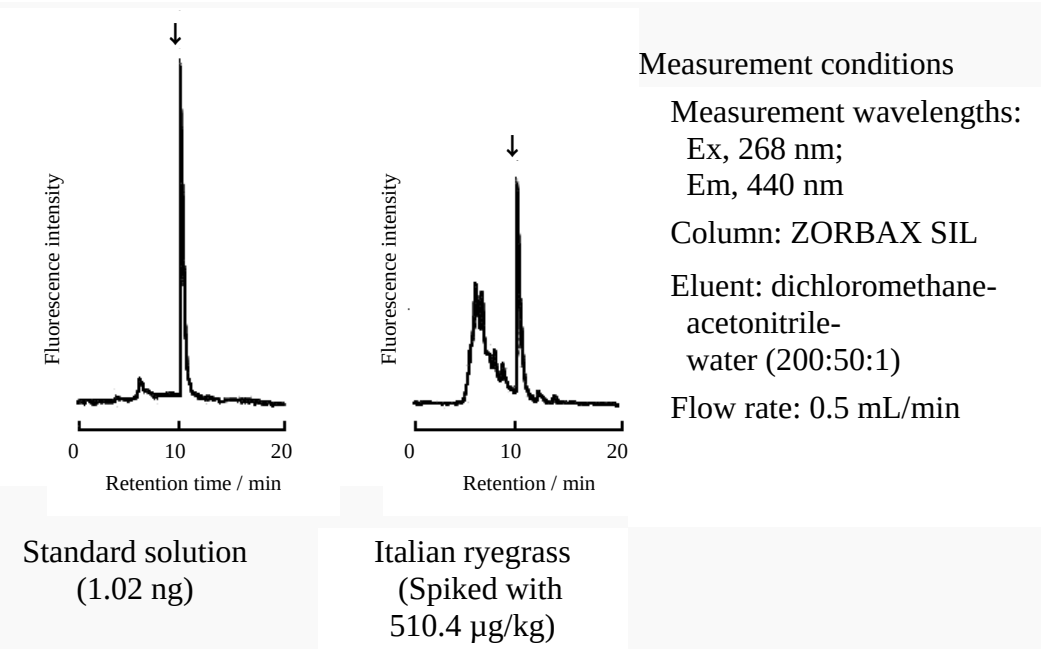


Figure 5.2.2-2 Chromatograms of lolitrem B
(Arrows indicate the peak of lolitrem B.)