Chapter 3
Endophyte Mycotoxins in Animal Health

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Abstract Fescue toxicosis and perennial ryegrass staggers are two of the most common toxic plant diseases plaguing livestock in the United States, and result from consumption of forage containing the endophyte-produced mycotoxins ergovaline and lysergic acid (fescue toxicosis) and lolitrem B (ryegrass staggers). Our group has developed analytical assays for detecting these compounds, which serve a dual purpose (1) high-performance liquid chromatography-fluorescence assays are used to measure these compounds in feed material in order to promote "safe feed" through diagnostic testing in a service laboratory environment, and (2) highly sensitive and specific liquid chromatography-tandem mass spectrometry assays are utilized to study the fate and metabolism of these compounds in a diversity of livestock matrices so that a more refined understanding as to the etiology of the diseases these compounds cause can be achieved. A discussion applying these techniques to both current and anticipated studies is given, with an emphasis on impacts to trade and food safety regulation.

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Abbreviations

HPLC	High-performance liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
SPE	Solid phase extraction
DCM	Dichloromethane
ACN	Acetonitrile
LOD	Limit of detection
LOQ	Limit of quantitation
ELISA	Enzyme-linked immunosorbent assay
ESI(+)	Electrospray ionization in the positive ion mode
MRM	Multiple reaction monitoring
APCI(+)	Positive atmospheric pressure chemical ionization
ppb	Parts per billion

3.1 Introduction

Plant toxins are chemical defenses which likely evolved to combat herbivore predation. As such, toxic plants are responsible for a portion of the morbidity and mortality affecting profitability in livestock production, the amount of which varies by region, toxic plant exposure, and management practices. Fescue toxicosis and perennial ryegrass staggers are two of the most common toxic plant diseases plaguing livestock in the United States, and result from consumption of forage containing endophyte-produced mycotoxins. Endophyte-infected pasture and hay are nutritious food resources (6–17% protein) for ruminants and pseudoruminants and can make up a significant portion of a herd's dietary regimen. For example, tall fescue (*Festuca arundinacea*) is the most widely grown pasture grass in humid areas of the southeastern and, to a lesser extent, the northwestern United States, with greater than 140,000 km^2 in production [1]. Perennial ryegrass (*Lolium perenne*) is a valuable pasture grass in temperate regions of the world, including the northwestern United States, Australia, and New Zealand. Both grasses are also fed as hay as a component of winter rations when adequate pasture becomes unavailable. Endophytic fungi have been deliberately promoted in both tall fescue and perennial ryegrass (*Neotyphodium coenophialum* and *N. lolii*, respectively) in order to combat insect predation and to produce more vigorous, drought-resistant plants. Some endophyte strains exert these benefits through the production of ergot and lolitrem alkaloids (Fig. 3.1), which, unfortunately, also cause deleterious effects in cattle and other herbivore species when endophyte-infected grasses are grazed or fed as hay [2]. (Other endophyte strains exist which are able to transfer the beneficial characteristics of this fungal–grass symbiosis without producing the ergot and lolitrem alkaloids that are detrimental to animal health [3, 4]. As development and release of

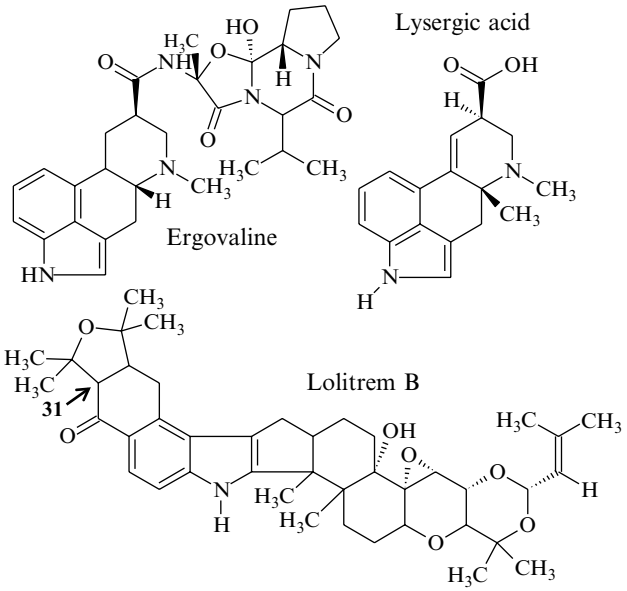

Fig. 3.1 Prominent ergot and lolitrem alkaloids found in endophyte-infected tall fescue and perennial ryegrass that are detrimental to animal health

these "novel" endophytes has taken place relatively recently, however, the economic impact of stock improvement versus cost of pasture replacement must be weighed by cattle managers to determine if this is a feasible option [5].)

It is estimated that the toxicological effects of ergot and lolitrem alkaloids cost between $0.5 billion and $1 billion in livestock losses annually in the United States alone [6], which have been categorized into three main diseases affecting animal health, namely, fescue foot, summer syndrome, and ryegrass staggers [2, 7]. "Fescue foot" typically occurs in cold environments, where tall fescue hay is fed as a significant portion of the diet, and is the result of the vasoconstrictive action of ergot alkaloids on blood vessels [8]. The alkaloids cause decreased blood flow to the extremities (hooves, ears, and tail), which become gangrenous, eventually resulting in euthanasia of the animal before it has reached full market potential. "Summer syndrome" is usually seen during hot, humid summer months when animals consume forage containing ergot alkaloids whose vasoconstrictive properties result in an inability of the animal to properly cool itself. This causes the animal to seek the coolness of shade and water, thereby spending less time grazing. Clinical signs observed in summer syndrome include reduced average daily gain, intolerance to heat, excessive salivation, rough hair coat, elevated body temperature, nervousness, lower milk production, and reduced conception rate [9]. "Ryegrass staggers" is a condition in which animals grazing on endophyte-infected perennial ryegrass pastures containing lolitrem B develop stilted gait, ataxia, tremors, and hypersensitivity

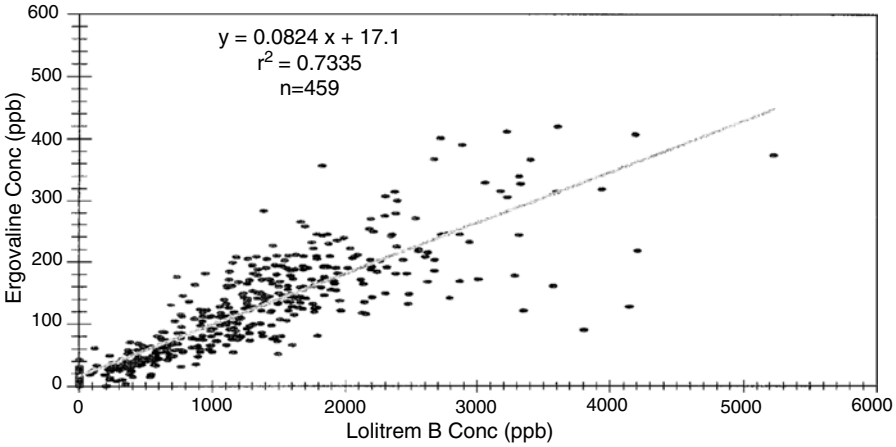

Fig. 3.2 Correlation between ergovaline and lolitrem B concentration in 459 perennial ryegrass samples (Adapted from Hovermale et al., 2001)

to external stimuli [10, 11]. Lolitrems have a long duration of action, yet their neurotoxic effects are completely reversible (Gallagher 1986); as such, livestock affected with ryegrass staggers regain normal muscle response within 3–4 days after being removed from infected feed and appear otherwise unaffected.

In order to better understand how endophyte-infected feed causes these maladies in food animals, three compounds have been identified for both monitoring in forage to promote safe feed and following in feeding studies so that the ultimate fate and disposition of these compounds can be realized. In tall fescue infected with ergot alkaloid-producing endophytes, ergovaline (Fig. 3.1) is the ergot alkaloid found in highest abundance [12, 13] and appears to be the most potent vasoconstrictor of the ergopeptides and ergolines tested to date [8, 14, 15]. Thus, it is the ergot alkaloid most often linked to fescue toxicosis (fescue foot and summer syndrome). In perennial ryegrass, lolitrem B (Fig. 3.1) is the main neurotoxin associated with ryegrass staggers [16] and acts by inhibiting large-conductance calcium-activated potassium channels [17], producing a characteristic tremoring response. For these reasons, ergovaline and lolitrem B were selected as the target toxins for establishing dietary threshold of toxicity recommendations in food animals [18] and for safe feed certification by service laboratories [19, 20]. Ergovaline is not the only ergot alkaloid present in tall fescue [12, 21], however, an examination as to the putative toxin(s) responsible for causing the maladies associated with fescue toxicosis is ongoing, with a particular focus on the breakdown product lysergic acid (Fig. 3.1) [22, 23]. Likewise, lolitrem B is one of the many end products in a complex indole-diterpene biosynthesis pathway which yields other structurally similar compounds in the plant [3]. In truth, grazing animals are potentially exposed to a myriad of alkaloids upon ingestion of endophyte-infected forage (e.g., Fig. 3.2). Future studies and discussions should therefore be modeled on exposure scenarios that more closely mimic these natural feeding conditions.

To this end, our group has developed analytical assays for detecting ergovaline, lysergic acid, and lolitrem B, which serve a dual purpose (1) high-throughput, high-performance liquid chromatography (HPLC)-fluorescence assays are used to measure these compounds in feed material in order to promote "safe feed" through diagnostic testing in a service laboratory environment, and (2) highly sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays are utilized to study the fate and metabolism of these compounds in a diversity of livestock matrices so that a more refined understanding as to the etiology of the diseases these compounds cause can be achieved. Better definition of compartmental values will also help optimize regulation of these compounds in animal by-products; thus, the service and research pursuits both feed off and support each other.

3.2 Analytical Procedures

3.2.1 Extraction of Endophyte Mycotoxins from Plant Material

3.2.1.1 Ergovaline

We have developed a method for extraction of ergot alkaloids from plant material based on previous studies [24–26], for subsequent analysis by HPLC-fluorescence or LC-MS/MS. Seed and straw samples are ground in a Cyclotec 1093 sample mill and passed through a 0.5-mm screen. One gram of the ground plant material is weighed into a glass screw-top tube. To each tube of sample, control, or reference material (as neat standard is expensive and difficult to synthesize, ground seed or straw is mixed in large batches at four target concentrations to generate material for use in a standard curve which is validated using >98% pure ergovaline (Forrest Smith, Auburn University)), 10 mL of chloroform plus 1 mL internal standard (1 μg/mL ergotamine in chloroform) and 1 mL of 0.001 N NaOH are added to deprotonate ergot alkaloids that may have been protonated in acidic conditions. The tubes are capped and mixed for 18–24 h in the dark, then centrifuged at 1,700 × *g*. Five milliliters of organic supernatant from the centrifugation step is applied to 500 mg/6 mL solid phase extraction (SPE) columns (United Chemical Technologies, Bristol, PA) containing Ergosil® (Analtech, Newark, DE) and anhydrous sodium sulfate (EMD Chemicals, Darmstadt, Germany). Ergovaline is extracted by conditioning with chloroform, followed by a 3:1 chloroform:acetone (v/v) wash and elution with 2.5 mL methanol. The eluent is dried under nitrogen at 50°C, then reconstituted in 0.5 mL methanol. Samples are mixed for 10 s, sonicated for 10 s, and centrifuged at 913 × *g* 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-assay and intra-assay variations are 5.7 and 3.7%, respectively [27].

3.2.1.2 Lysergic Acid

In addition to ergovaline, lysergic acid (Fig. 3.1) has been proposed as a causative agent in fescue toxicosis [22] and is present in tall fescue [28–30]. Lysergic acid is extracted from plant material by weighing 1 g of sample (dried, ground to 0.5-mm particle size) into glass screw cap tubes, to which 10 mL of 1:1 water:acetonitrile (v/v) is added and rotated for 16 h in the dark at room temperature. Samples are subsequently centrifuged for 10 min at 2,000 × *g*; then 5 mL of the liquid layer are adjusted to pH 5.0–5.5 with 10% acetic acid. Cation-exchange SPE cartridges (Discovery DSC-SCX, Sigma-Aldrich, St. Louis, MO) are preconditioned with 3 mL of methanol, followed by 3 mL of 0.1-M HCl and two 3-mL portions of pure water. Acidified extracts are loaded onto the preconditioned columns, and columns are washed with duplicate 3-mL aliquots of water. Lysergic acid is eluted with 3 mL of 95:5 methanol:ammonium hydroxide (v/v), the solvent removed using a centrifugal evaporator at room temperature, and the dried residue reconstituted in 200 μL of 50:50 methanol:0.05-M phosphate (pH 9.5) (v/v). Sample extracts are then sonicated for 30 s and transferred to a centrifuge filter and centrifuged at 10,000 × *g* for 5 min. Filtrates are transferred to HPLC vials and sealed for analysis. This extraction method resulted in recoveries of 64% and 79% for 30- and 150-ng/g spikes into endophyte-free seed, respectively, and recoveries of 61 and 77% for 30- and 150-ng/g spikes into endophyte-free straw, respectively [29].

3.2.1.3 Lolitrem B

Initially, lolitrem B was extracted and further purified using column chromatography with silica gel, reversed-phase thin-layer chromatography, and HPLC [16]. The first large-scale isolation used ground perennial ryegrass seed and solvent extraction with petroleum ether, then purification with liquid-liquid partitioning and flash chromatography [31]. Another method for the purpose of lolitrem B quantitation involves solvent extraction and filtering before HPLC analysis [27]. Plant material is ground as described above for the ergovaline and lysergic acid extractions. Three milliliters of a 2:1 chloroform:methanol (v/v) mixture is added to 0.2 g of sample, control, or reference material, capped and rotated for 18–24 h in the dark. (Due to the same cost and availability circumstances described above for the ergovaline standard, our lab uses straw or seed reference material mixed in large batches at four concentrations that is validated using highly purified lolitrem B (AgResearch, Ltd, New Zealand) to establish a calibration curve.) Next, the samples are centrifuged at 2,000 × *g* for 10 min, and 1.6 mL of supernatant is pulled off and dried under nitrogen at ambient temperature. One milliliter of dichloromethane (DCM) is added to the evaporated supernatant, capped and sonicated for 10 s, followed by mixing for 10 s. An additional 1 mL of DCM is added, and the sample is again sonicated and vortexed for 20 s to ensure the entire sample is dissolved. CUSIL 500-mg/6-mL SPE cartridges (United Chemical Technologies, Bristol, PA) are loaded onto a positive pressure manifold and preconditioned with

2 mL DCM. The samples are loaded onto the SPE, followed by a 2-mL DCM wash. A 0.5-mL wash of elution solution (4:1 DCM:acetonitrile (ACN) (v/v)) is added to the cartridges, and positive pressure is applied after dripping is no longer observed. The sample is then eluted with 3.0 mL of elution solution, and the eluent is collected in glass culture tubes. The SPE columns are allowed to stop dripping and then dried to force remaining liquid out of the columns. These tubes are capped and mixed, and 1.5 mL is transferred to amber HPLC vials and sealed for analysis by HPLC-fluorescence. The percent recovery for this method is 91.5% for plant material. Inter- and intra-assay variations are 14.3/9.3% and 8.3/5.9% for straw/seed, respectively [27].

3.2.2 Extraction of Endophyte Mycotoxins from Animal Matrices

3.2.2.1 Ergovaline

The best method for extracting ergovaline from animal matrices (blood, feces, urine, and ruminal fluid) involves cleanup with silica-based C18 SPE columns. When extracting for ergovaline, tissue matrices are typically pretreated with a dilute base to ensure optimum recovery, as treatment with strong acids or bases will completely hydrolyze the amide bond. Jaussaud et al. (1998) reported recovery rates from 90 to 102% for ergovaline extracted out of ovine plasma using a sodium hydroxide pretreatment and a liquid–liquid extraction with diethyl oxide. Ergovaline can be extracted from dried feces following the same methodology used for plant material [24, 28]. Extraction of ergovaline from urine and ruminal fluid can be performed by placing it in chloroform buffered with K_3PO_4 and adding ergotamine as an internal standard, then rotating for 5 h in the dark. The supernatant is then added to an SPE-containing Ergosil® and anhydrous sodium sulfate and extracted as described above for plant material. The final ruminal fluid extract is dried under nitrogen and reconstituted in methanol for analysis by HPLC [28].

3.2.2.2 Lysergic Acid

The presence of lysergic acid (Fig. 3.1) in body matrices is a good indicator that the grass animals are consuming contains lysergic acid and/or ergot alkaloids, as the lysergic acid moiety is common to all ergot alkaloids and has been found as a breakdown product in ruminal fluid and urine in feeding studies. For instance, lysergic acid was present in ruminal fluid, urine, and endophyte-infected tall fescue in feeding experiments with cattle [29] and sheep [28] and in urine and endophyte-infected tall fescue fed to horses [30]. Lysergic acid can be extracted by acidifying the matrix, taking it through an SPE (Discovery DSC-SCX, Sigma-Aldrich) extraction procedure involving preconditioning with methanol and 0.1-M HCl, washing with pure water, and elution with a methanol:ammonium hydroxide (95:5 v/v)

solution [29]. Acidifying the matrix before SPE cleanup helps to extract the compound from complex matrices by protonating the carboxylic acid group at pH lower than 3.44 while selecting for deprotonation at pH above 9. (The pK_a of the carboxylic group is 3.44, while the –NH group has a pK_b of 7.68 and is protonated at pH less than 2.) Percent recoveries for high-spiked (150 ng/g) samples of ruminal fluid, urine, and feces were 81, 88, and 87%, respectively. For a low spike of 30 ng/g, the percent recoveries of ruminal fluid, urine, and feces were 80, 85, and 81%, respectively.

3.2.2.3 Lolitrem B

While chlorinated solvents are best for extracting lolitrem B from grass and seed, Miyazaki et al. (2004) were able to use a 9:1 (v/v) hexane:ethyl acetate solvent mixture for extracting lolitrem B from bovine fat and other tissues, followed by a 9:1 (v/v) hexane:ethyl acetate prewash and wash on Sep-Pak Plus Silica SPE columns (Waters, Milford, MA), with elution using a 7:3 (v/v) hexane:ethyl acetate solution. The eluent was dried under nitrogen and reconstituted in 85:15 DCM:ACN before analysis via HPLC-fluorescence and HPLC-MS. Our group has successfully used this extraction procedure for bovine feces and a similar method for extracting lolitrem B and its metabolites from bovine urine. Alternatively, lolitrem B quantification in bovine feces is also possible using the same extraction method described above for quantitating lolitrem B in plant material [27].

With the extraction methods detailed above for ergovaline and lysergic acid in blood, feces, urine, and ruminal fluid and lolitrem B in fat, tissues, feces, and urine, detection of endophyte mycotoxins in animal matrices is possible and can be used as a diagnostic tool to confirm cases of endophyte toxicosis. The feeding trials conducted in sheep, cattle, and horses found fecal ergovaline and urinary lysergic acid to be the primary excretory products formed [28–30]. From these studies, we can conclude that a fecal sample extracted for ergovaline and a urine sample extracted for lysergic acid would be the best tools for clinical diagnosis of fescue toxicity. Studies like these are still needed for lolitrem B in order to determine the best matrix and extraction method to use as a tool for diagnosis of ryegrass staggers.

3.2.3 HPLC-Fluorescence Analysis of Endophyte Mycotoxins

HPLC-fluorescence is currently the most frequently used platform for quantification of ergovaline and lolitrem B in diagnostic laboratories which certify “safe feed” [20] or provide data to aid in the diagnosis of endophyte toxicosis in clinical cases [27], as it remains a cost-effective and robust tool with the capacity for high-throughput applications.

3.2.3.1 Ergovaline

The current protocol in our laboratory for HPLC analysis of ergovaline involves reversed-phase chromatography with fluorescence detection (excitation and emission wavelengths of 250 nm and 420 nm, respectively) and a gradient pump program run at a flow rate of 1.0 mL/min with 30% ACN and 2 mM ammonium carbonate in purified water as mobile phase A and ACN as mobile phase B, as follows: 0–5.5 min at 99% A, decreased linearly from 5.5 to 7.5 min to 35% A, held at 35% A from 7.5 to 9.5 min, then raised linearly to 99% A from 9.5 to 10.5 min and held for another 1.5 min before cycling to the next sample. A Gemini C18 3-μ column (Phenomenex, Torrance, CA) is used in conjunction with a guard column cartridge (Phenomenex) of similar packing. The retention time for ergovaline is 8 min, while that for ergotamine is 9 min. In addition, the introduction of Kinetex core-shell columns (Phenomenex) has recently provided an alternative in column selection, giving better peak separation, resolution, and shorter run times. We are currently using this technology in our lab to analyze for ergovaline with a 4.6 × 100-mm, 2.6-μ, 100 Å C18 column run at a flow rate of 1.8 mL/min and an injection volume of 10 μL. The pump program is the same as for the Gemini C18 column, except it is scaled to a total run time of 4.5 min.

Using the TotalChrom data system (Perkin Elmer, Waltham, MA), a standard curve is constructed from reference material of concentrations around 100; 400–500; 900–1,000; and 2,000 ng/g of plant material. A linear regression fit of the peak area versus the amount of analyte injected is used to determine the amount of ergovaline in unknown samples. The limit of detection (LOD) is 31 ng/mL, and the limit of quantitation (LOQ) is 100 ng/mL for forage samples. The LOD for ergovaline in rumen fluid is 10 ng/mL. While this method may be sufficient for analysis of plant material for regulatory purposes, it is not sufficient as a research tool to determine total distribution and metabolism of ergovaline in a feed study with livestock where analysis of body matrices requires a much lower LOD/LOQ. For example, ergovaline was previously extracted from plasma by two groups using a liquid diethyl oxide extraction and subsequently quantitated by HPLC-fluorescence; they determined their LOQ to be 3.5 ng/mL [32] and LOD to be 1.2 ng/mL [33]. Both extractions required a large amount of sample (4 mL plasma), and while they were good for determining the kinetic properties of ergovaline after a single intravenous dose, the actual amount of ergovaline ingested by livestock on a daily basis in typical feeding experiments would not be detected or quantified, based on data which found actual serum levels of ergovaline to be 0.7–3.8 pg/mL (pregnant mares grazing on endophyte-infected tall fescue pastures with a daily dose of approximately 1 mg/day ergovaline) [34]. Instead, Lehner et al. (2008) assayed their sera by LC-MS/MS which had an LOQ of 1 pg/mL. We review the usefulness of LC-MS/MS for these types of samples below. Based on these studies, we also suggest that better data may come from analyzing serum, instead of plasma, as there will likely be less interference since the blood has already been allowed to clot, allowing any unnecessary components to be removed.

Additionally, HPLC-fluorescence can be used where separation and purification of mixtures of ergot alkaloids and their metabolites are needed, particularly before use of instrumentation such as high-resolution mass spectrometry. For example, this technique was used to isolate metabolic products from ergotamine incubations in mice [35]. Isolation of ergotamine, its epimer, and seven transformation products was accomplished by manual peak collection via monitoring on a photodiode array detector (254 nm), cleanup of the incubation matrix on C18 SPE cartridges, dry-down under nitrogen, and reconstitution in methanol before analysis by mass spectrometry.

3.2.3.2 Lysergic Acid

Lysergic acid can be analyzed by HPLC-fluorescence with the same parameters used for ergovaline analysis, with a few exceptions. Better detection and quantification can be accomplished by using a 0.05-M phosphate-buffered mobile phase and a 5-μ C18 column, rather than the 3-μ column size used for ergovaline. Lodge-Ivey et al. (2006) reported limits of quantitation and detection for multiple matrices for lysergic acid. Seed, straw, and feces had LOQ/LOD values of 24.2/7.26, 14.5/4.34, and 36.0/10.80 ng/g, respectively. Ruminal fluid and urine LOQ/LODs were 5.5/1.64 and 18.4/5.52 ng/mL, respectively. Alternatively, ergoline molecules like lysergic acid can be measured using an enzyme-linked immunosorbent assay (ELISA) which was developed with antibodies against lysergol [36]. However, this ELISA has some limitations when the goal is to detect all ergot alkaloids, since it has variable specificity to individual alkaloids. In particular, it exhibits low binding affinity for the ergopeptides ergotamine, ergocryptine, ergocornine, and ergocristine [23].

3.2.3.3 Lolitrem B

Our group performs lolitrem B quantitation by HPLC-fluorescence detection using normal phase separation and an isocratic mobile phase (DCM:ACN:H_2O 4:1:0.02 (v/v)) run at 0.5 mL/min for 15 min [16, 37, 38]. Lolitrem B is detected using a fluorescence detector set with an excitation wavelength of 268 nm and an emission wavelength of 440 nm. A Zorbax Rx-SIL, 5-μ, 4.6×250-mm analytical column (Agilent Technologies, Santa Clara, CA) is used in conjunction with a hand-packed silica guard column. The retention time of lolitrem B is 8.3 min. Using the TotalChrom data system, a standard curve is constructed from reference material of concentrations around 500, 900, 2,000, and 4,000 ng/g of plant material. A linear regression fit of peak height versus the amount of analyte injected is used to determine the amount of lolitrem B in unknown samples. The LOD is 30 ng/mL and LOQ is 100 ng/mL for plant material. HPLC-fluorescence is sufficient for regulatory purposes, but the metabolism of lolitrem B is still largely unknown, and expensive analytical standards are necessary for quantifying lolitrem B in a variety of matrices. Like ergovaline, this will require the sensitivity and specificity of analytical tools like LC-MS/MS.

Table 3.1 Precursor and product ions used in MRM analysis of commonly tested ergot alkaloids[a]

Analyte	Precursor ion (*m/z*)[b]	Product ion (*m/z*)
Lysergol	255.1	240.2, 197.2
Ergine	268.1	223.2, 208.2
Lysergic acid	269.3	223, 167, 44
Ergometrine	326.2	223.2, 208.2
Ergovaline	534.2	223.2, 208.2
Ergosine	548.4	223.2, 208.2
Ergocornine	562.2	223.2, 208.2
α-Ergocryptine	576.4	223.2, 208.2
Ergotamine	582.2	223.2, 208.2
Ergocristine	610.4	592.4, 223.2

[a]Adapted from Lehner et al. (2004) and Sulyok et al. (2007)
[b]All precursor ions are given as the $[M+H]^+$ ion

3.2.4 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis of Endophyte Mycotoxins

3.2.4.1 Ergovaline

A need exists for a fast, highly specific, highly sensitive method of detection for ergot alkaloids which can be easily utilized in a variety of matrices. In recent years, LC-MS/MS has become a prominent tool for identifying and quantifying ergot alkaloids as it capitalizes on these characteristics, specifically electrospray ionization in the positive ion mode (ESI (+)) using multiple reaction monitoring (MRM). Detection of ergovaline is best done by MRM because the fragmentation pattern of the product ions is similar for most of the commonly tested ergot alkaloids (Table 3.1). The most common product ions produced are *m/z* 223 and *m/z* 208 (Fig. 3.3), representing the lysergic acid and demethylated lysergic acid moieties, respectively [39]. Representative spectra of the two main compounds associated with fescue toxicosis (ergovaline and lysergic acid) and LC-MS/MS conditions used in our laboratory are presented in Fig. 3.4. Analysis of ergot alkaloids in food products by LC-MS/MS has LOQs of 0.17–2.78 ng/g and LODs of 0.02–1.2 ng/g [40], up to three orders of magnitude below those of HPLC-fluorescence.

When using LC-MS/MS to detect and quantify ergot alkaloids, two major variables must be considered: (1) pKa values of 4.0 to 6.2 dictate that amines of rings 2 and 4 (Fig. 3.3a) be charged in acidic solution and neutral at alkaline pH. Thus, it is common practice to use weak volatile bases in the mobile phase to enhance detection [40]. Mobile phases similar to those described for HPLC-fluorescence are sufficient for mass spectrometry analysis. (2) Ergot alkaloids form epimers that do not necessarily fragment consistently (Table 3.2) [41]. In particular, some epimers may favor a different fragment, such as ergometrinine, which favors the *m/z* 208.2 over the *m/z* 223.2 fragment. Epimers also increase in concentration the longer they are suspended in organic solvents or held at room temperature. For instance, ergovaline was shown to

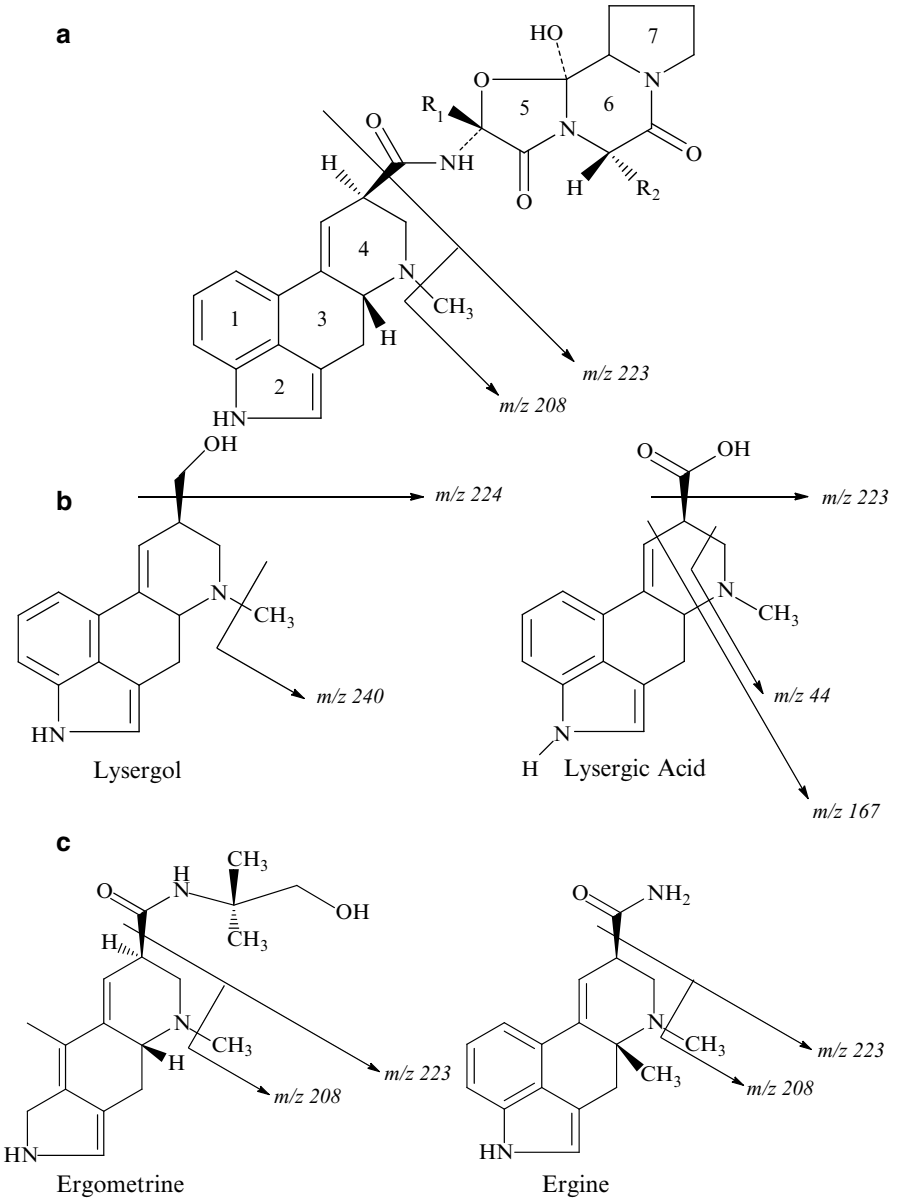

Fig. 3.3 (**a**) Typical fragmentation pattern for ergot alkaloids that contain the general peptide structure (ergovaline, ergotamine, ergocornine, ergocryptine, and ergonovine). (**b**) Lysergol and lysergic acid give slightly different fragmentation patterns. (**c**) Ergometrine and ergine show the same fragmentation pattern of *m/z* 223 and *m/z* 208 but do not have the same general peptide structure as represented in (**a**) (Adapted from Lehner et al. 2004)

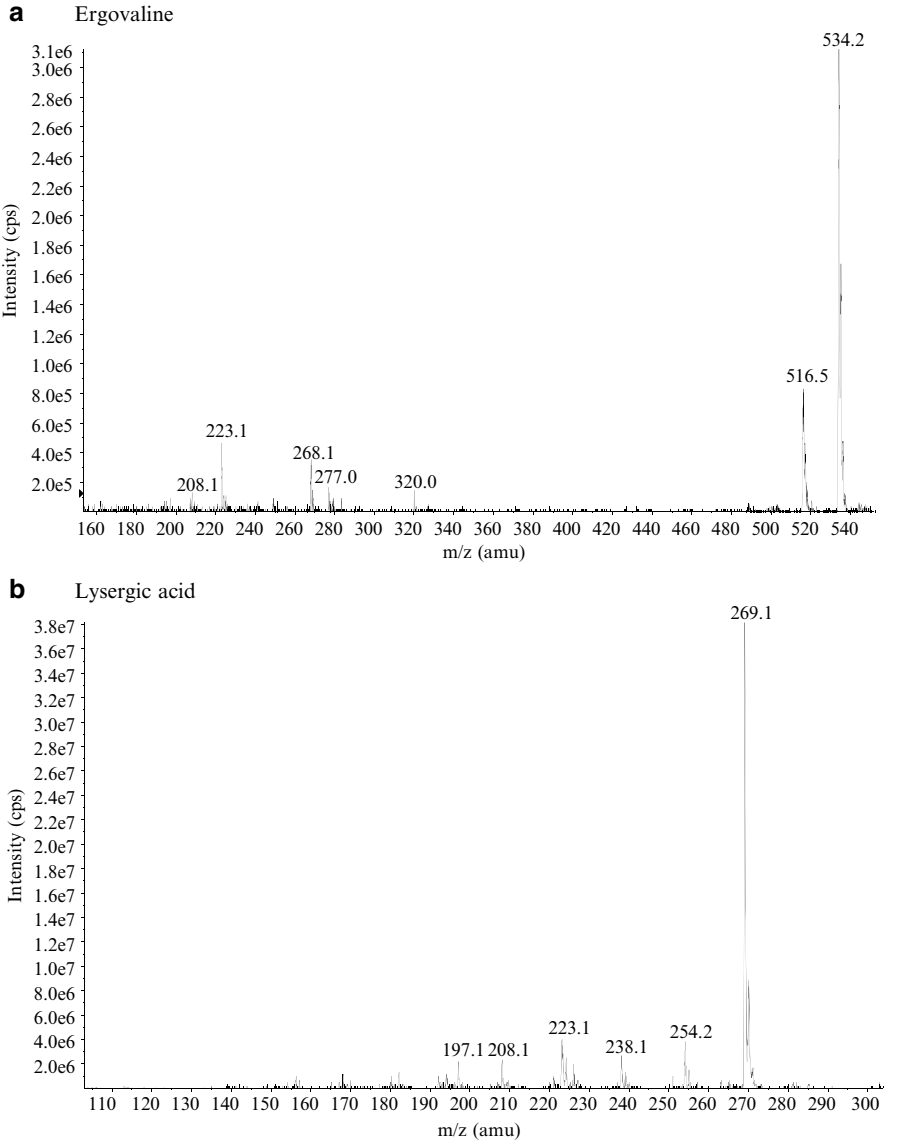

Fig. 3.4 ESI(+)-enhanced mass spectrometry spectra of ergovaline prepared in a 50:50 (v/v) mix of 2 mM ammonium carbonate and acetonitrile (**a**) and D-lysergic acid prepared in acetonitrile (**b**). Methods: A positive enhanced mass spectra scan was performed using a 3,200-QTRAP hybrid triple-quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA) with a Turbo V electrospray ionization source operated at 600°C, ion spray voltage of 5.5 kV, declustering potential of 61 V, and a collision energy of 10 eV. A linear gradient (0–100% B over 30 min, holding for 10 min at 100% B, and equilibrating for 10 min at 100% A) of 30% acetonitrile, 2 mM ammonium carbonate in water (mobile phase A), and acetonitrile (mobile phase B) was run on a 2.6-μ, C18, 100 Å, 100×4.60-mm Kinetex column using a flow rate of 1.4 mL/min. For (**a**), the common ergot fragments of *m*/*z* 149, 208, 223, 249, 269, 277, 320, 488, and 516 are visible, as seen in Lehner et al. (2004 and 2005). For (**b**), the common ergot fragment *m*/*z* 208 is visible, as is the parent *m*/*z* of 268

Table 3.2 Ergot alkaloid epimer precursor and product ions used in MRM analysis[a]

Analyte	Precursor ion (*m/z*)[b]	Product ion (*m/z*)
Ergometrinine	326.2	208.2, 223.2
Ergosinine	548.4	223.2, 208.2
Ergocoroninine	562.2	544.2, 223.2
α-Ergocryptinine	576.4	558.4, 223.2
Ergotaminine	582.2	223.2, 208.2
Ergocristinine	610.4	592.5, 223.2

[a]Adapted from Sulyok et al. (2007)
[b]All precursor ions are given as the $[M+H]^+$ ion

epimerize at 37°C in 0.1-M phosphate-buffered 9% aqueous fetal bovine serum solutions and in water, methanol, and acetonitrile, reaching an epimerization equilibrium between 1 and 19 hours [42]. Further, ergot alkaloid epimers generally chromatograph separately from their non-epimerized counterparts [35, 43], thereby eliminating part of the total concentration in the original sample to be measured, resulting in concentrations lower than what the sample truly contains. To this end, epimer formation can cause major variability in quantitation by affecting peak areas and intensity as well as retention times if analyzing standards or samples that have been suspended in solvents for an extended period of time. Therefore, it is of paramount importance to keep this in mind when handling standards and samples and in performing experiments where animals are dosed using a liquid solution of these toxins.

3.2.4.2 Lolitrem Compounds

Currently, mass spectrometry by positive atmospheric pressure chemical ionization (APCI (+)) is the ionization mode of choice for analysis of lolitrem B [44]; it has also been used to identify the lolitrem B biosynthesis pathway in plants and new lolitrem-like compounds [3]. A Phenomenex Prodigy ODS (30), 5-μ, 150×4.6-mm column was used with mobile phases consisting of 40% aqueous ACN, 0.1% acetic acid (A) and ACN and 0.1% acetic acid (B) run at 1 mL/min using a gradient beginning with 20% B, rising linearly to 50% B at 20 min, then to 100% B at 40 min and recycling after 60 min [3]. The mass spectrometer was operated with nitrogen sheath and auxiliary gas set to 40 and 10 psi, respectively. Source voltage was 6 kV, capillary temperature was 200°C, and vaporizer temperature was set to 450°C.

Our lab has adapted this mass spectrometry method for the purpose of quantifying lolitrem B in bovine matrices such as urine, serum, fat, and feces, using a shorter run time. Quantitation is accomplished on an LC-MS/MS system via APCI (+) and MRM detection using the transitions 686.4/237.9, 686.4/196.3, 686.4/628.4, and 686.4/238.3. The transition 686.4/237.9 gives the best R^2 value (e.g., $R^2=0.9994$ for lolitrem B-spiked urine) when running a calibration curve; therefore it is used for quantitation, while the other transitions are used for qualitative confirmation. Additional quality control parameters (LOD, LOQ, intra- and inter-assay variation) are currently being defined. MRM analyses are conducted using an autosampler

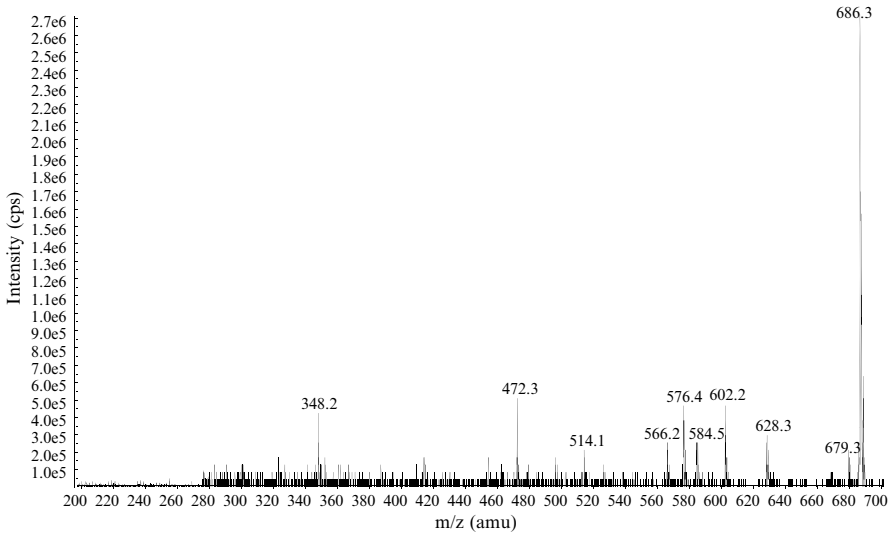

Fig. 3.5 APCI(+)-enhanced mass spectrometry spectrum of lolitrem B standard prepared in acetonitrile. Methods: A positive enhanced mass spectrometry scan was used to acquire this spectra on a 3,200-QTRAP hybrid triple-quadrupole/linear ion trap mass spectrometer with a Turbo V atmospheric pressure chemical ionization (APCI) source operated at 450°C and nebulizer current of 6 μA, using a declustering potential of 20 V and a collision energy of 10 eV. LC conditions from Young et al. (2009) were used with the exception of flow rate, which was 0.5 mL/min. Common fragments specific to lolitrem B are *m/z* 576, 602, and 628 (Nielsen and Smedsgaard 2003; Young et al. 2006; Young et al. 2009)

cooling tray at 4°C, a flow rate of 0.5 mL/min, and a linear gradient of 20–100% B over 20 min, using the same mobile phases described above in Young et al. (2009). Lolitrem B has a retention time of 14.5 min using this method. Mass spectrometer conditions are as follows: declustering potential = 61 V, entrance potential = 7 V, collision cell exit potential = 4.0 V, collision energy = 51 V, ion spray voltage = 5,500 V, temperature = 450°C, nebulizer gas = 65 psi, turbo gas = 0 psi, curtain gas = 30 psi, and nebulizer current = 6 μA. For metabolite mining, an EMS scan is performed using the full-length, 60-min gradient described in Young et al. (2009) (Fig. 3.5).

3.3 Impacts of Endophyte Mycotoxins on Animal Health

3.3.1 Physiological Impacts and Metabolic Endpoints

3.3.1.1 Ergot Alkaloids

Ergot alkaloids act as α-adrenergic and serotonergic agonists which stimulates contraction of smooth muscle cells, resulting in peripheral vasoconstriction [7, 45]. In cold temperatures in which peripheral vasoconstriction is already occurring,

additional vasoconstriction induced by ergot alkaloids can result in tissue ischemia, necrosis, and sloughing of extremities (fescue foot). In hot climes, peripheral vasoconstriction causes general malaise and reduced average daily weight gains (summer slump). The ergopeptides can also alter prolactin secretion by binding to and blocking dopamine receptors [7]; suppressed serum prolactin concentration is, therefore, often used as an indicator of fescue toxicosis in livestock. Depressed serum prolactin in prepartum cattle can result in reduced milk yield but has negligible effects once lactogenesis occurs. Cows grazing endophyte-infected fescue are also reported to have reduced pregnancy rates [46] and increased rates of early embryonic death [47], leading to reproductive efficiency problems. Mares consuming endophyte-infected fescue exhibit increased gestation lengths, agalactia, foal and mare mortality, and weak and poorly developed foals [48]. These late gestational effects are the reasoning behind the zero tolerance level for ergot alkaloids advised for mares in the last 2–3 months of pregnancy. When combined, these performance and reproductive impacts on animal health cause significant monetary losses for livestock producers, estimated to be upwards of $1 billion in the United States alone [6].

In order to determine the toxic moiety/moieties responsible for these maladies, metabolism of ergot alkaloids has been studied in a variety of animal models, using a myriad of analytical tools. The use of radiolabeled compounds has shown biliary (fecal) excretion to be the primary route of elimination in monogastric models and humans, with a small amount detected in the urine [49, 50]. More recent work has shown lysergic acid to be a metabolic breakdown product of ergot alkaloids in the ruminal fluid and urine of sheep and cattle [22, 28], with the possibility of conjugated parent molecules being excreted in the urine as well. A feeding study conducted in sheep with endophyte-infected tall fescue containing toxic ergovaline levels (0.610 mg/kg) fed over 28 days examined feed, ruminal fluid, urine, and feces for ergovaline and lysergic acid [28]. The authors recovered 35% of the dietary ergovaline in the feces and 248% of the dietary lysergic acid in the urine and feces. They also observed an increase of lysergic acid in the ruminal fluid over time. These data indicate that ergovaline is being metabolized to lysergic acid by ruminal microbes, which is then passed into the urine for excretion. In another study where horses were fed endophyte-infected tall fescue seed containing 0.5 mg/kg ergovaline and 0.3 mg/kg lysergic acid over 21 days, a similar pattern was observed whereby urinary excretion of lysergic acid was the primary route of elimination, with fecal excretion of ergovaline playing a more minor role [30].

For the proportion of ergot alkaloids that do enter systemic circulation and pass through the liver, CYP3A is the main cytochrome P450 subfamily responsible for metabolism, with N-dealkylation and mono- and dihydroxylation being the main oxidative reactions carried out by these enzymes [35, 43, 51–53]. An equine study examined the effects of a single IV dose of 15 μg/kg bwt ergovaline in plasma and found the elimination half-life and total clearance of ergovaline to be 57 min and 0.020 L/min kg, respectively [32]. While the study of Bony et al. (2001) and that of DeLorme et al. (2007) provide pieces to begin putting together a model for the absorption, distribution, metabolism, and excretion of ergot alkaloids (Fig. 3.6), a

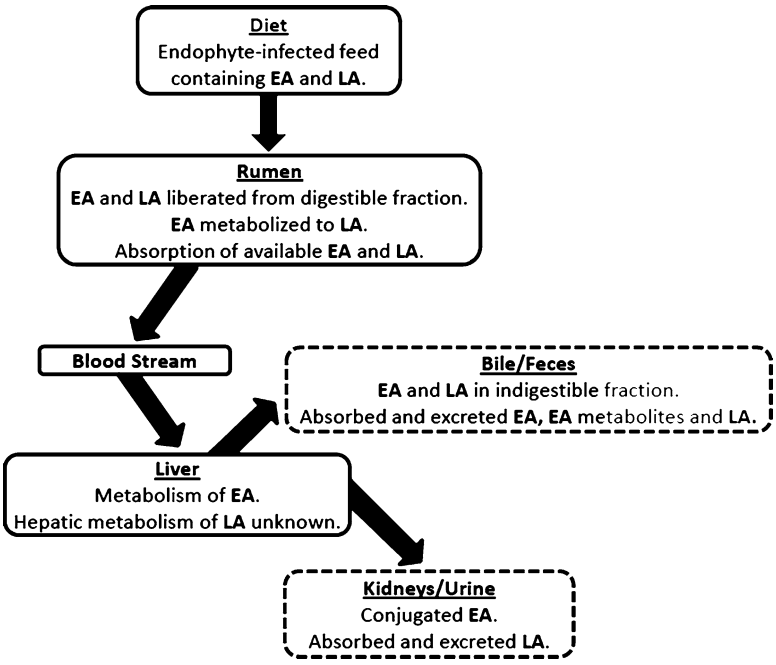

Fig. 3.6 Proposed model for the absorption, distribution, metabolism, and excretion of ergot alkaloids and lysergic acid. Compartments with broken lines signify routes of excretion. *EA* ergot alkaloids, *LA* lysergic acid (Adapted from DeLorme et al., 2007)

multicompartmental toxicokinetic study utilizing the specificity and sensitivity of LC-MS/MS for metabolite mining is needed for a more detailed understanding of the metabolic fate of these compounds in livestock.

3.3.1.2 Lolitrem B

The molecular site of action of lolitrem B is on large-conductance calcium-activated potassium channels, which was first shown in human embryonic kidney cells [17] and later in mice deficient for this ion channel (*Kcnma1*$^{-/-}$) [54]. Specifically, the β-4 subunit is responsible for modulating motor control and is associated with the ataxia observed upon ingestion of this toxin [54]. Seven other members of the lolitrem family of compounds have been examined in a similar manner and have also been shown to inhibit this channel to varying degrees [55]. Lolitrem B was found to be one of the most potent inhibitors; the presence of an isoprene unit appears to be a determinant of an individual molecule's potency, as is an α-oriented hydrogen atom at position 31 (indicated in Fig. 3.1). However, to date, no detailed toxicokinetic study on the fate and metabolism of lolitrem B once ingested has been carried out.

In addition, public health concerns over the safety of meat and other by-products from food animals which have consumed endophyte mycotoxins in imported ryegrass straw have been raised [37]. Specifically, the presence of lolitrem B in fat of exposed animals has been questioned since lolitrem B is somewhat lipophilic. Based on pilot studies, 3–10% of the ingested lolitrem B dose is sequestered in the fat (with no detectable lolitrem B found in skeletal muscle, liver, kidney, heart, or cerebrum) [37, 56]. Thus, a chronic toxicokinetic study mimicking extended lolitrem B feed intake would be valuable to establish definitive compartmental values. Utilizing LC-MS/MS capabilities, such a study could determine (1) whether lolitrem B accumulates in adipose tissue and if it reaches steady-state concentrations; (2) if lolitrem B is retained in adipose tissue until metabolic liberation (similar to polychlorinated biphenyls) or if it is depleted once dietary exposure to lolitrem B is removed; (3) the relationship of level of dietary lolitrem B and lolitrem B concentrations found in adipose and other tissues; and (4) the metabolic pathway(s) of lolitrem B, all of which would be useful parameters to define in the interest of food safety regulation.

3.3.2 Dose Response Studies: Impacting the Forage Trade

Feeding trials have been conducted to establish the threshold of toxicity for ergovaline and lolitrem B in feed material for cattle and sheep [18, 57]; evidence from clinical cases provides an estimate for threshold values in horses [19] (Table 3.3). No threshold levels have been determined for camelids; however, anecdotal evidence of poor milk production (ergot alkaloids) and intention tremors and hypermetria (lolitrem B) have been reported and associated with consumption of endophyte-infected feed (personal communications, A. Morrie Craig and Linda Blythe, DVM). Ergovaline dietary levels of 400–750 parts per billion (ppb) and 500–800 ppb for cattle and sheep, respectively, and 1,800–2,000 ppb lolitrem B for both species are approximated threshold values for the disease syndromes caused by these mycotoxins. Environmental variables, including summertime heat and wintertime cold, could influence the development of fescue toxicoses at fixed dietary concentrations. Such uncertainty surrounding the environment-dose interaction is the rationale behind the broad range of threshold dose values listed for ergovaline. Additionally, investigations as to the bioaccumulation potential of these compounds are warranted and could shift the parameters by which these compounds are regulated in food products from dietary exposure to residue analyses.

Regarding variables in the plant, the crown and seed heads of the plant concentrate both toxins relative to the stem, so it is advised to avoid overgrazing pastures and to harvest grass hay above the crown, before seed head production has occurred or after seed has been harvested. It takes one to three weeks for clinical signs of these diseases to appear in livestock, so a history of the animal's diet, including whether or not the straw was fed as 100% or only part of the ration and the duration feed was consumed, is a key component to making a diagnosis. If the suspected feed material is available, testing is recommended at a certified testing laboratory (e.g.,

Table 3.3 Threshold values for fescue toxicosis (ergovaline) and ryegrass staggers (lolitrem B) in livestock

Livestock species	Ergovaline (ppb)	Lolitrem B (ppb)
Cattle	400–750	1,800–2,000
Sheep	500–800	1,800–2,000
Horses	300–500[a]	ND
Camelids	ND	ND

[a]Except in the last 60–90 days of pregnancy when the threshold is 0 ppb
ND not determined

http://oregonstate.edu/endophyte-lab/). A review of typical cases and interactions between livestock producers/veterinarians (both domestic and international) and testing laboratories is described in Blythe et al. (2007a). In addition, hay producers/exporters are encouraged to test any tall fescue and perennial ryegrass forage products which may contain endophyte in order to ensure the material being distributed is safe for livestock to consume.

In conclusion, the methods described herein are useful both for the promotion of safe livestock feed through service laboratories and as clinical tools for diagnosing cases of endophyte toxicoses. Additionally, they are a research asset which will allow for a better understanding of the diseases caused by these mycotoxins so that development of more effective preventative and/or therapeutic measures can be realized. Lastly, it is worth noting that animals are often exposed to a mixture of ergovaline, other ergot alkaloids, lolitrem B, and a myriad of other plant toxins in endophyte-infected feed materials [27], yet experimental investigation as to the additive or multiplicative effects of these toxins on the development of disease has not been undertaken. Further, the possible impact of bioaccumulation of these toxins under varying exposure conditions on both toxicity and as residues in food products has not been thoroughly investigated and could affect the manner by which these compounds are regulated.

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