

## The Role of the Oregon State University Endophyte Service Laboratory in Diagnosing Clinical Cases of Endophyte Toxicoses

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**ABSTRACT:** The Oregon State University Colleges of Veterinary Medicine and Agricultural Sciences instituted the Endophyte Service Laboratory to aid in diagnosing toxicity problems associated with cool-season grasses in livestock. The endophyte (*Neotyphodium coenophialum*) present in tall fescue (*Festuca arundinacea*) produces ergopeptine alkaloids, of which ergovaline is the molecule used to determine exposure and toxicity thresholds for the vasoconstrictive conditions “fescue foot” and “summer slump”. Another vasoconstrictive syndrome, “ergotism,” is caused by a parasitic fungus, *Claviceps purpurea*, and its primary toxin, ergotamine. “Ryegrass staggers” is a neurological condition that affects livestock consuming endophyte (*Neotyphodium lolii*)-infected perennial ryegrass (*Lolium perenne*) with high levels of lolitrem B. HPLC-fluorescent analytical methods for these mycotoxins are described and were used to determine threshold levels of toxicity for ergovaline and lolitrem B in cattle, sheep, horses, and camels. In addition, six clinical cases in cattle are presented to illustrate diagnosis of these three diseases.

**KEYWORDS:** endophyte, ergovaline, lolitrem B, ergot alkaloids

### ■ INTRODUCTION

The origin of the Oregon State University (OSU) Endophyte Service Laboratory began when Pacific Northwest veterinarians asked for a way to determine if ergot alkaloids were the cause of the vasoconstriction and suppressed reproduction that they were seeing in the field, which these compounds are known to be responsible for.<sup>1</sup> Analytical methods using HPLC-fluorescence were developed and refined for testing feed material.<sup>2–4</sup> However, threshold levels of toxicity were needed to correlate alkaloid levels in feed with clinical disease so that preventative “safe feed” practices could be instituted in livestock management. A series of studies determined the threshold levels for both the ergot alkaloid ergovaline, **1** (Figure 1), in tall fescue (*Festuca arundinacea*), and the perennial ryegrass (*Lolium perenne*) toxicant causing ryegrass staggers, lolitrem B, **2** (Figure 1), in various animals.<sup>5–8</sup>

The relevance of this issue to the Pacific Northwest is due to the fact that 70% of the world’s supply of cool-season grass seed, including perennial ryegrass and tall fescue, is grown there. Historically, the grass seed industry burned the straw residue after harvesting the seed. In the late 1990s, field burning was phased out and a new export hay/straw industry developed, which currently brings in \$350 million annually for the states of Oregon and Washington. The ports of Portland and Tacoma ship more than 33000 containers of compressed residual straw per year, primarily to the Far East (Japan and Korea), where straw constitutes approximately 55% of their fiber needs. Unfortunately, this straw has the potential of harboring three fungal species, two of which are endophytic (*Neotyphodium coenophialum* in tall fescue and *Neotyphodium lolii* in perennial ryegrass) and one that is parasitic (*Claviceps purpurea* in a variety of hay/straw species). *N. coenophialum* primarily produces the ergot alkaloid ergovaline, **1**, whereas there are five ergot alkaloids associated with *C. purpurea*, of which ergotamine, **3**, is usually found in the largest quantity. Lolitrem B, **2**, is the main

tremorgenic alkaloid found in endophyte (*N. lolii*)-infected perennial ryegrass.

As exports were increasing, a crisis developed in 2000 when Japanese officials and veterinarians reported over 5400 cases of ryegrass staggers, fescue abortions, and agalactia associated with feeding perennial ryegrass and tall fescue straw.<sup>9</sup> Shipping of these straw products was stopped, and a number of ships carrying straw exports were turned around midocean to return to American ports. Within months, a solution was found between the Japanese Ministry of Agriculture and the U.S. authorities, namely, that all shipments of straw were to be certified to be below the toxic threshold levels established for the toxins associated with disease from both tall fescue and perennial ryegrass. The function of the OSU Endophyte Service Laboratory was defined at that particular time and persists to date. To ensure that the shipments are below threshold levels, allotments of straw are tested and a certificate is issued stating alkaloid concentration(s) to guarantee “safe feed”. Analysis is by HPLC-fluorescence, which can distinguish between the neurotoxic and the vasoconstrictive alkaloids.<sup>3,4,10–12</sup> If the levels are above the threshold of toxicity, the straw is diluted with endophyte-free straw until it is safe to feed.

Recently, a new feed market has been developing in the Middle East for goats, camels, and fat-tailed sheep, as nations there have increasingly decided to prioritize their water resources for human use and consumption. As a consequence, they have begun importing the majority of their livestock feed as opposed to growing it domestically. New threshold levels of toxicity need to be established for native livestock species consuming tall fescue

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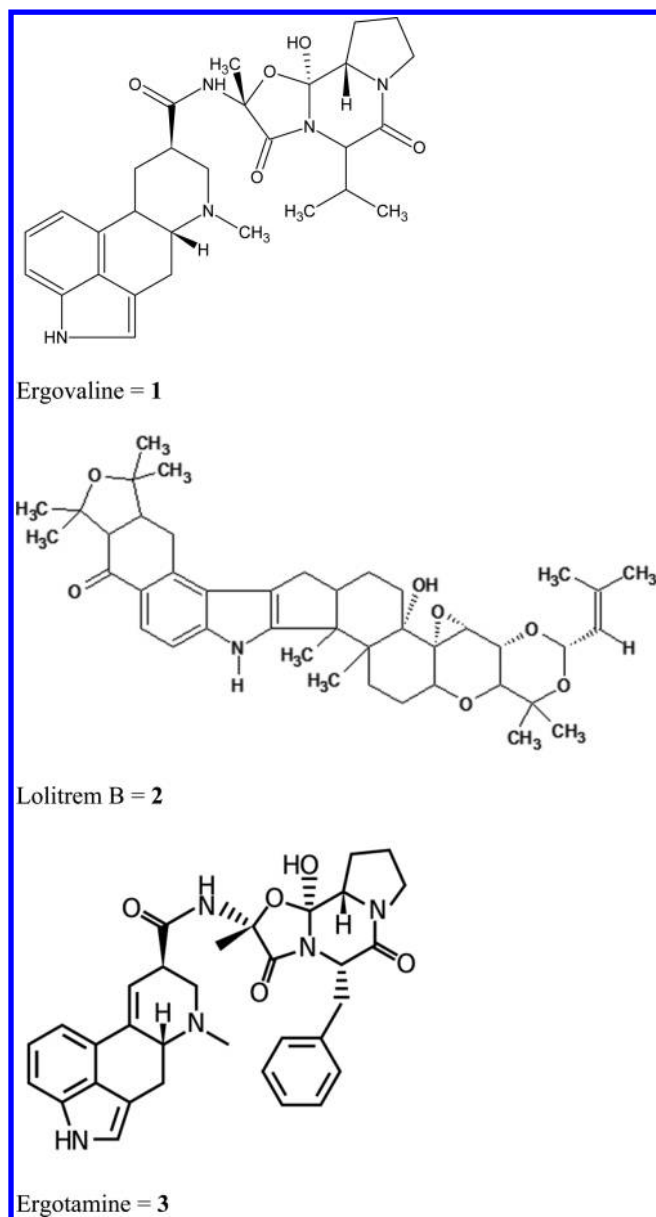
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**Figure 1.** Chemical structures of the compounds ergovaline (1), lolitrem B (2), and ergotamine (3).

and perennial ryegrass straw, with particular attention paid to replicating the environmental conditions in which these animals exist; such a study has been conducted for camelids.<sup>6</sup>

The OSU Endophyte Service Laboratory<sup>13</sup> receives an average of 3497 test requests per year (years 2005–2013) to test feed material for the alkaloids discussed above. During 2005–2013, an average of 226 accessions/year were clinical cases submitted principally by veterinarians who suspected feed material was causing vasoconstrictive disease, reproductive problems, or ryegrass staggers. The remaining majority of the samples were submitted by the Ag-Fiber industry with the goal of ensuring “safe feed”. A summary of the extraction and HPLC detection methodologies currently in use and six examples of clinical cases follow.

## EXTRACTION AND DETECTION METHODOLOGY

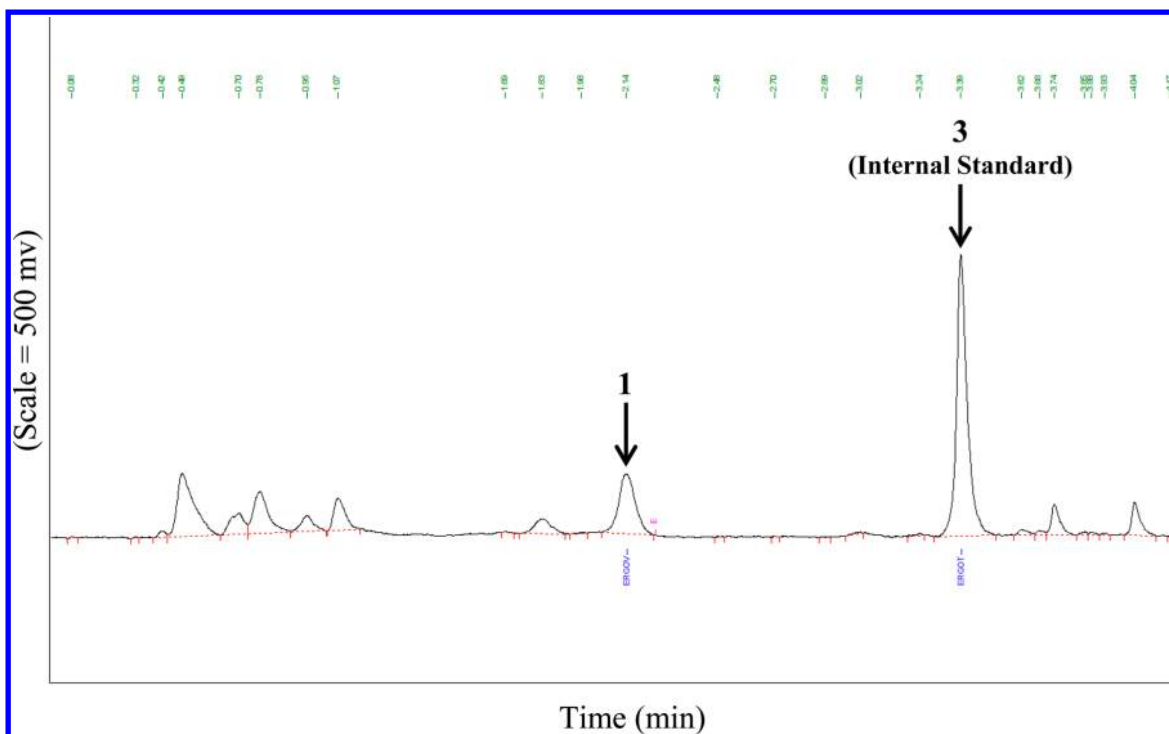
**Extraction of Endophyte Mycotoxins from Plant Material. Ergovaline and the Ergot Alkaloids.** A method for

the extraction of ergot alkaloids from plant material was developed on the basis of previous studies for subsequent analysis by HPLC-fluorescence.<sup>2,3,10,14</sup> As “neat” standard is expensive and difficult to synthesize, ground seed or straw is mixed in large batches at four target concentrations to generate reference material for use in a calibration curve, which is validated using >98% pure ergovaline, **1**, provided by Forrest Smith, Auburn University. Seed and straw samples are ground in a Cyclotec 1093 sample mill and passed through a 0.5 mm screen. To each tube containing 1 g of sample, control, or reference material are added 10 mL of chloroform plus 1 mL of internal standard (0.661 mg/L ergotamine, **3**, in chloroform) and 1 mL of 0.001 M NaOH; the tubes are capped and mixed for 18–24 h in the dark and then centrifuged at 650g. Five milliliters of organic supernatant is applied to a 500 mg/6 mL solid phase extraction (SPE) column containing 0.5 g of Ergosil and 0.5 g of anhydrous sodium sulfate. Ergovaline, **1**, and the ergot alkaloids are extracted by adding 5 mL of sample eluent to the SPE contained in a positive pressure manifold, followed by a 2 mL 4:1 acetone/chloroform (v/v) wash and elution with 2.5 mL of methanol. The eluent is dried under nitrogen at 50 °C and then reconstituted in 0.5 mL of methanol. Samples are mixed for 10 s, sonicated for 10 s, and centrifuged at 650g for 5 min. Samples are transferred to amber HPLC vials and sealed for analysis. The percent recovery for this method is 91% for seed and plant material; inter- and intra-assay variations are 5.7 and 3.8%, respectively.

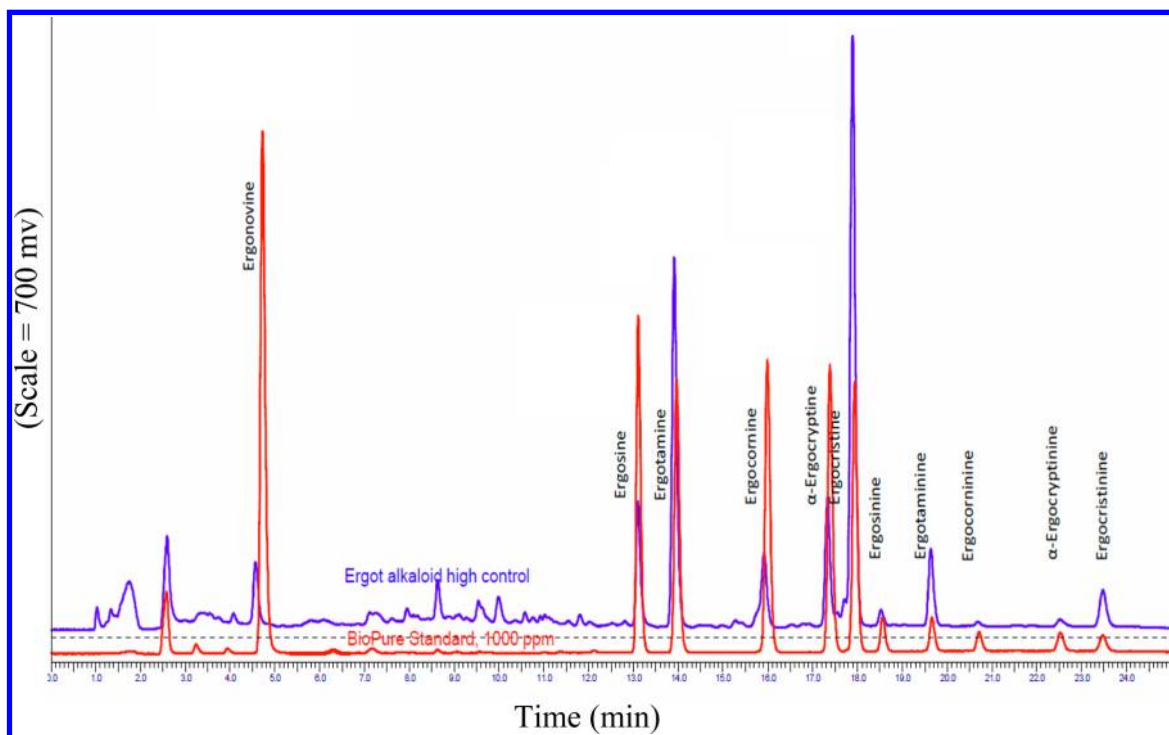
**Extraction of Lolitrem B.** Lolitrem B, **2**, quantitation involves solvent extraction and filtering before HPLC analysis.<sup>4,10</sup> Plant material is ground as described above, and then 3 mL of a 2:1 chloroform/methanol (v/v) mixture is added to 0.200 g of sample, control or reference material, capped, and mixed for 18–24 h in the dark. Due to the same cost and availability circumstances described above, the Endophyte Service Laboratory uses straw or seed reference material mixed in large batches at four concentrations that is validated using purified lolitrem B, **2**, provided by Ag Research, Ltd., New Zealand, to establish a calibration curve. Next, the samples are centrifuged at 650g for 10 min, and 1.6 mL of supernatant is dried under nitrogen at ambient temperature. One milliliter of dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) is added to the evaporated supernatant, capped, and sonicated for 10 s, followed by vortexing for 10 s. An additional 1 mL of CH<sub>2</sub>Cl<sub>2</sub> is added, and the sample is again sonicated and vortexed for 20 s each to ensure the entire sample is dissolved. CUSIL 500 mg/6 mL SPE cartridges are loaded onto a positive pressure manifold and preconditioned with 2 mL of CH<sub>2</sub>Cl<sub>2</sub>. The samples are loaded onto the SPE, followed by a 2 mL CH<sub>2</sub>Cl<sub>2</sub> wash. A 0.5 mL wash of elution solution (4:1 CH<sub>2</sub>Cl<sub>2</sub>/acetonitrile (CH<sub>3</sub>CN) (v/v)) is added to the cartridges, and positive pressure is applied. The sample is then eluted with 3 mL of elution solution; SPE columns are dried to force all remaining liquid out of the columns. The eluent is mixed, and 1.5 mL is transferred to amber HPLC vials and sealed for analysis. The percent recovery for this method is 91.5% for plant material. Inter- and intra-assay variations are 8.1/8.5 and 6.2/6.8% for straw/seed, respectively.

## HPLC-Fluorescence Analysis for Ergot Alkaloids.

**Ergovaline, 1.** The current protocol in our laboratory for HPLC analysis of ergovaline, **1**, involves reversed-phase chromatography with fluorescence detection (excitation and emission wavelengths of 250 and 420 nm, respectively) and a gradient pump program run at a flow rate of 1.8 mL/min with 35% CH<sub>3</sub>CN and 2 mM ammonium carbonate in purified water as mobile phase A and CH<sub>3</sub>CN as mobile phase B, as follows:



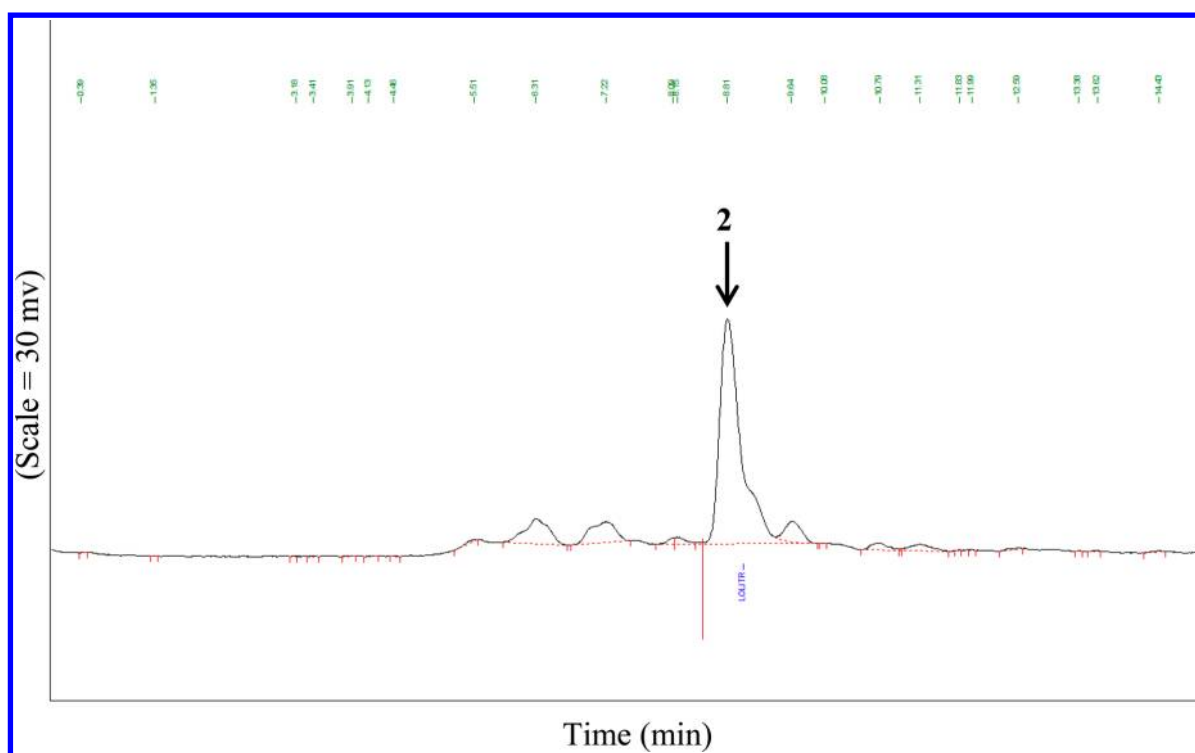
**Figure 2.** HPLC-fluorescence chromatogram of tall fescue seed (500 ppb) extracted for ergovaline, 1, by the Endophyte Service Laboratory, Oregon State University. Ergotamine, 3, is added as an internal standard to all materials being tested.



**Figure 3.** HPLC-fluorescence chromatogram of pure standard (red trace) and tall fescue high control seed (blue trace) extracted for the five ergot alkaloids associated with ergot (ergosine, ergotamine, 3, ergocornine,  $\alpha$ -ergocryptine, and ergocristine) by the Endophyte Service Laboratory, Oregon State University. (The -inine epimers for these five alkaloids elute subsequent to the main compounds.)

equilibrate from 0 to 0.5 min at 99% A, hold at 99% A for 1.0 min, then decrease linearly from 1.5 to 2.3 min to 35% A, hold at 35% A from 2.3 to 3.1 min, then raise linearly to 99% A from 3.1 to 3.4 min, and hold for another 1.3 min before cycling to the next sample. A column (either 100 mm  $\times$  4.6 mm i.d., 2.6  $\mu$ m, Kinetex C18 or 100 mm  $\times$  4.6 mm i.d., 2.7  $\mu$ m, Brownlee SPP C18) is

used in conjunction with a guard column cartridge of similar packing. The retention time for ergovaline, 1, is 2 min, whereas that for ergotamine, 3, is 3 min. The limit of detection (LOD) of ergovaline, 1, is 31 ppb, whereas the limit of quantitation (LOQ) is 100 ppb. Figure 2 shows an example of a HPLC-fluorescence chromatogram for ergovaline, 1, produced using this assay.



**Figure 4.** HPLC-fluorescence chromatogram of perennial ryegrass straw (2500 ppb) extracted for lolitrem B, 2, by the Endophyte Service Laboratory, Oregon State University.

**Ergot Alkaloids.** For ergot analysis, fluorescence detection is the same as above but a different chromatography configuration is used. At a flow rate of 0.9 mL/min, mobile phases of 1 mM ammonium carbonate (A) and  $\text{CH}_3\text{CN}$  (B) are run through a gradient pump program as follows: equilibrate from 0 to 5 min at 75% A, then decrease linearly to 65% A from 5 to 15 min, hold at 65% A from 15 to 20 min, and then decrease linearly to 25% A from 20 to 25 min. Compounds analyzed in this assay and their LOD/LOQ values are ergosine, ergotamine, 3 (11/38 ppb), ergocristine (14/50 ppb), ergocornine (11/41 ppb), and  $\alpha$ -ergocryptine (13/49 ppb). Figure 3 illustrates an example of a HPLC-fluorescence chromatogram for the ergot alkaloids examined for cases of ergot using this assay.

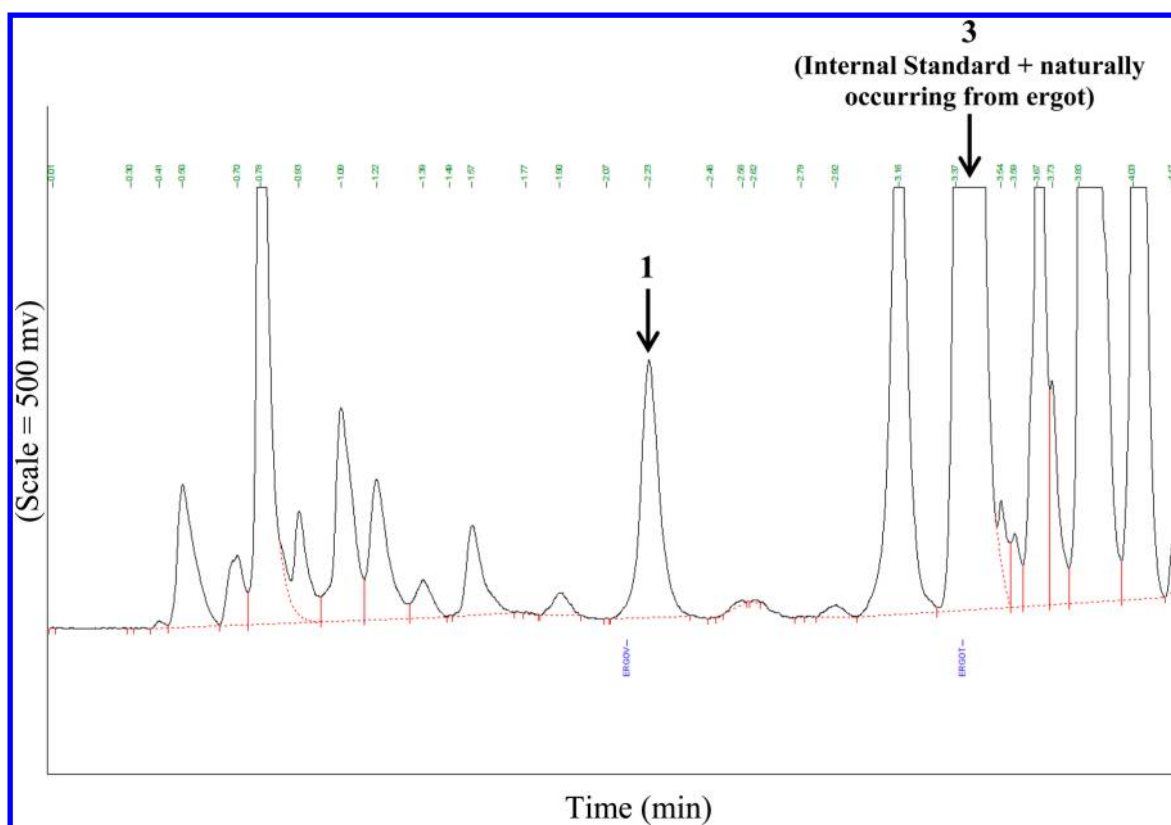
**HPLC Analysis Protocol for Lolitrem B, 2.** The Endophyte Service Laboratory performs lolitrem B, 2, quantitation by HPLC-fluorescence detection using normal phase separation and an isocratic mobile phase ( $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CN}/\text{H}_2\text{O}$  4:1:0.02 (v/v)) run at 0.5 mL/min for 15 min.<sup>15–17</sup> Lolitrem B, 2, is detected using a fluorescence detector set with an excitation wavelength of 268 nm and an emission wavelength of 440 nm. A 250 mm  $\times$  4.6 mm i.d., 5  $\mu\text{m}$ , Zorbax RX-SIL analytical column is used in conjunction with a hand-packed silica guard column. The retention time of lolitrem B, 2, is 8.3 min. The LOD is 30 ng/mL and the LOQ, 100 ng/mL, for plant material. Figure 4 shows an example HPLC-fluorescence chromatogram of straw reference material extracted for lolitrem B, 2, using this method.

## ■ CASE REPORTS

The following documents six clinical cases of endophyte toxicoses in herds of cattle in the Pacific Northwest, USA and Canada, coupled with toxin levels analyzed by the Endophyte Service Laboratory. Often, these values were confirmatory of the tentative diagnosis. In addition, these values helped validate the

threshold levels that had been determined by experimental feeding trials.

**Case 1.** A crossbred beef cow/calf operation of 1800 head from Harney County, eastern Oregon, was supplemented in January with endophyte-infected tall fescue straw in addition to their winter range. They were owned by the same family but were kept divided into three herds, A, B, and C. Two of the three herds with 1040 animals (herd A) and 260 cows (herd B) were fed from common stacks of tall fescue straw. Tall fescue straw was fed initially as the sole source of fiber, supplemented with residual fall pasture grazing starting November 25. Herd A had added alfalfa hay at 20% of the ration to their younger cows and at a 50:50 ratio to the older cows from December 1 to January 17. Herd B had free access to a protein supplement (28%), and both herds A and B had access to mineral salts. On January 17, all feeding of the tall fescue stopped. Herd C with 500 cows was fed 100% fine fescue straw until November 27 and then changed on December 10 to 50% meadow hay and 50% fine fescue. This herd returned to a full ration of fine fescue from December 31 to January 15. Clinical signs of lameness and swollen feet were first noted by the owners in cows from herds A and B on December 25, but the cause was not yet understood. These signs progressed to dry gangrene of the feet, often with subsequent fracture of the necrotic toes or lower limbs. By March 5 in herd A, 449 animals had died or were euthanized due to fescue foot, 56 were lame, and 12 had uterine prolapses. In herd B, 36 were dead or destroyed, 49 were lame, and 3 had uterine prolapses. Abortions and birth of weak or dead calves occurred in the majority of the pregnant cows that were affected. Herd C had no clinical problems. The minimum environmental temperatures from November 25 to December 25 ranged from 1 to 30 °F, with 16 days of minimum temperatures below 10 °F. Core samples of the remaining tall fescue were taken on January 17, with subsequent analyses for ergovaline, 1, concentration. The mean of 10 core



**Figure 5.** HPLC-fluorescence chromatogram of pellets associated with loss of 13/40 cattle (case 4). Sample was flagged for suspected ergot contamination by the Endophyte Service Laboratory, Oregon State University, due to the large ergotamine, 3, peak eluting in the ergovaline, 1, assay.

samples was 329.7 ppb, with a standard deviation of 90.7 ppb. Whereas some core samples had low levels of 116 and 222 ppb, eight samples had values from 340 ppb to a high of 406 ppb. Forty tons of tall fescue straw had been consumed in November and December, prior to testing. Within a month, a total of 600 cows had died, were humanely euthanized, or aborted.

**Case 2.** During January, 1500 steers in Lethbridge, Alberta, Canada, were supplemented with silage and wheat and barley grain. Clinical signs of dry gangrene of the ears and feet were evident, with sloughing of the hooves being the most serious. In sum, 45 steers were euthanized or died. The grain was infected with *C. purpurea* and contained 3473 ppb total ergot alkaloids. This total was derived from peaks of 262 ppb of ergosine, 545 ppb of ergotamine, 3, 753 ppb of ergocornine, 613 ppb of  $\alpha$ -ergocryptine, and 1299 ppb of ergocristine.

**Case 3.** In January, 330 steers, bulls, and pregnant beef cows, on frozen pastureland in Coburg, OR, USA, were supplemented with tall fescue straw plus pellets. The pellets were said to be made from perennial ryegrass screenings combined with steamed corn. Five bins of pellets were tested, as well as the straw bales. The straw bales had <100 ppb of ergovaline, 1, which is below the LOD for our method. It was determined that the five bins of pellets had more than ryegrass screenings, as evidenced by chromatographic analyses, which showed ergovaline, 1, concentrations ranging from 800 to 1100 ppb; these values are more consistent with tall fescue screenings. One bin of pellets underwent an ergot analysis, which had a value of 820 ppb of ergotamine, 3. Initial examination of the lameness by a local veterinarian was mistaken for wire cuts on the lower limbs. Within days, multiple cows were affected with vasoconstrictive dry gangrene. Eventually, 44 animals lost hooves, ears, and tails, resulting in their deaths.

**Case 4.** Forty cattle in a cow/calf operation were housed in a pen in rural Oregon in early January. They were being fed pellets, seed screenings, and haylage. Thirteen of these developed lameness and dry gangrene of their distal limbs and were euthanized. It was determined that the vasoconstrictive lesions were due to ergot toxicosis caused by *C. purpurea*. Pellets were contaminated with 54916 ppb total ergot alkaloids. These included ergotamine, 3 (36858 ppb), ergocristine (8016 ppb),  $\alpha$ -ergocryptine (3494 ppb), and ergosine (4684 ppb) (Figure 5).

**Case 5.** Thirty pregnant cows in their second trimester were on grass seed fields in late December. Pellets made of tall fescue grass seed screenings were spread in piles over the field. After 2 weeks of ingestion, two animals aborted and three animals had open bleeding from their distal limbs and were in extreme pain. Feeding of the pellets was stopped and samples were sent to the Endophyte Service Laboratory for analysis. The affected animals also developed vasoconstrictive lesions in their feet; five were euthanized, four lost their tails, two became lame, and two aborted. Analysis of the screenings detected ergovaline, 1, at 2096 ppb.

**Case 6.** Seventeen Hereford  $\times$  Red Angus male and female calves weighing between 136 and 181 kg had been weaned and moved into confinement in the Willamette Valley, Oregon. They were fed free-choice perennial ryegrass straw and started on a grain ration, the amount of which was gradually increased over a 1.5 week period to 1.82 kg/calf/day. After 1 week, some of the calves developed head tremors and a staggering, stiff gait. The owner incorrectly suspected grain overload and treated them with a mineral oil drench, procaine penicillin G (IM), and dexamethasone (20 mg IM). Response to this treatment was minimal. The calves were not removed from the perennial ryegrass straw until day 23 following the initiation of feeding and

the death of one of the calves. Samples of the turf-quality perennial ryegrass straw being fed contained  $3711 \pm 243$  ppb of lolitrem B, 2 (the threshold of toxicity in cattle is between 1800 and 2000 ppb of lolitrem B, 2). The ruminal contents of the necropsied calf had a lolitrem B, 2, concentration of 185 ppb, as compared to a clinically normal calf from the same herd with 25 ppb of lolitrem B, 2, in its ruminal fluid. Eight of 17 of the calves developed concurrent acute pulmonary emphysema, which had not been reported. An experimental study with four healthy calves fed perennial ryegrass straw with a similar lolitrem B, 2, concentration showed ryegrass staggers in all animals after 5 days of the feeding trial, but no lung lesions.<sup>18</sup>

## SUMMARY

The overriding importance of the Endophyte Service Laboratory is to ensure “safe feed” is exported throughout the world, as well as to local markets. The clinical cases presented here of lameness, dry gangrene, sloughing of ears and tails, and abortions in livestock highlight the fact that, only through feed analysis, can the causative agent of disease be determined. In a similar manner, definitive diagnosis of ryegrass staggers requires analysis of feed even though cessation of feeding does result in remission of clinical signs. For tall fescue toxicosis, levels of ergovaline, 1, at 300–350 ppb are the threshold for development of clinical disease in horses and cattle.<sup>5</sup> With extreme low environmental temperatures during winter months, these values may be lower. Care should be taken not to feed endophyte-infected tall fescue straw or seed screenings in the winter months unless they have been analyzed for ergot alkaloid toxins. Threshold values for cattle of lolitrem B, 2, in feed are between 1500 and 2000 ppb;<sup>8</sup> camels have shown clinical signs in experimental feeding trials at 1000 ppb when fed continuously for 60 days.<sup>6</sup> Table 1 details

**Table 1. Threshold Levels for Fescue Toxicosis (Ergovaline, 1) and Perennial Ryegrass Staggers (Lolitre B, 2)**

	ergovaline, 1	lolitre B, 2
camels	not determined	500 ppb
cattle	300–500 ppb <sup>a</sup>	1800–2000 ppb
horses	300–500 ppb <sup>b</sup>	not determined
sheep	500–800 ppb <sup>a</sup>	1800–2000 ppb

<sup>a</sup>Depending on weather. <sup>b</sup>Brood mares should have no detectable ergovaline, 1, in their feed.

current threshold levels for livestock species for these two endophyte toxicoses. In addition, in the Pacific Northwest, cases of ergotism due to *C. purpurea* contamination of feed have been increasing. Feeding trials need to be conducted to establish threshold of toxicity levels in livestock species for the collection of ergot alkaloids that cause this disease.

## AUTHOR INFORMATION

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### Notes

The authors declare no competing financial interest.

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