AN ABSTRACT OF THE THESIS OF

Lia D. Murty for the degree of Master of Science in Pharmacy presented on November 21, 2012.

Title: Correlation of Fecal Ergovaline, Lolitrem B, and their Metabolites in Steers Fed Endophyte Infected Perennial Ryegrass Straw

Abstract approved:

A. Morrie Craig

Perennial ryegrass (PRG, *Lolium perenne*) is a hardy cool-season grass that is infected with the endophytic fungus *Neotyphodium lolii*, which enables the plant to be insect repellant and drought resistant, lowering the use of insecticides and fertilizers. However, this fungus produces the compound lolitrem B (LB, *m/z* 686.4) which causes the tremorgenic neurotoxicity syndrome 'ryegrass staggers' in livestock consuming forage which contains <2000 ppb LB. Ergovaline (EV, m/z 534) is a vasoconstrictor normally associated with tall fescue (*Festuca arudinacea*), but has also been found in endophyte-infected PRG. Past research has shown a strong linear correlation between levels of LB and EV in PRG. The purpose of this study was to examine the linear relationship between EV and LB in feces and determine common metabolites. To accomplish this, four groups of steers (*n*=6/group) consumed endophyte- infected PRG over 70 days consumed the following averages of LB and EV: group I 2254ppb LB/633 ppb EV; group II 1554ppb LB/ 373ppb EV, group III 1011ppb LB/259ppb EV, and group IV 246ppb LB/<100ppb EV. Group I in week 4 was inadvertently given a washout period at which time the steers consumed the amount of LB and EV given to group IV (control). Both feed and feces samples were extracted using difference solid phase extraction methods and quantified by

HPLC-fluorescence for LB and EV. Concentrations of EV and LB obtained through HPLC-fluorescence in both PRG and feces showed a linear relationship. Additional screening for metabolites was conducted LC-MS/MS and showed possible oxidation and reduction metabolites for both toxins.

Correlation of Fecal Ergovaline, Lolitrem B and their Metabolites in Steers Fed Endophyte Infected Perennial Ryegrass Straw

by
Lia D. Murty

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APPROVED:

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Lia D. Murty, Author

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LIST OF ABBREVIATIONS

HPLC	High pressure liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
PRG	Perennial ryegrass
LB	Lolitrem B
EV	Ergovaline
NaOH	Sodium Hydroxide
K_3PO_4	Potassium phosphate
LOD	Limit of detection
LOQ	Limit of quantitation
MRM	Multiple reaction monitoring
EMS	Enhanced mass spectrum (MS)
IDA	Independent data acquisition
EPI	Enhanced parent ion scan (MS/MS)
ppb	Parts per billion
IACUC	Institutional Animal Care and Use Committee
OB	obstetrics
OSU	Oregon State University
AB	Applied Biosystems
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
ESI	Electrospray ionization
CYP450	Cytochrome P 450 enzymes

CHAPTER 1. INTRODUCTION

1.1 General Introduction

Ergovaline and Lolitrem B are two alkaloid toxins commonly known in large animal medicine, specifically cattle. These two compounds originate from endophyte fungus in perennial ryegrass (PRG). Ergovaline (EV) is normally associated with tall fescue, however it is also found in endophyte infected perennial ryegrass. EV is a vasoconstrictor and can cause gangrenous clinical signs as well as reproductive issues for cows. Lolitrem B is potent neurotoxin which causes 'ryegrass staggers' in cattle and other animals. The long term effects and metabolic implications are not completely understood with LB in combination with ergovaline. Hovermale *et al.* (2001) showed that these two compounds exhibit a linear relationship in PRG but no clinical signs of EV toxicity are seen when both EV and LB are consumed at toxic levels. The reason for this is not well understood and understanding how these two toxins are metabolized and excreted may help veterinarians treat both clinical diseases in cattle and other animals.

1.2 Aim and Objectives

The present study aims to determine the correlation of EV and LB excreted in the feces of steers fed PRG by quantifying both compounds in fecal samples by HPLC-fluorescence and identifying possible metabolites by LC-MS/MS analyses. The objective was to provide a better understanding on clinical disease in bovine species that consume PRG and help better understand the fate and metabolism of LB which is largely unknown.

1.3 Outline of Thesis

The thesis consists of five chapters. Chapter 2 is the literature review which focuses on general properties of EV and LB. The structure, chemistry, sources, and metabolism of both compounds are discussed as well as analytical procedures common for analyzing both compounds in plant and animal matrices. Chapter 3 covers materials and methods for the animal study and analytical procedures used for the data that resulted in chapter 4. Chapter 4 discusses the findings of EV and LB in bovine fecal samples along with LC-MS/MS data of possible metabolites. Chapter five provides a discussion and conclusion of the present study and the contribution to our knowledge of endophyte diseases in cattle.

CHAPTER 2. LITERATURE REVIEW

Parts of the section Analytical Procedures were published in Recent Advances in Phytochemistry: Phytochemicals, Plant Growth, and the Environment, Vol. 42, Chapter 3: Endophyte Mycotoxins in Animal Health., 2012.

2.1. General properties and toxicity of ergovaline

2.1.1 Structural and chemical properties

EV is part of a larger group of secondary fungal metabolites known as ergot alkaloids. These compounds all share a common ergolene ring and a tricyclic peptide portion of the molecule [1, 2]. The common ergopetine structure is shown in figure 2.1 with important carbons labeled that are key in chemical transformations of ergot alkaloids. Ergot alkaloids are colorless crystals and readily soluble in various organic solvents; however, insoluble or only slightly soluble in water. Ergots which contain the double bond at C-9 and C-10 rapidly epimerize with respect to the centre of symmetry at C-8.

Biological and physiochemical properties vary between the C-8 epimers. From pH 4.8 for ergocorninine to pH 6.2 for ergometrinine. They are positively charged at the N-6 position in the ergoline ring when under acidic conditions [1].

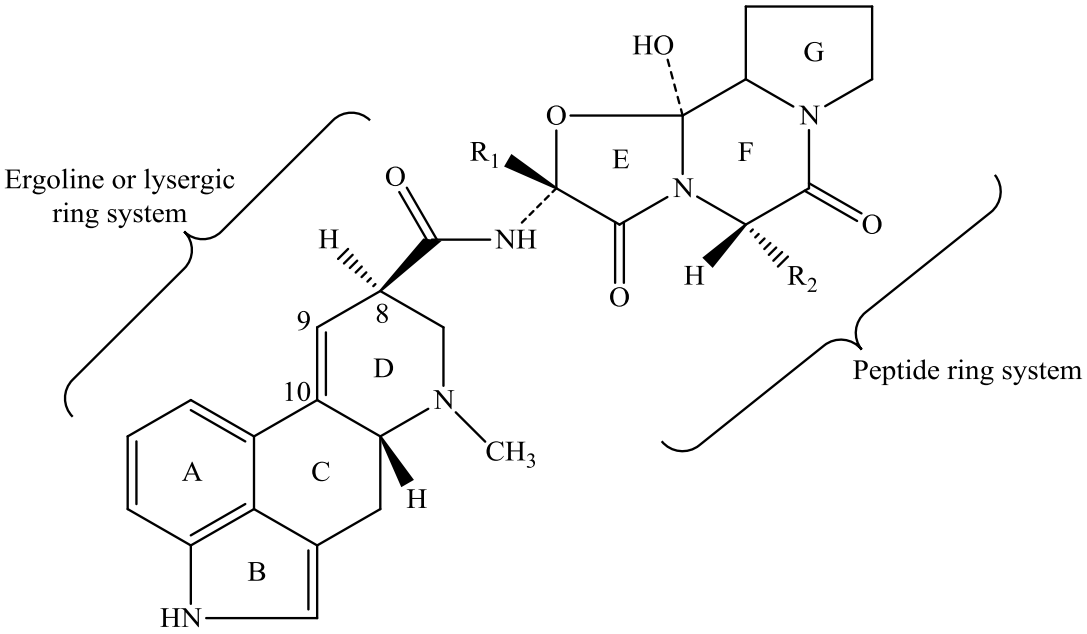


Figure 2.1 Common structure of ergopeptine compounds [1, 3].

2.1.2 Formation and Sources

Ergovaline is a compound that is produced in tall fescue or perennial ryegrass by the genus *Claviceps purpurea.* It is an ergopeptine and falls in the group of ergot alkaloids that are commonly associated with tall fescue. Tall fescue originated from Western Europe and most likely came to the United States as a seed contaminant. The most common cultivar in the US is the "Kentucky 31" which was released commercially in 1942. Soon after commercial release, this grass was very popular because of its tolerance to adverse weather conditions, pest resistance, and high protein levels. Tall fescue is grown best in temperate climates like that of Oregon [4].

2.1.3 Animal Toxicity

Ergovaline has been a common problem since the increase in the use of tall fescue grass for animal forage early in the 1940's. Soon after the popularity of the grass increased, the "fescue problem" began. Clinical signs of EV and ergot alkaloid toxicity can be classified into three categories. First, during lower temperatures during winter months "fescue foot" was noticed in livestock. This is a gangrenous condition seen in feet and or tails of affected animals. Secondly, fat necrosis has been shown in animals that consumed high endophyte tall fescue forage. This condition is caused by hard fat masses in adipose tissue forming around intestines and causing digestive upset and reproductive problems. This clinical sign of fescue toxicosis lead early researchers to investigate the influence endophyte toxins had on lipid metabolism in animals. The third category of clinical signs is called "summer slump" which is a period during warm temperatures when animals show poor weight gain, intolerance to heat, excessive salivation, rough hair coat, elevated body temperatures, nervousness, lower milk production, and reduced conception rate. For example, cattle experiencing summer slump may lay in mud puddles or under shade for the majority of the day[4, 5].

The mechanism of toxicity for EV was not well understood until the early 1990s. Porter et al. (1990) showed that endophyte infected feed altered dopaminergic and serotonergic metabolites after steers consumed the infected feed. The precursor to this study was the findings in Berde (1978) that endophyte consumption was known to reduce serum prolactin levels. Prolactin secretion is known to be inhibited by dopamine but

increased by serotonin. A common test is to measure serum prolactin levels to determine exposure to EV or other ergot alkaloids [6, 7]. It was not until Dyer 1993 that evidence of EV acting on serotonin receptors was published [5, 8].

2.2 General properties and toxicity of Lolitrem B

2.2.1 Structural and chemical properties

The lolitrem compounds belong to a class of compounds known as indole-diterpenes. These compounds are from a large class of compounds from the fungal secondary metabolites of Trichocomaceae (*Aspergillus and Penicilium* spp.) and Clavicepitaceae (*Neotyphodium/Epichloe* and *Claviceps* spp.). Lolitrem B is also considered a paxilline like compound and is often studied simultaneously with paxilline [9]. These compounds while diverse all share a common moiety of a cyclic diterpene skeleton (paxilline) core and an indole group shown in figure 2.4 [10, 11]. The rings A and B represent the indole core and rings C-F represent the diterpene skeleton common to lolitrem compounds.

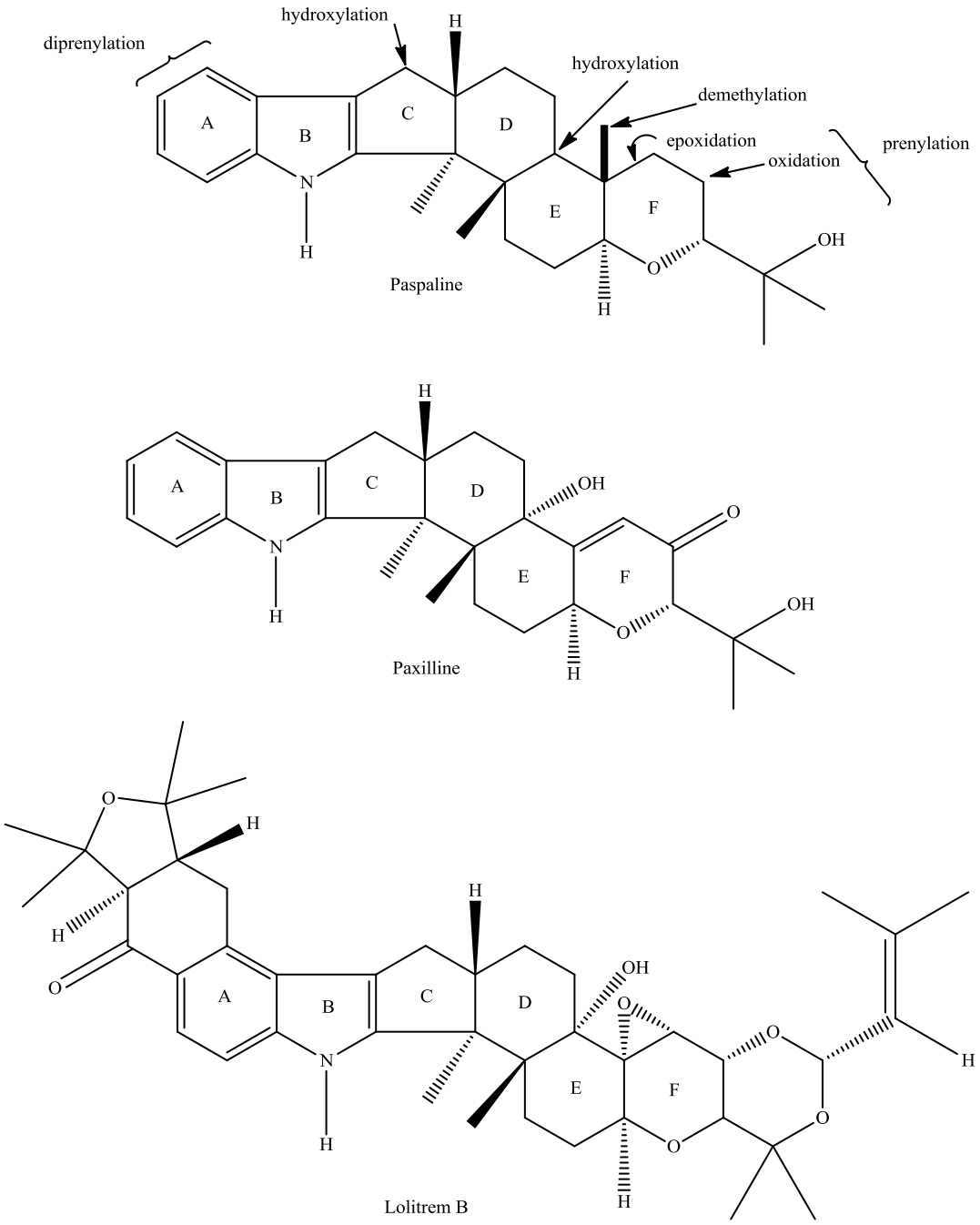


Figure 2.2. **Lolitrem Compounds.** Strucutre of paspaline, paxilline and lolitrem with the indole and diterpene core structures labeled as rings A-F. The structure of paspaline indicates likely sites of enzymatic conversions proposed in Saikia *et al.* 2008 [11].

This core structure specifically on paspaline is the precursor, is where enzymatic conversions are likely to occur in biosynthetic pathways of more complex indole-diterpenes [11].

2.2.2 Formation and Sources

Lolitrem B along with other lolitrem compounds is a potent neurotoxin produced by the endophytic fungus *Acremonium lolii* L. and is present in PRG (*lolium perenne* L.) [12, 13]. PRG is common in New Zealand and Oregon because it is easily adaptable to a wide range of soils and climate conditions. It is a persistent crop which also serves as a good nutritive forage for livestock. The presence of endophyte fungus in PRG allows the grass to be insect resistant causing enhanced plant growth and prevents overgrazing by herbivores [14].

2.2.3 Animal Toxicity

In livestock animals consumption of PRG can lead to a condition known as "ryegrass staggers" which was first reported in 1880 in New Zealand. The clinical signs reported were ataxia and tremors. Animals show impaired motor coordination and can cause significant management issues and animal losses [15]. Other clinical signs are headshaking, abnormal staggering gait, and stumbling, collapsing, and severe muscle spasms. These clinical signs can take 7-12 days to occur in sheep and cattle [16]. Clinical signs of ryegrass staggers usually follow seasonal patterns and show the highest occurrences in late summer and early fall. Mortality is not usually from the consumption of PRG but attributed to drowning in creeks, fractures limbs, or falls due to the staggering that PRG causes [14, 16].

Lolitrem compounds were later isolated and determined the causative agent of "ryegrass staggers." These compounds show long duration of actions and usually completely reversible neurological effects. Other physiological effects such as increased blood pressure, heart rate and respiration rate all suggested that LB affects large conductance calcium activated potassium ion (BK) channels[17]. Paxilline has been shown to inhibit the large conductance calcium activated potassium ion channels and is commonly studied with LB because of structural and mode of action similarities [17]. To determine the mechanism of toxicity by LB, Dalizel *et al.* (2005) used patch clamping and embryonic human kidney cells to look at the α subunit of BK channels and the effects of LB. This study did show that LB inhibited the α subunit. However, the question still unanswered in this study was whether the inhibition was happening in the central nervous system (CNS) or directly on muscles.

Imlach *et al.* (2008) was able to determine which β subunit lolitrem B inhibited and answer the question posed in Dalizel *et al.* (2005) whether the inhibition was occurring at the CNS or by direct effect on muscles [15]. Since the α subunit of BK channels is diverse and present in many tissues it is paired with one of four β subunits. They are present in smooth muscle, ovaries, testis, and neural tissues and labeled β1-4 respectively. Imlach *et al.* (2008) tested their hypothesis by using mice deficient in either β1 or β4 to determine exactly where LB inhibits BK channels. This study found that LB toxicity requires the β-4 subunit present in the neural tissues. Interestingly, LB was shown to have five times the affinity for the BK channels than paxilline did. LB was also shown to have a longer duration of action than paxilline. [15].

2.3 Analytical Procedures

2.3.1 Extraction from Plant Material

Initially, LB was extracted by multiple liquid-liquid extractions and further purification was accomplished using column chromatography with silica gel, reversed phase thin layer chromatography and analytical HPLC [18]. The first large-scale isolation of LB used ground perennial ryegrass seed [19] and a series of solvent extractions including petroleum ether, alcohol, acetonitrile, water, and dichloromethane (DCM). The final step involved flash chromatography using the same solvents. Another method for the purpose of LB quantitation involves liquid-liquid extraction and filtering before HPLC analysis [20].

Current methods for extraction and quantification of ergot alkaloids in plant material are based on previous studies [21, 22], 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. The curve 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 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 hours in the dark, then centrifuged at 1,700 x *g*. Five ml of organic

supernatant from the centrifugation step is applied to solid phase extraction (SPE) columns containing Ergosil® and anhydrous sodium sulfate. EV 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 seconds, sonicated for 10 seconds, and centrifuged at 913 x *g* for 5 minutes. 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 3.8%, respectively.

2.3.2 Extraction from Animal Matrices

The best method for extracting EV from animal matrices (blood, feces, urine and ruminal fluid) involves clean-up with silica-based C-18 SPE columns. When extracting for EV, tissue matrices are typically pre-treated 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 90-102% for EV extracted out of ovine plasma using a sodium hydroxide pretreatment and a liquid-liquid extraction with diethyloxide. EV can be extracted from dried feces following the same methodology used for plant material [21, 23]. Extraction of EV 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 hours in the dark. The supernatant is then added to an Ergosil® SPE column, 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.

While chlorinated solvents are best for extracting LB from grass and seed, Miyazaki *et. al.* (2004) was able to use a 9:1 (v/v) hexane:ethyl acetate solvent mixture for extracting LB from bovine fat and other tissues, followed by a 9:1 (v/v) hexane:ethyl acetate pre-wash 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 LB and its metabolites from bovine urine. LB quantification in bovine feces is also possible using the same extraction method described above for quantitating LB in plant material [20].

With the extraction methods detailed above for ergovaline and lysergic acid in blood, feces, urine and ruminal fluid and LB 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. For example, the feeding trials conducted in sheep, cattle and horses found fecal ergovaline and urinary lysergic acid to be the primary excretory products formed [23-25]. 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 LB in order to determine the best matrix and extraction method to use as a tool for diagnosis of ryegrass staggers.

2.3.3 HPLC-Fluorescence Analysis of Endophyte Mycotoxins

The current protocol for HPLC analysis of ergovaline involves reverse phase chromatography with fluorescence detection (excitation and emission wavelengths of 250 nm and 420 nm, respectively) and a gradient pump program using ammonium carbonate in aqueous acetonitrile and acetonitrile as mobile phases. Additional methods of analysis can be found in Hovermale (2001) and Craig (1994).

Using the TotalChrom data system (Perkin Elmer, Waltham MA), a standard curve can be constructed from reference plant material of concentrations around 100, 400-500, 900-1000, and 2000 ng/g of plant material. The concentrations of plant material can be determined using purified standards; however, using plant material for day to day operations provides a cost benefit and allows for lower cost of analysis. 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 to 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 diethyloxide extraction and subsequently quantitated by HPLC-fluorescence; they determined their LOQ to be 3.5 ng/ml [26] and LOD to be 1.2 ng/ml [27]. Both extractions required a large amount of sample (4 mL plasma) and, while they were good

for determining the kinetic properties of EV after a single intravenous dose, the actual amount of EV 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) [28]. Instead, Lehner et. al (2008) assayed their sera by LC-MS/MS which had a 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 [29]. Isolation of ergotamine, its epimer, and seven transformation products was accomplished by manual peak collection via monitoring on a photodiode array detector (254 nm), clean-up of the incubation matrix on C18 SPE cartridges, dry down under nitrogen and reconstitution in methanol before analysis by mass spectrometry.

LB quantitation by HPLC-fluorescence detection uses 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 minutes [18, 30] . LB 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 x 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 minutes. Using the TotalChrom data system, a standard curve is constructed from reference material of concentrations around 500, 900, 2000, and 4000 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 LB is still largely unknown, and expensive analytical standards are necessary for quantifying LB in a variety of matrices. Like EV, this will require the sensitivity and specificity of analytical tools like LC-MS/MS.

2.3.4 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis

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. It capitalizes on these characteristics, specifically, electrospray ionization in the positive ion mode (ESI (+)) using multiple reaction monitoring (MRM). Detection of EV is best done by MRM because the fragmentation pattern of the product ions is similar for most of the commonly tested ergot alkaloids (Table 2.1). The most common product ions produced are *m/z* 208 and 223 (Figure 2.2), representing the lysergic acid and demethylated lysergic acid moieties, respectively [31]. Representative spectra of the two main compounds associated with fescue toxicosis (EV and lysergic acid), and LC-MS/MS

conditions used in our laboratory, are presented in Figure 2.2. Analysis of ergot alkaloids in food products by LC-MS/MS have LOQs of 0.17-2.78 ng/g and LODs of 0.02-1.2 ng/g [1], 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 (Figure 2.2A) 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 [32]. 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 2.1) [33]. 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, EV was shown to 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 1-19 hours [34]. Further, ergot alkaloid epimers generally chromatograph separately from their non-epimerized counterparts [29, 35], 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.

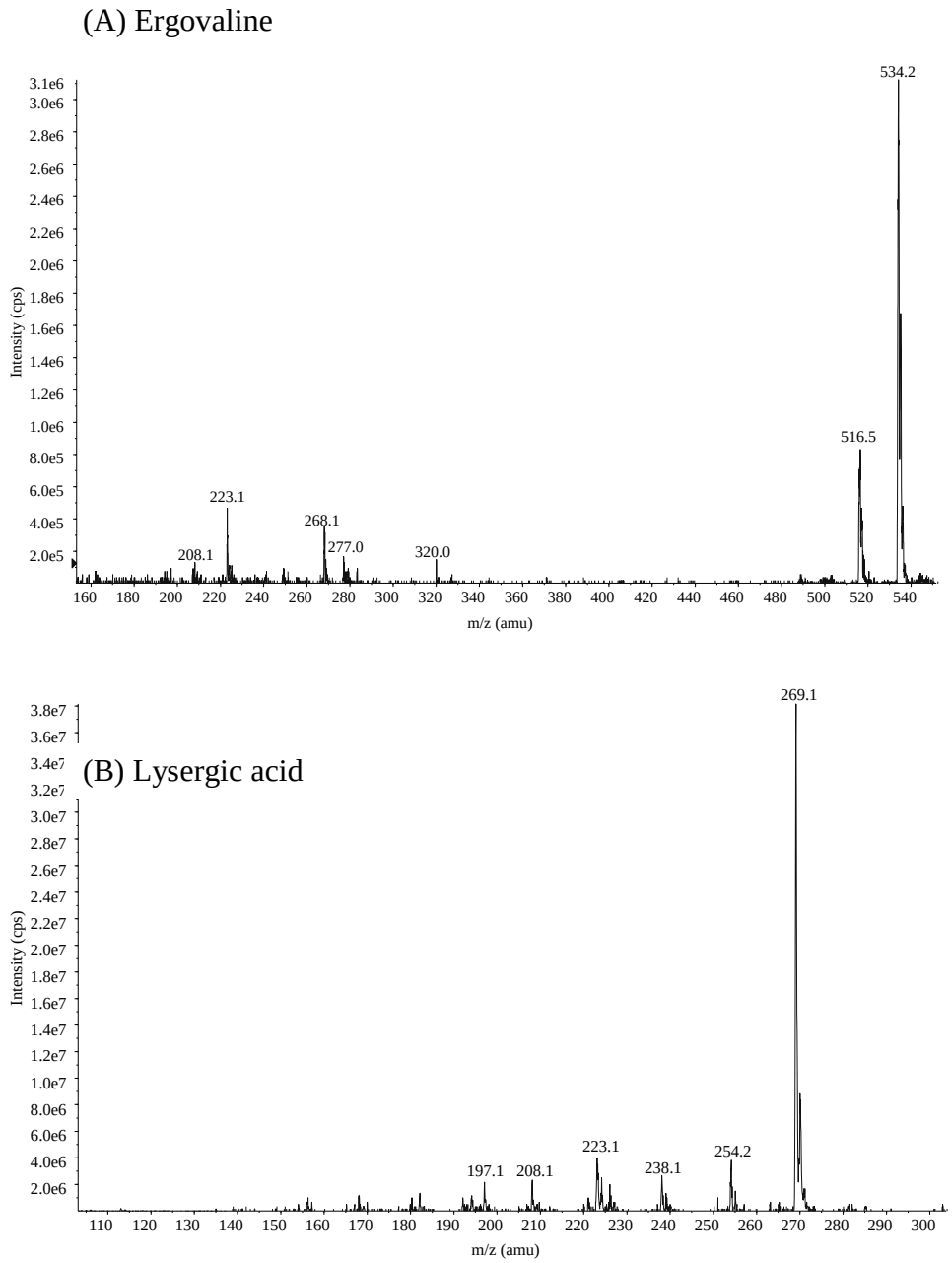


Figure 2.3. ESI(+)-enhanced mass spectrometry spectra of ergovaline prepared in a 50:50 (v/v) mix of 2 mM ammonium carbonate and acetonitrile (a) and D-lysergic acid prepared in acetonitrile (b). Methods: A positive enhanced mass spectra scan was performed using a 3200 QTRAP hybrid triple-quadrupole/linear 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 2.6µ, C18, 100Å, 100 x 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, 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 2.1. 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.

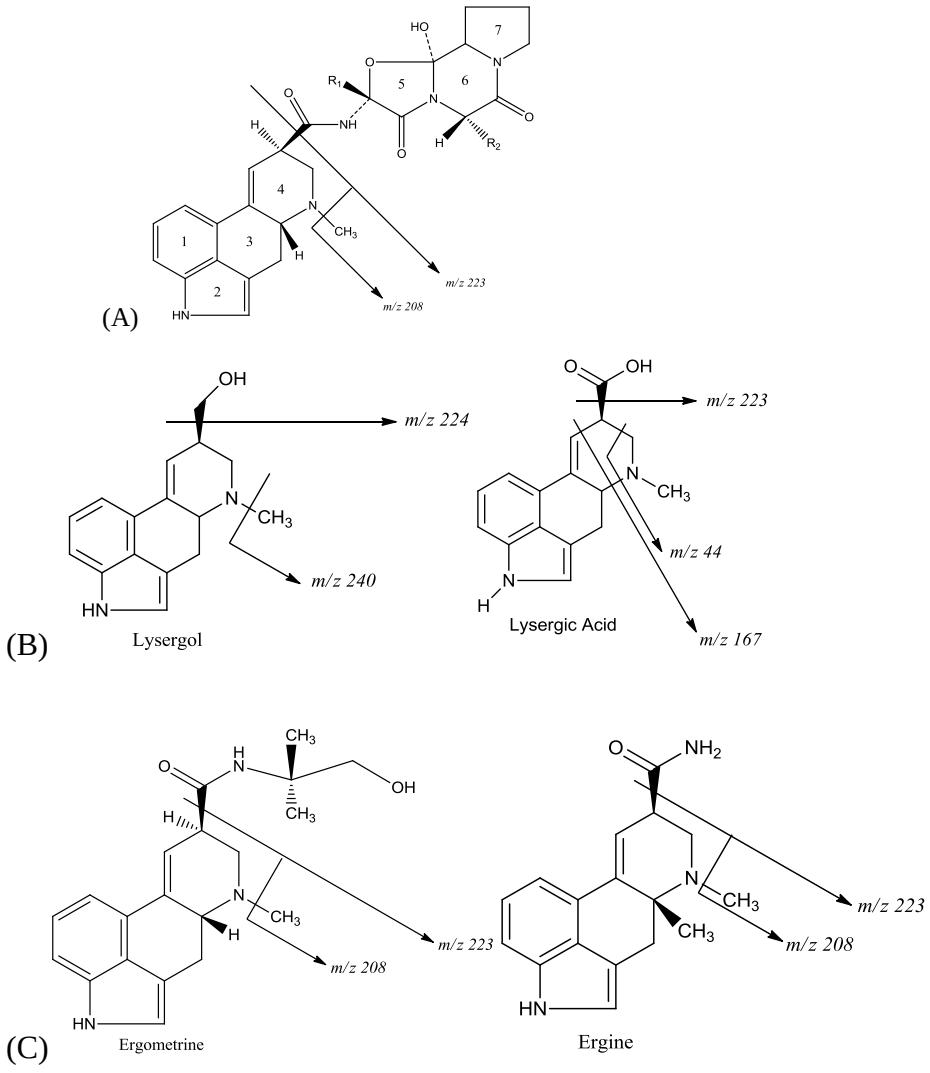


Figure 2.4. General Ergot Alkaloid Peptide Structures. (A) Typical fragmentation pattern for ergot alkaloids that contain the general peptide structure (ergovaline, ergotamine, ergocornine, ergocryptine, ergocrystine, and ergonovine). (B) Lysergol and lysergic acid give slightly different fragmentation 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).

Currently, mass spectrometry by positive atmospheric pressure chemical ionization (APCI (+)) is the ionization mode of choice for analysis of LB [36]; it has also been used to identify the LB biosynthesis pathway in plants and new lolitrem-like compounds [37]. A Phenomenex Prodigy ODS (30), 5 µ, 150 x 4.6 mm column was used with mobile phases consisting of 40% aqueous acetonitrile, 0.1% acetic acid (A) and acetonitrile, 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 [37]. 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^{o}C, and vaporizer temperature was set to 450^{o}C. For metabolite mining, an EMS scan is performed using the full-length, 60 min gradient described in Young et. al (2009) (Figure 2.4).

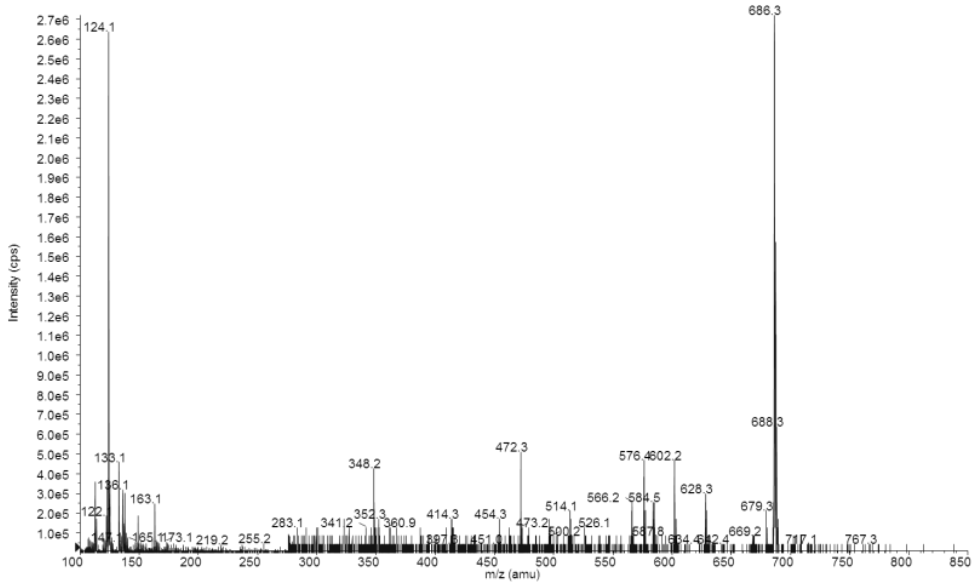


Figure 2.5. APCI(+)-EMS spectrum of lolitrem B standard prepared in ACN. Methods: A positive enhanced mass spectrometry (EMS) scan was used to acquire this spectra on a 3200 QTRAP hybrid triple-quadrupole/linear ion trap mass spectrometer with a Turbo V atmospheric pressure chemical ionization (APCI) source operated at 450°C and nebulizer current of 6µA, 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, 628, 686 [37-39]

2.4 Ruminal Metabolism

2.4.1 Ergovaline and Ergot Alkaloids

Bovine species are unique in the fact that they have a rumen which houses microogranisms that are capable of metabolizing toxins and thus sometimes making ruminants less susceptible to toxins [40]. Metabolism of EVis still being studied however some researchers have been able to generalize the effects of ergot alkaloids on cattle metabolism. One study in an attempt to better understand ergot metabolism found total ergot concentrations to be mostly in the duodenum (67%) and the feces (~24%) while no ergot alkaloids were detected in milk or blood. It was also postulated here that the pH of the rumen may play an important role in metabolism by causing the ergots to transform to their respective –inine isomers which are less pharmacologically active and therefore less toxic [41]. The pH of the rumen is slightly acidic (5-6) which does make this highly probable [42].

Cattle liver incubations with ergotamine showed through HPLC that the parent molecule was converted to more hydrophilic molecules but also decreased over time suggesting further metabolism. These hydrophilic molecules were identified as mono and

di-hydroxylated metabolites that were hydroxylated on the peptide structure of the parent molecule. In these incubations dexamethasone, a CYP3A inducer, was also used suggesting CYP3A has a role in detoxifying ergot alkaloids [35].

Settivari *et al.* (2006) later identified the genes specifically associated with endophyte infected feed that are responsible for detoxification and antioxidant activity in rat liver. This study showed that CYP2C13 and CYP2E1 were upregulated as well as 4-hydroxyphenlypyruvic acid dioxygenase, NADH dehydrogenase, epoxide hydrolase, catalase, and tryptophan 2,3-dioxygenase. All of these genes play a key role in detoxification of xenobiotics and were all upregulated in the presence of ergot alkaloids. Interestingly, glutathione synthetase responsible for glutathione biosynthesis was down regulated [43]. Conversely, a study by Tanaree *et al.* (2012) found bovine species showed up regulation of glutathione S-transferase M1 [44]. This suggests key differences in species when faced with ergot metabolism.

Loline alkaloids are a class of compounds produced by the same fungus that makes EV and LB. However, these compounds are saturated pyrrolizidines that are very unusual in structure with an ether linkage that connects two bridge head carbon atoms. Lolines have not been implicated as toxins in livestock but as potent insect replant found in grasses. Gooneratne *et al.*,(2012) studied the excretion of loline alkaloids in urine and feces of sheep. However, this study found that there was only a small amount excreted in the feces, but this could have also been from interference of multiple compounds that eluted at the same time as known lolines. Total loline excreted in the feces was estimated at 9.9%. Authors also stated this recovery was probably underestimated [45]. N-acetyl

loline has been suggested to enhance the effect of EV on cattle [45]. Klotz *et al.* (2008) showed that alone, neither N-acetylloline or lysergic acid produced contraction responses in bovine lateral saphenous veins. However, upon addition of EV, a vasconstricive response was generated when in combination with either N-acetylloline or lysergic acid. The responses of the single doses of toxins versus the mixtures was different. Therefore there exists additive properties of alkaloids. However, there is still no documented synergistic evidence of alkaloids with different mechanisms of action like those of the lolitrems and ergopeptides.

2.4.2 Lolitrem B and Indole Compounds

Research into the exact genes that are upregulated or downregulated with respect to LB are to our knowledge unknown. LB has been shown to accumulate in the fat tissue of both sheep and cattle [30, 46]. However, toxicokinetic data on LB is limited, and investigations into the enzymes responsible for metabolism of the parent molecule have not been conducted. However, since LB does belong to the indole class of compounds, this section aims to examine the metabolism 3-methylindole (3-MI) which is toxic to ruminants and has been heavily studied.

An indole compound commonly associated with ruminant lung toxicity is 3-methylindole which is derived from dietary tryptophan during ruminant fermentation. Cytochrome P450 monooxygenases are the main enzyme responsible for the bio-activation of 3-MI to its toxic intermediate. Conversely, the major route of detoxification of 3-MI has been proposed as through glutathione conjugation through formation of a

electrophilic imine methide intermediate which can also trap water and tautomerize to 3-methyloxindole, a urinary metabolite [47].

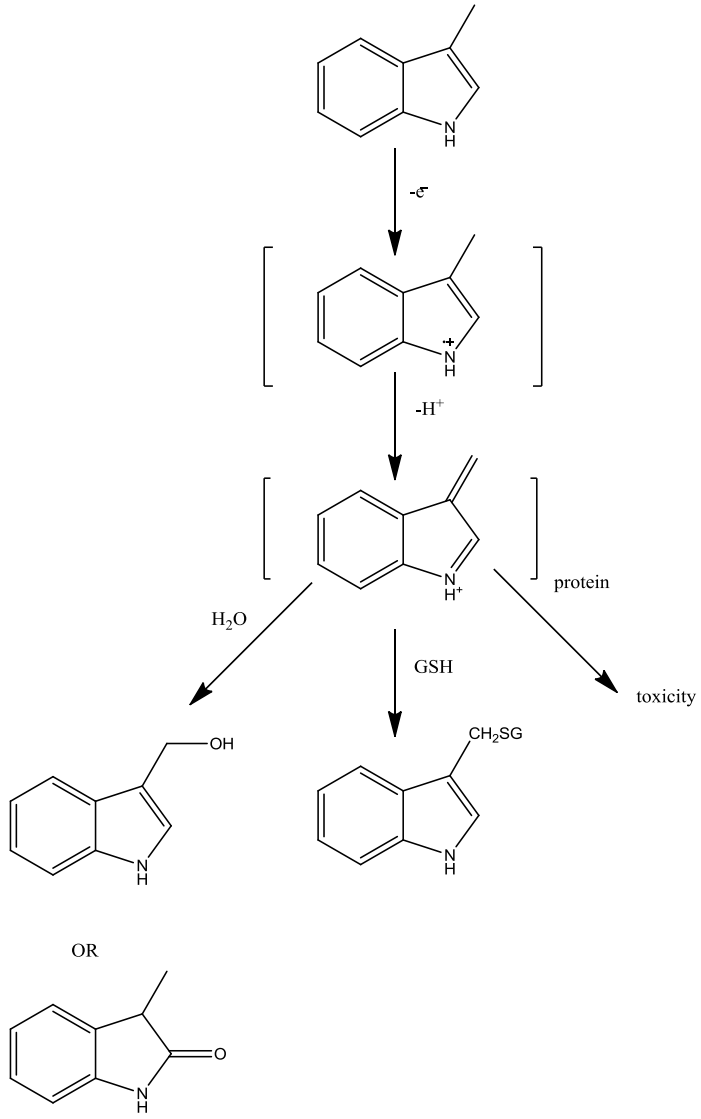


Figure 2.6. Metabolism of 3-methylindole by ruminal species proposed by Nocerini et. al (1985) [47].

Lanza *et al.* (2001) showed that 3-MI was metabolized by a number of CYP450 enzymes, but in very specific routes. CYP 1A1 and 1A2 both were found to produce the dehydrogenation, oxidation, and indole 3-carbinol metabolites. CYP 2F1 and 2F3 were found to selectively produce the dehydrogenation metabolites. CYP1B1 was shown to

produce I3C and 3MOI (shown in figure 2.7). CYP 2A6, 2C19, and 2D6 were shown to produce I3C and 3MOI. CYP 2B6, 3A4, and 3A5 only produced 3MOI. Interestingly, CYP2E1 was shown to selectively produce 3MOI, which is also a common upregulated CYP450 in ergot alkaloid metabolism [43, 48].

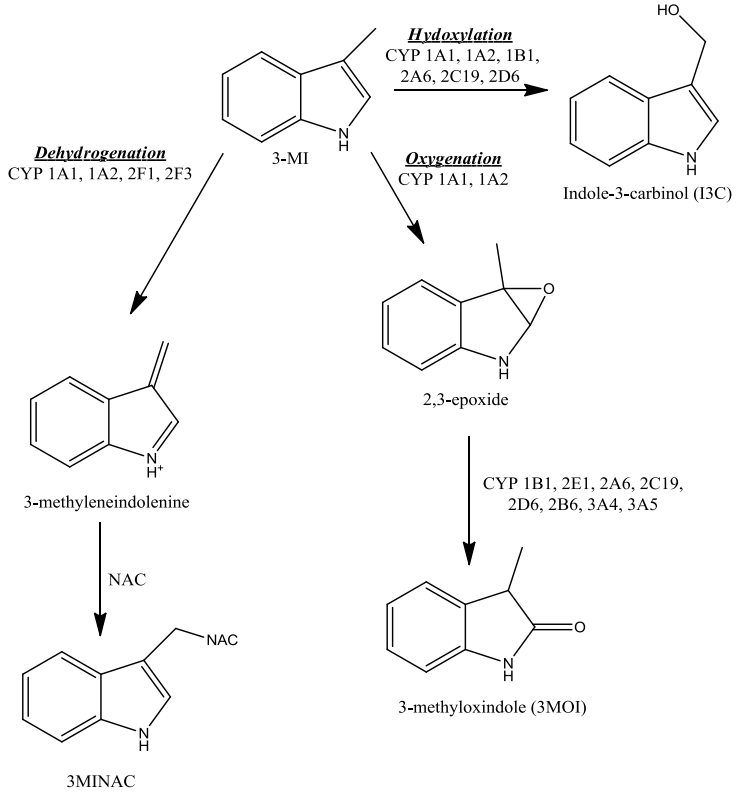


Figure 2.7. Proposed metabolism pathway of 3-MI by CYP450 enzymes. 3-methyleneindolenine was unstable and determined after trapping with NAC [48].

In an unpublished study conducted at our laboratory, a microarray of the liver samples collected from the same study that was used to analyze fecal samples for LB and EV, showed 6 potential biomarkers for perennial ryegrass toxicosis: stearoyl-CoA desaturase, acyl-CoA synthesase short-chain family member 2, glutathione peroxidase 4, diazepam binding inhibitor GABA receptor modulator, arginase (liver), and LAG

homolog ceramide synthetase 6. Gene ontology showed that oxidation and reduction genes were upregulated in all groups which suggests CYP450 activation. Additionally, lipid, cholesterol, and sterol biosynthetic pathways were shown to be upregulated. Additionally ceramide synthesis was shown to be upregulated in the presence of LB. However, since EV was measured in this experiment it is not known whether the genes being upregulated for fatty acid synthesis are from EV or a combination of the two toxins [49]. The influence of glutathione in the detoxification of EV and LB when consumed together has not been studied as a possible explanation to why only LB clinical symptoms are seen and not EV. Settivari *et. al.* (2006) indicated that glutathione synthetase was down regulated in rat liver genomic analysis. This gene is responsible for glutathione synthesis, oxidoreductase activity, cholesterol biosynthesis, and lipid metabolism [50]. In Tanaree *et al.* (2012), a study with four bovine species found that there was an upregulation of glutathione S-transferase M1. This gene is responsible for the detoxification of xenobiotics through conjugation with glutathione [44]. Li *et al* (2012) found an upregulation of glutathione peroxidase 4 and upregulation of ceramide biosynthesis. Ultimately gluthathione metabolites could help answer questions pertaining to the influence of glutathione on metabolism of LB and EV. There is obviously a difference in metabolism between species which can offer an explanation to the clinical disease signs shown when both toxins are consumed.

2.4.3 Additional characteristics of ruminal metabolism

Ruminants possess characteristic metabolic properties because of the rumen which houses a consortium of anaerobic microbes that possess the ability to detoxify a number of compounds. However, ovine and bovine have been shown to possess different capabilities for metabolizing toxins. Wachenheim *et al* (1992) showed that the ovine rumen was significantly different from the bovine rumen when forced to metabolize pyrrolizidine alkaloids (PA) found in tansy ragwort (*Senecio jacobaea*). This was demonstrated by infusing PAs directly into the liver of sheep which showed the same clinical disease signs when PAs were consumed by cattle[51]. Rumen microbiota was the key component to sheep being more resistant to PA toxicity. By HPLC-fluorescence this was further exemplified by conducting *in vitro* experiments with whole rumen fluid (WRF) from sheep, goats, and cows. The sheep showed that PAs disappeared after 4 hrs, goats after 3hr, and cow WRF took 19hrs to transform PAs [52].

Structural similarities between the pyrrol ring in PAs and the rings of nitro-aromatic compounds led researchers to investigate the degradation capacities of ovine ruminal microbes as a possible tool for bioremediation. De Lorme *et. al.*, (2009) showed that seven out of twenty-one ruminal bacteria species were capable of degrading TNT. However, one aspect lacking in this study was LC-MS/MS data to determine if ring cleavage was occurring. Eaton *et al* (2011) showed that ovine ruminal microbes were also capable of degrading RDX, another explosive compound known to contaminate multiple military sites across the country. Similar to the TNT experiment, the results for RDX degradation experiments lacked LC-MS/MS data and any indication of the types of

metabolites being formed. Not until Eaton *et al* (2012) was LC-MS/MS data obtained and it was concluded that RDX was being metabolized by ovine ruminal microbes to nitroso intermediates MNX, DNX, and TNX. Additionally, an ion of *m/z* 149 was proposed as ring cleavage product as a result of the intermediate metabolite MEDINA[53, 54].

The differences seen in metabolism of sheep versus cattle shows that the rumen and the certain microbes present play a key role in metabolism and may offer a better understanding of toxic mixtures when considering the synergistic properties of LB and EV. There is sufficient evidence from enzyme and genomic studies conducted with ergot alkaloids and 3-methylindole that similar routes of excretion and metabolism may be happening in the gastrointestinal tract and circulatory system. This study aims to better understand why clinical signs of LB are seen and not EV while both are consumed at toxic levels.

CHAPTER 3. Materials & Methods

3.1 Chemicals and Equipment

HPLC and LC-MS/MS grade acetonitrile, methanol, dichloromethane, chloroform and reagent grade ammonium carbonate were purchased from J.T. Baker (Phillipsburg, N.J.). Lolitrem B analytical standard was purchased from AgResearch Limited, Ruakura Research Centre (Hamilton, New Zealand). Ergotamine tartrate was purchased from Sigma-Aldrich (St. Louis, MO). Ergovaline tartrate analytical standard was purchased from and prepared by Dr. Forrest Smith, Department of Pharmaceutical Sciences, Auburn University (Auburn, AL).

3.2 Animals and treatment

A detailed experimental design can be found in Blythe *et al.*, (2012). Briefly, 24 steers under one year of age, with an average weight of 348kg, were purchased from commercial feedlots in Oregon. Animals were castrated and treated with anthelmnthics prior to shipping. Upon arrival, steers were placed onto pasture for 10 days with open access to the barn and water. All twenty four steers were initially placed onto pasture for 10 days with open access to the barn. During this time steers were examined by a veterinarian and those affected were treated for dermatophytosis (ringworm), infectious bovine keratoconjunctivitis (IBK, pink eye) and bovine respiratory disease (BRD) or "shipping fever." Following this initial acclimation period they were randomly separated into 4 pens of 6 steers each. Steers were allowed one week to adjust to a perennial ryegrass straw (LB <300ppb) diet prior to the start of the study.

Animals were fed twice daily *ad libitum* for the duration of the experiment with open access to water and mineral blocks. Feed samples and orts were taken each day, dried, and stored at -20°C until analysis of LB and EV concentrations as described below. During each fecal sample collection steers were weighed using a scale built into the chute. All procedures were approved by the Institutional Animal care and Use Committee at Oregon State University (IACUC #4031).

3.3 Perennial Ryegrass

Endophyte infected PRG was obtained at various concentrations of lolitrem B. Four rations were formulated to different concentrations. Group I was designated as high group which was aimed to feed on approximately 2000ppb LB, group II was designated the medium group of approximately 1500ppb LB, Group III was the low group with approximately 1000ppb LB, and Group IV was designed as the control group which was fed less than 300ppb LB morning and evening. Feed samples were analyzed each week by HPLC-fluorescence analysis for LB and EV concentrations as described below.

3.4 Sample collection and preparation.

Fecal samples were collected once per day on days -7, -1, twice daily on days 0-3, once daily on days 4-7, then weekly for the duration of the trial (days 14-70). Fecal samples were obtained while steers were in a squeeze chute from the rectum. Samples were immediately placed into freezer bags on ice, and then transported to a chemical hood where they were spread on weight boats and allowed to air dry for 4-5 days. After

drying, samples were stored at -20°C until analysis. Dried feed, orts, and feces samples were prepared for HPLC-fluorescence and LC-MS/MS analysis by grinding in a Tecator 1093 Cyclotec 1093 sample mill and passed through a 0.5mm screen. Orts and feed samples were collected every day from each group; feces samples were pooled according to group and time point. All samples were submitted to the Endophyte Service Laboratory at OSU (ISO/IEC 17025, http://oregonstate.edu/endophyte-lab/) for quantitation of LB and EV.

Solid phase extraction was then utilized for sample clean up. For LB, 0.200 g of dried, ground PRG straw, orts, or pooled feces was weighed out in duplicate, then turned in the dark for 18-24 hours at room temperature in 3ml of extraction solution (chloroform:methanol 2:1 (v/v)). The tubes were then centrifuged at 1,700 x *g* for 5 minutes and 1.6 ml of the supernatant was transferred to clean glass culture tubes. The solvent was evaporated under a flow of nitrogen at ambient temperature. Two 1 ml additions of dichloromethane were used to dissolve the sample. CUSIL (United Chemical Technologies, Bristol, PA) SPE cartridges were loaded onto a positive pressure manifold and conditioned with 2 ml dichloromethane. The sample was then applied, followed by a 2 ml dichloromethane wash. Next, a second wash of 0.5ml of elution solution (dichloromethane: acetonitrile 4:1 (v/v)) was added. LB and its metabolites were eluted with 3.0 ml elution solution which was captured and sealed for analysis. [20].

For EV, solid phase extraction of dried, ground PRG straw, orts, and pooled feces was accomplished using a method adapted from Craig *et al.* (1994), Rottinghaus *et al.* (1990), and Hovermale *et al.* (2001). One gram of sample was added to 10 ml of

chloroform, 1 ml internal standard (1µg/mL ergotamine tartrate) and 1 ml of 0.001 N NaOH. The tubes were capped and mixed for 18-24 hours in the dark. The tubes were then centrifuged at 1,700 x *g* and 5 ml of the supernatant was loaded onto Ergosil (United Chemical Technologies, Bristol, PA) SPE columns. The column was washed with 2 ml of acetone:chloroform (4:1 v/v) solution and EV and metabolites were eluted with 2.5 ml methanol. The eluate was collected, dried under nitrogen at 50 ºC and reconstituted with 0.5 ml methanol. After sonication and mixing the extract was transferred to amber HPLC vial and sealed for analysis [20, 55, 56].

3.5 HPLC-fluorescence analysis

Analyses were performed using a Series 200 autosampler (PerkinElmer Instruments, Shelton, CT) coupled to an LS 40 fluorescence detector (Perkin Elmer). Data collection was completed using an interface to a PC-based data system which consisted of a 900 Series Interface and Total Chrom Workstation (PerkinElmer).

LB was detected using at an excitation wavelength of 268 nm and an emission wavelength of 440 nm. An analytical column Zorbax RX-SIL 4.6 x 250mm, 5-µm particle size (Agilent, Santa Clara, CA) was used in conjunction with a hand packed silica guard column. The mobile phase, dichloromethane-acetonitrile-purified water (400:100:2), was run isocratically at a flow rate of 0.5 ml/min. Lolitrem B eluted at 8.3 minutes with a total run time of 15 minutes. A standard curve was constructed from reference PRG material and then a linear regression fit of the peak height versus the amount of analyte injected was used to quantitate LB in unknown samples.

For EV, the procedure was adapted from a previously described method [56]. Using a flow rate of 1.0 mL/min, separation was achieved via gradient elution using 65% H_2O, 35% ACN, and 0.02% ammonium carbonate (mobile phase A) and ACN (mobile phase B) as follows: 0-3.5 minute 99%A:1%B; 3.5-5.5 minute a linear change to 35% A:65% B; 5.5-7.5 minute 30% A:70% B; 7.5-8.5 minute a linear change to 99% A:1% B; 8.5-12 minutes 80% A:20% B. An analytical column packed with Gemini 4.6x 150mm, 5-µ particle size was used in conjunction with a guard column cartridge with similar packing (Phenomenex, Torrance, CA). EV was detected using an excitation wavelength of 250 nm and an emission wavelength of 420 nm. EV eluted at 8 minutes and the ergotamine at 9 minutes with a total run time of 12 minutes. A standard curve was constructed from tall fescue reference material and a linear regression fit of the peak area versus the amount of analyte injected was used to quantitate ergovaline in unknown samples.

3.6 Liquid chromatography-mass spectrometry analysis

The LC-MS/MS system used consisted of a PerkinElmer Series autosampler, Perkin Elmer LC 200 micropump, a Peltier Series 200 cooling tray, and an AB 3200 Q TRAP system (Applied Biosystems, Foster City, CA). Both compounds were analyzed in positive ionization mode. LB and its metabolites were analyzed using atmospheric chemical ionization (APCI), while EV and its metabolites were analyzed with electrospray ionization (ESI). Multiple reaction monitoring (MRM) transitions were

obtained using the quantitative optimization in Analyst 1.4.2; mass spectrometer settings are shown in table 3.1.

TABLE 3.1
Mass spectrometry parameters for metabolite identification and analysis of feces samples

Parameter	Multiple Reaction Monitoring		Enhanced Mass Spectrum	
	Lolitrem B	Ergovaline	Lolitrem B	Ergovaline
Curtain Gas (psi)	30	10	30	30
Nebulizer Current (µA)	6	N/A[a]	6	N/A[a]
Ion Spray Voltage (V)	N/A[a]	5000	N/A[a]	5000
Temperature (°C)	450	550	450	550
Gas 1 (psi)	65	55	65	55
Gas 2 (psi)	0	55	0	55
Declustering Potential (V)	61	36	25	20
Entrance Potential (V)	7	9	10	10
Cell Exit Potential (V)	4	4	N/A[a]	N/A[a]
Collision Energy (V)	51	85, 53, 41[b]	10	10
Q1	686.41	534.04	N/A[a]	N/A[a]
Q3	196.3, 237.9, 628.4	207.0, 208.1, 223.1	N/A[a]	N/A[a]

[a]Parameter not required in specific method of analysis.

[b]Collision energies are listed in the same order as their respective Q3 values.

The HPLC settings were as follows: for LB a Prodigy ODS(30), 5 µ, 150 x 4.6 mm (Phenomenex, Torrance, CA, USA) column was used with 40% acetonitrile in water, 0.1% acetic acid (mobile phase A) and acetonitrile with 0.1% acetic acid (mobile phase B) flowing at 0.65mL/min at a gradient as follows: held for 5 minutes at 20% B

then raised linearly to 100% B over 5 minutes and held for 5 minutes before finishing at 20% B. For EV the same column and mobile phases as described above for HPLC-fluorescence quantitation of EV were used. However, the gradient profile was altered such that the system was held for 5 minutes at 99% A then decreased linearly to 35% A over 10 minutes and held at 35%A for 2 minutes before equilibrating for 6 minutes at 99%A.

Potential metabolites in feces were identified using an enhanced mass spectrum, independent data acquisition, and enhanced product ion scans (EMS IDA EPI) method with the same equipment and mobile phases mentioned previously for the MRM methods. HPLC conditions were the same except for the gradient used. For LB the gradient was as follows: 10 minutes at 100%A, then raised linearly to 100% B over 55 minutes and held at 100%B for 5 minutes before re-equilibrating at 100%A for 5 minutes at a flow rate of 0.65 mL/min. For EV, the gradient was as follows: 2 minutes at 99% A, linearly decreased to 35%A over 60 minutes, held at 35%A for 5 minutes and equilibrated again at 99%A for 5 minutes.

The EMS experiments for LB were conducted using a scan of all ions in the mass ranges of 150-630 and 625-1500 amu using a cell exit potential (CEP) of 5.54 V. An EPI (MS/MS) scan was triggered for ions greater than 1000 counts. The EMS scans for EV were done in two ranges of 100-280 and 275-1500amu with CEPs of 4.74 and 7.54 V, respectively. For both toxins, EMS parameters were the same for EPI except three EPI experiments were done with different collision energies of 47, 61, and 63. All CEP values for EPI experiments were 3.62. Additional settings are shown in Table 1. An EPI scan of

1.46 µM LB in ACN and 1nM EV standard using this LC-MS/MS method are shown in figure 1.

Metabolite screening and identification was conducted using Lightsight Software Version 2.0 (Applied Biosystems).

3.7 Statistical analysis.

Statistical analysis of all HPLC-fluorescence and LC-MS/MS data was done using a one way analysis of variance (ANOVA) followed by a Bonferroni's test (GraphPad Prism 5.0 , GraphPad Software, La Jolla, CA). Metabolite Q1/Q3 transitions obtained from EMS IDA EPI and Lightsight were added to the respective MRM method. From the MRM peak area , a semi-quantitative analysis of metabolites was accomplished. Epimers eluting with the same retention times as to ergovaline and its epimer, the peak areas were added together. For determination of a linear relationship between LB and EV, the concentrations of LB and EV in feces were compared across all groups using a Pearson's test of correlation to determine coefficients.

Chapter 4. Results

4.1 Animal Health and Staggers

The average body weight for each group increased linearly with R^2 values of 0.72, 0.93, 0.88, 0.89 respectively for Group I-IV. One way ANOVA showed an P value of 0.0069 (GraphPad Prism 5.0 , GraphPad Software, La Jolla, CA). Bonferroni's multiple comparison test showed $P < 0.05$ when group I was compared to groups III and IV.

Feed consumption was measured in pounds by adding the amount of hay and grain given to each group and subtracting the orts in pounds measured the following morning. Feed consumption was monitored a total of 71 times before and during the experiment. One way ANOVA of lbs of feed consumed showed a $P < 0.0001$ across all four groups. Bonferroni's multiple comparison tests showed $P < 0.05$ for group I vs II & III, group II vs III & IV, and group III vs IV.

On day 24 of the experiment an increase in diarrhea was noticed and one steer from each group was tested for Coccidia parasites using a fecal floatation test at the Oregon State University Veterinary Diagnostic Laboratory (Corvallis, OR). Group I showed no oocysts or eggs, Group II, III, and IV showed 125, 50, and 675 oocysts per gram of feces, respectively.

4.2 Perennial Ryegrass and Orts Quantitation by HPLC-Fluorescence

Table 4.1 shows the results of LB and EV concentration in the feed for each group. For both EV and LB, one-way ANOVA analysis with a Bonoferroni's correction

gave P<0.0001 and P <0.05, respectively. Thus, feed concentrations of EV and LB were different between all four groups.

HPLC-fluorescence quantitation of the feces showed significant differences between groups for LB concentration (P value < 0.0001, Figure 2A). was sampled a total of 70 times and averages for both EV and LB during the entire experiment are shown in table 2. For both EV and LB, one way ANOVA analysis followed by Bonoferroni's post test showed P< 0.0001 and P<0.05, respectively. This showed that the concentrations for EV and LB were statistically different for all groups.

Table 4.1: Average concentration of lolitrem B and ergovaline in perennial ryegrass feed and orts determined by HPLC-fluorescence.

Group	Lolitrem B in perennial ryegrass (ng/g)[a]	Lolitrem B in orts (ng/g)[a]	p value[b]	Ergovaline in perennial ryegrass (ng/g)[a]	Ergovaline in orts (ng/g)[a]	p value[b]
I	2256 ± 166	2484 ± 348	0.0169	638 ± 77	741 ± 49	< 0.0001
II	1554 ± 213	1598 ± 263	0.4821	373 ± 119	463 ± 167	0.0961
III	1012 ± 197	1793 ± 212	0.0015	259 ± 53	357 ± 58	0.0002
IV	247 ± 175	211 ± 138	0.3111	<100	<100	**N/A**

[a]Results reported as mean ± standard deviation (*n*=60)
[b]Results from students t-test comparing concentrations of lolitrem B and ergovaline in perennial ryegrass feed and orts.

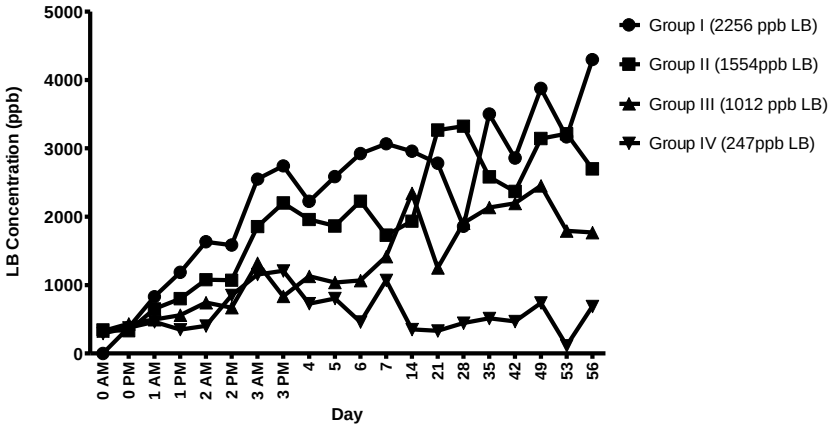
4.3 Feces Samples Quantitation by HPLC-Fluorescence

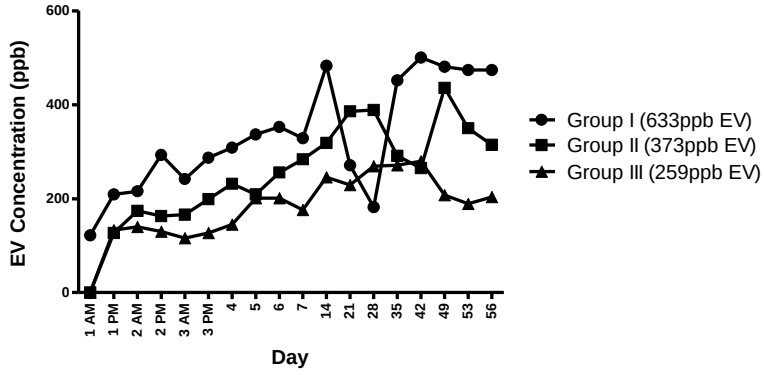
HPLC-Fluorescence data showed significant differences between groups in LB concentration (P value <0.0001). Group I showed peak LB concentrations on days 3 PM (2743 ppb), 7 (3065 ppb), 25 (3502 ppb), 49 (3877 ppb), and 56 (4297 ppb). Group II showed similar maximum concentrations on days 3PM (2200ppb), 6 (2227ppb), 28

(3323ppb), and 53 (3213ppb). Group III (1012ppb) showed maximum concentrations on days 3AM (1324 ppb), 14 (2344 ppb), and 49 (2454 ppb) while group IV (247 ppb) showed maximum concentrations at day 3PM (1209 ppb), 7 (1071 ppb), 49 (741 ppb), and 56 (690 ppb). Concentration curves of LB in feces by group is shown in figure 5A. The LB concentration for group I was not completely eliminated during the one week inadvertant washout period, but it did decline to the level seen in group III.

Quantitation of EV by HPLC-fluorescence detection in bovine feces showed statistically different concentrations for all groups ($p < 0.0001$, Figure 4.1). All fecal samples for Group III showed values of EV <100ppb, while group I showed peak EV concentrations on days 2 PM (293 ppb), 6 (353 ppb), 14 (483 ppb), and 42 (481 ppb); group II showed peak EV concentrations on days 2 AM (174 ppb), 4 (232 ppb), 28 (389 ppb), 49 (436 ppb). Group IV showed peak EV levels on days 2AM (140 ppb), 5 and 6 (201 ppb), 14 (246 ppb), and 42 (280 ppb). During the one week washout period for group I levels of EV did decrease to the levels seen for group III.

Figure 4.1 Lolitrem B and ergovaline concentrations (ppb) in feces by HPLC-fluorescence. Group IV EV levels were below the limit of quantitation. For each group, 6 feces samples for group I, III, and IV and 5 samples for group II, were pooled and analyzed in duplicates by HPLC-fluorescence.





4.4 LC-MS/MS Feces Metabolite Analysis

Using the EMS-IDA-EPI and MRM LC-MS/MS methods previously described for LB, possible metabolites were identified using product ions found in the fragmentation of LB standard (Figure 4.2), specifically *m/z* 196 and 238.

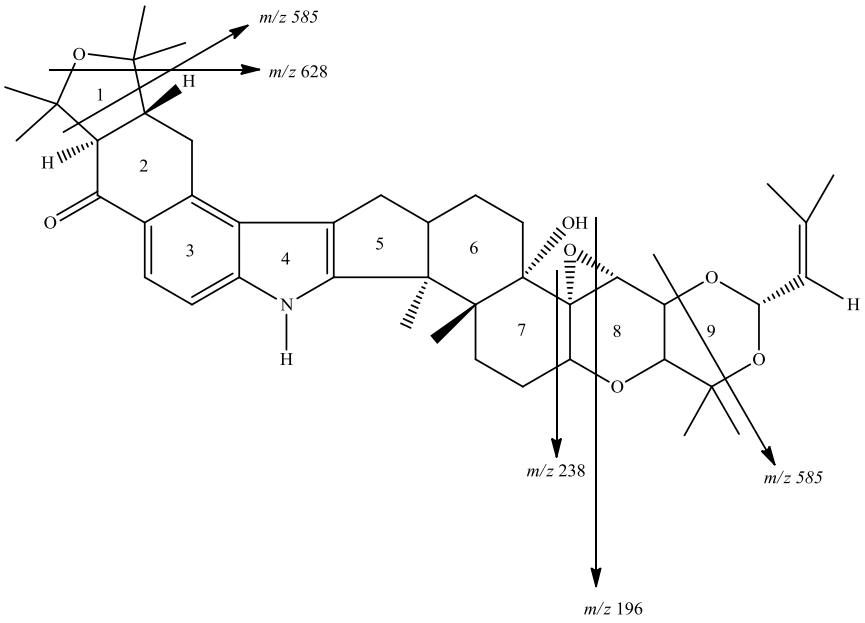


Figure 4.2. Structure and fragmentation pattern of lolitrem B.

Lightsight analysis was used to help identify additional metabolites. This software provides lists of possible metabolites based on common losses, common product ions, and matching molecular ion peaks to common metabolites based on parent molecule. Through Lightsight analysis, peaks consistent with an *m/z* of 603.4, 669.4, 672.4, 687.4, 688.4 and 702.4 were detected in multiple samples and showed fragmentation to the 196 and 238, daughter ions common to LB (Appendix A). This information was used to create a more tailored MRM method in order to identify trends in these metabolites. Peak areas were obtained for each of the compounds: a semi-quantitative analysis showed that there were significant differences in peak area between all four feeding groups for peaks consistent with *m/z* 687.4, 688.4, and 702.4 ($p < 0.0001$, Table 4.3). The metabolite *m/z* 688.4 eluted at two difference times 12.6 and 13.2 minutes; peak areas of both peaks showed a significant difference between all four groups ($p < 0.0001$). Upon further investigation with Light Sight, possible metabolites identified through MS/MS (Appendix

A) are consistent with deamination and reduction, and/or amine hydrolysis, demethylation and oxidation or hydrogenation, and oxidation (*m/z* 702.4). Possible structures for m/z 688.4 are shown in Appendix E based on literature of known metabolism of 3-MI. Bonferroni's multiple comparison test results (table 4.2) show the significance is the greatest between all groups when looking at *m/z* 702.4

Table 4.2
Bonferroni's Multiple Comparison Test of Lolitrem B metabolites from MRM peak area analysis

	m/z 688.4 (13min)		m/z 688.4 (12 min)		m/z 687.4		m/z 702.4	
	P < 0.05		P < 0.05		P < 0.05		P < 0.05	
I vs II	No	ns	No	ns	No	ns	No	ns
I vs III	Yes	**	Yes	*	Yes	*	No	ns
I vs IV	Yes	*	Yes	*	Yes	*	Yes	***
II vs III	Yes	***	Yes	***	Yes	**	No	ns
II vs IV	No	ns	No	ns	No	ns	Yes	***
III vs IV	Yes	***	Yes	***	Yes	***	Yes	***

Table 4.3

Lolitrem B metabolites determined by enhanced mass spectra and Lightsight analysis

Parent[a]	Common Fragments[a]	Proposed Biotransformation(s)[b]	Q1/Q3[c]	p Value[d]
702.12	644.2, 238.1, 196.1	oxidation	702.4/237.9	<0.0001
687.52	629.3, 571.2, 238.1, 237.1	deamination, reduction AND/OR primary amide hydrolysis	687.4/237.9	<0.0001
688.4 (a)	630.0, 238	demethylation, oxidation AND/OR hydrogentation	688.4/237.9	<0.0001
688.4 (b)	630.0, 238	demethylation, oxidation AND/OR hydrogentation	688.4/237.9	<0.0001

[a]Identified using enhanced mass, independent data acquisition, and enhanced product ion scan (EMS IDA EPI).
[b]Proposed biotransformation obtained using Lightsight version 2.0.
[c]Transitions obtained from EMS IDA EPI method and then used in multiple reaction monitoring method (MRM) to obtain peak areas for statistical analysis.
[d]Values calculated using peak areas obtained from transition in Q1/Q3 column, one way analysis of variance, and Bonferroni's post test.

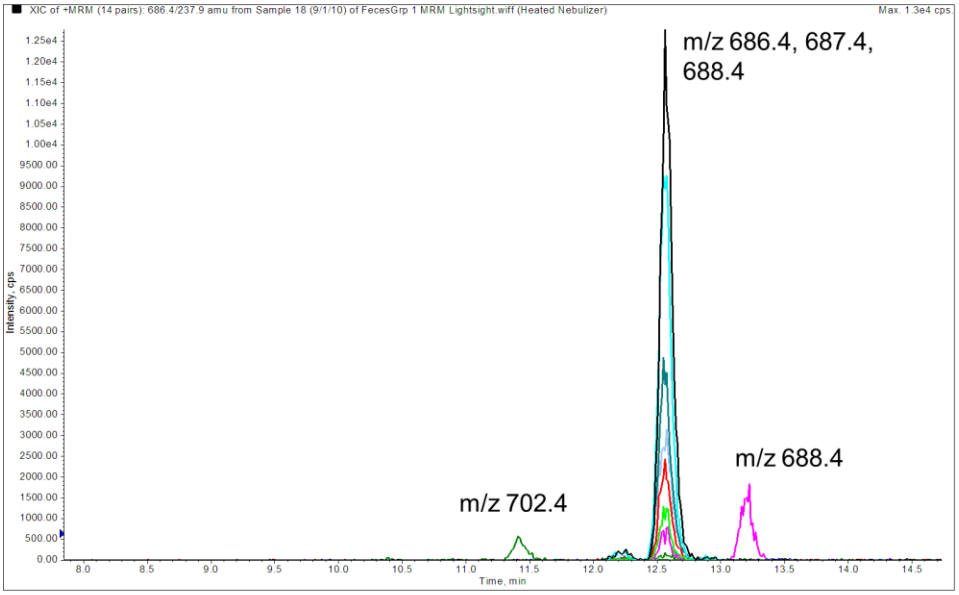


Figure 4.3 Shows a sample MRM of Loltirem B metabolites identified by EMS IDA EPI and Lightsight.

Using the EMS-IDA-EPI and MRM LC-MS/MS methods described above for EV, possible metabolites were identified using the common and most abundant ergot alkaloid product ions of *m/z* 208 and 223. The structure in figure 4.3 shows the common site of fragmentation that gives *m/z* 223 and 208. [57]. Secondary and less abundant ions used for identification were *m/z* 249 and 516. All possible metabolites obtained using the EMS IDA EPI method are shown in table 4.5. From this data MRM transitions were added to the method already used for EV MRM analysis, in order to conduct a semi-quantitative analysis using peak area and ANOVA. Additionally, Q1/Q3 values used for semi-quantitative analysis are also shown in table 4.5.

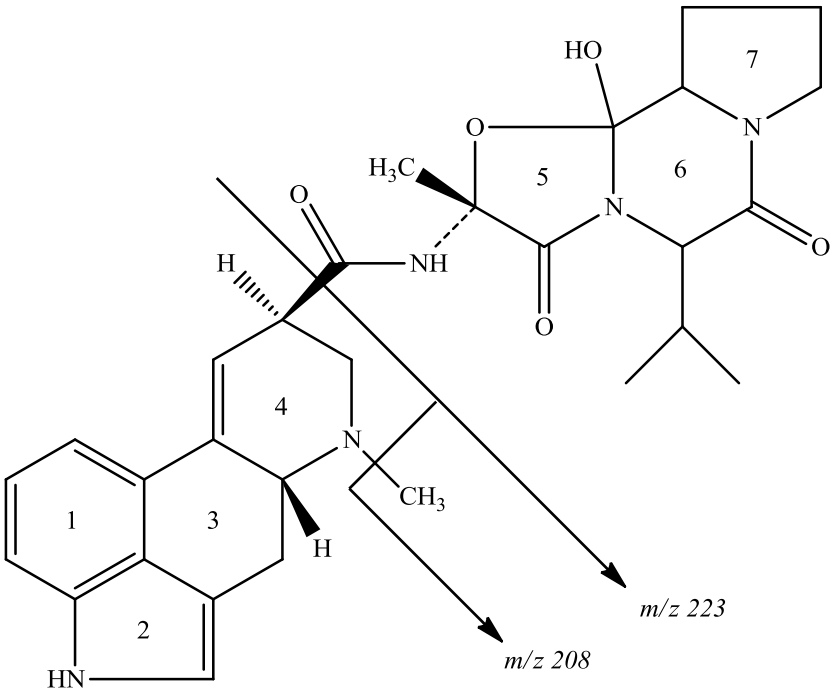


Figure 4.4. Structure and fragmentation pattern of ergovaline.

The possible metabolite of m/z 564 was seen only in group IV (<100 ppb EV). However, this is also a common fragment of ergotamine which was used as an internal standard. However, since it is predominately present in group IV it is also possible that the ion is in fact the oxidation to acid of a methyl group on ergovaline [58]. Certain metabolites showed epimer peaks similar to ergovaline (2 peaks), however an epimer for the purpose of this study was only used when the retention times matched those of EV parent and its epimer. Figure 9 also shows that *m/z* 550 and 532 show a total of 4 peaks suggesting 2 epimers and possible phase II metabolism. It should also be noted that there are noticeable multiple peaks for metabolites suggesting ion source fragmentation and phase II metabolism. From the MS/MS (Appendix B) obtained for the metabolites 516, 532, 536, 55, 564, and 566 it can be shown that metabolism is most likely occurring on

rings 5,6, and 7 of the structure shown in figure 2B. This is shown by the common fragments of 223 and 208 being retained in most cases.

Table 4.4
Bonferroni's Multiple Comparison Test For Ergovaline Metabolites from MRM peak area analysis.

m/z	598.8		516		532		535/224	
	P < 0.05	Summary[a]	P < 0.05	Summary[a]	P < 0.05	Summary[a]	P < 0.05	Summary[a]
I vs II	No	ns	No	ns	Yes	***	No	ns
I vs III	Yes	*	Yes	***	Yes	***	Yes	***
I vs IV	Yes	***	Yes	***	Yes	***	Yes	***
II vs III	No	ns	No	ns	No	ns	No	ns
II vs IV	Yes	***	Yes	***	Yes	***	Yes	***
III vs IV	No	ns	Yes	**	Yes	***	Yes	**
m/z	534.8/224		537		539		535/223	
	P < 0.05	Summary[a]	P < 0.05	Summary[a]	P < 0.05	Summary[a]	P < 0.05	Summary[a]
I vs II	No	ns	Yes	*	Yes	*	Yes	*
I vs III	Yes	***	Yes	***	Yes	***	Yes	***
I vs IV	Yes	***	Yes	***	Yes	***	Yes	***
II vs III	No	ns	Yes	*	Yes	**	No	ns
II vs IV	Yes	***	Yes	***	Yes	***	Yes	***
III vs IV	Yes	***	Yes	***	Yes	**	Yes	*
m/z	816.9		610.8		552		611.9	
	P < 0.05	Summary[a]	P < 0.05	Summary[a]	P < 0.05	Summary[a]	P < 0.05	Summary[a]
I vs II	No	ns	No	ns	No	ns	No	ns
I vs III	Yes	**	Yes	***	Yes	**	No	ns
I vs IV	No	ns	Yes	***	No	ns	No	ns
II vs III	Yes	**	Yes	***	Yes	**	Yes	***
II vs IV	No	ns	Yes	***	No	ns	No	ns
III vs IV	No	ns	No	ns	No	ns	No	ns
m/z	802.9		824.7		550		596.8	
	P < 0.05	Summary[a]	P < 0.05	Summary[a]	P < 0.05	Summary[a]	P < 0.05	Summary[a]
I vs II	No	ns	No	ns	Yes	***	No	ns
I vs III	No	ns	Yes	**	Yes	***	Yes	**
I vs IV	Yes	***	------	------	Yes	***	Yes	***
II vs III	No	ns	No	ns	Yes	*	Yes	**
II vs IV	Yes	**	------	------	Yes	***	Yes	***

III vs IV	No	ns	------	------	Yes	***	No	ns

[a]Indicates how significant the differences were between the groups(ns = not significant). More "*" indicates stronger significance.

Table 4.5

Ergovaline metabolites determined by enhanced mass spectra and Lightsight analysis

Parent[a]	Common Fragments[a]	Proposed Biotransformation(s)[b]	Q1/Q3[c]	p Value[d]	Possible Epimers[e]
492[f]	225, 239, 251	tri-demethylation			
516	249, 223	dehydration	516/223	<0.0001	+
532	249, 221	dehydrogenation	532/221	<0.0001	
535	(A) 224, 209 (B) 223, 208 (C) 224, 208	deamination, reduction AND/OR primary amide hydrolysis	(A) 534.8/224 (B) 535.1/223 (C) 535.1/224	(A) <0.0001 (B) <0.0001 (C) <0.0001	+
536	223, 209	demethylation, oxidation AND/OR hydrogenation	536/223	<0.0001	+
537	509, 437	*Unknown*	537/437	<0.0001	
539	450.8, 479.9	*Unknown*	539/450.8	<0.0001	+
550	249,221,208	oxidation	550/221	<0.0001	
552[g]	534, 523	hydrolysis	551.9/533.9	0.0007	
564[h]	223, 208	oxidation (CH_3) to acid (COOH)			+
566[f]	223	dioxidation			
596.8	569, 537, 209	loss of cyclohexyl ring, oxidation, glutamine conjugation AND/OR loss of NH_3, sulfonation	596.8/568.7	<0.0001	+
598.8	570.7, 538.9	tri-demethylation and taurine conjugation	598.8/570.7	<0.0001	
610.8	551, 533, 249	*Unknown*	610.8/532.9	<0.0001	
611.9[g]	594, 534	loss of H_2O, oxidation, and sulfonation AND/OR di-keto, tri-oxidation	611.9/533.9	0.0001	
615[f]	551, 175	sulfonation			
802.9	363, 292, 226, 207	loss of cyclohexyl ring and bis-glucuronidation	802.9/363	0.0002	
816.9	562, 256	loss of cycohexyl ring, dioxidation, CO, and glutathione conjugation	816.9 / 256.1	0.0008	
824.7[g]	726, 563	loss of NH_3 and glutathione conjugation	824.7 / 563.0	0.02	
841[f]	823, 223	glutathione conjugation			

[a]Identified using enhanced mass, independent data acquisition, and enhanced product ion scan (EMS IDA EPI).
[b]Proposed biotransformation obtained using Lightsight version 2.0.
[c]Transitions obtained from EMS IDA EPI method and then used in multiple reaction monitoring method (MRM) to obtain peak areas for statistical analysis.
[d]Values calculated using peak areas obtained from transition in Q1/Q3 column, one way analysis of variance, and Bonferroni's post test.
[e]Possible epimers indicated with a "+" because of matching retention times to ergovaline and its epimer.
[f]Not enough data to compare all groups.
[g]Not enough data for group III (control)
[h]Only identified in group III and very little in group I

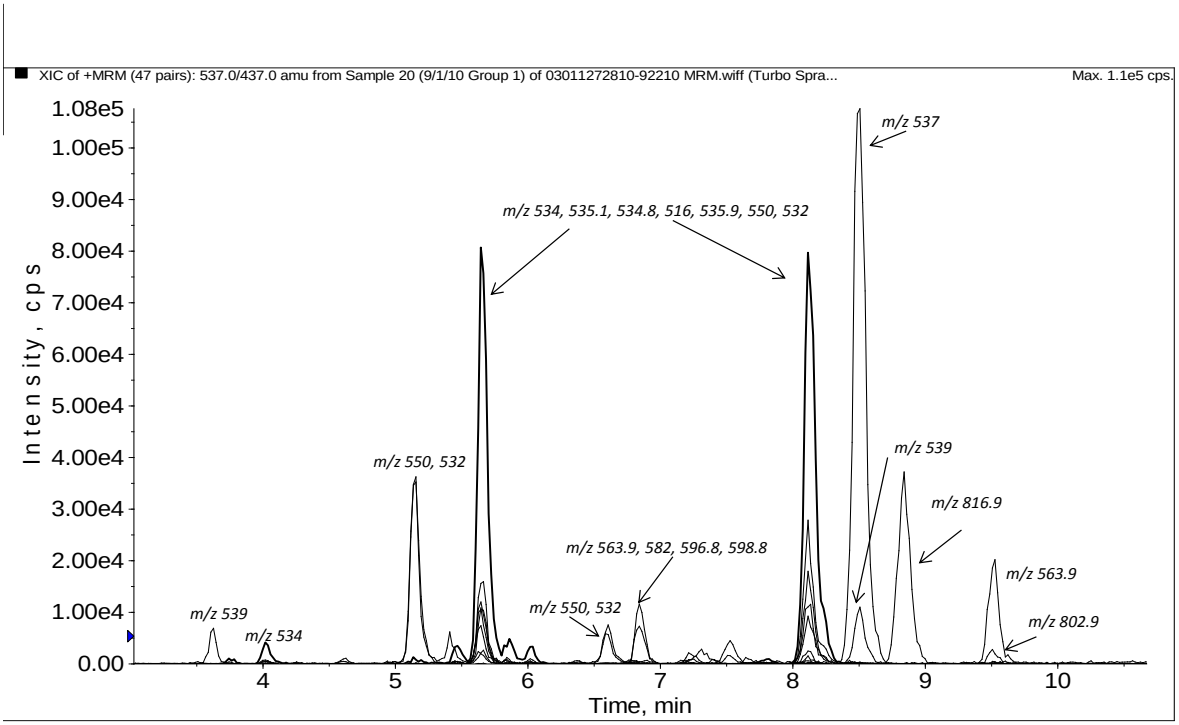


Figure 4.5 Shows a sample MRM developed for EV metabolites using the MS/MS data obtained in the EMS IDA EPI method.

Chapter 5. DISCUSSION

5.1 Animals

The results of the clinical trial showed that both groups I and II developed clinical signs of ryegrass staggers during the course of the 56 day feeding experiment. This is in contrast with previous findings that LB at a dose of 1500ppb does not cause ryegrass staggers [59]. The possible factors influencing this outcome could be age, time of year, and rumen microbiota.

5.2 Feed intake and weight gain

Ergot alkaloids have been associated with decreased feed intake and average daily weight gain [60]. In this study the average weight gain of group I compared to group III and IV did show small statistically significant differences. Alkaloids from both PRG and tall fescue have been studied in relation to their effect on weight gain. Some studies have found that weight gain and feed intake do not differ with varying alkaloid concentrations. A study done during winter months did conclude that weight gain and feed intake did not differ [61]. The results in this study showed from the feed intake monitoring all four groups did show differences in feed intake; however, there was no significant difference between the feed intake of group I and group IV, high and control respectively.

5.3 Coccidia Tests

The results of the unplanned coccida fecal flotation tests are intriguing and suggest a need for future studies designed to look specifically at this effect. Group I

(2256 ppb LB/ 638 ppb EV) showed no oocysts while group IV, the group consuming the least amount of LB and EV showed the most oocysts per gram of feces. Thus, it appears that increased alkaloid concentration in the feed may actually inhibits growth of coccidia. Coccidia is a common problem in young calves which also associated with diarrhea and reduced weight gain [62]. Calves that are more at risk usually become infected with coccidia parasites after being grouped together or moved into a contaminated environment[63]. The decreased weight gain seen in our study could have been complicated by this factor. In Girgis, *et. al.*, (2010) effects of *Fusarium* mycotoxins in were studied with a coccidia challenge given initially and at 4 weeks of age after receiving between 0.5-6.5 µg/g of deoxynivalenol (DON), 15-acetyl-DON and zearalenone which were present in a naturally contaminated chicken feed. This study showed naturally contaminated feed did have the potential to modulate the immune system response to coccidial infections. [64-68]. The potential for EV or LB to act as anti-protozoa compounds has not been explored.

5.4 HPLC-Fluorescence Feed and Feces Data

Investigation into a linear relationship between LB and EV in PRG was repeated according to Hovermale *et. al.* (2001). Briefly, a linear regression and Pearson tests were performed resulting in an R^2 value and Pearson coefficients, Groups I, II, III showed R^2/Pearson coefficients of values 0.7965/0.834, 0.4213/0.902, and 0.3731/0.776 respectively (group IV had no detectable EV and so was not analyzed). The R^2 values demonstrate that the linear relationship between LB and EV is strongest at high

concentrations. The R^2 value calculated previously was 0.7335; thus, both studies show a similar relationship between the toxins despite their being performed ten years apart [69]. The Pearson coefficients are all very close to +1 indicating a linear equation describes the relationship of EV and LB most accurately in PRG samples.

When examining the LB and EV linear relationship in feces, groups I, II, and III showed R^2/Pearson coefficients of 0.8193/0.834, 0.8727/0.902, and 0.6778/0.776, respectively. The Pearson coefficients appear to be similar to the PRG straw, but the R^2 values in the feces seem to be more consistent across dose groups. In summary, however, the linear relationship between the two toxins appears to carry over from feed to excretion. Fecal sample quantitation by HPLC-fluorescence did show similar patterns to the amount of either LB/EV in the feed; those fed the highest amount of alkaloids excreted the highest amount of LB/EV in the feces (Figure 4.1). Over the one week washout, LB levels in the feces dropped from 3000 ppb to 2000 ppb LB then quickly rebounded to 3000 ppb after return to 2256 ppb LB in the feed. EV levels dropped from 500 to 200 ppb and took one more week to regain previous levels in the feces (450 ppb). For fecal excretion of LB, group II did reach the same concentration as that seen in group I, which helps explain why staggers were observed in those two groups, while groups III and IV showed no clinical signs and feces LB levels remained below 2000 ppb for the duration of the experiment. Analysis of serum, urine, and fat samples from this study need to be completed; this will allow for a more extensive toxicokinetic evaluation of these two compounds. However, previous research suggests that feces are likely the primary route of excretion for EV, as it was found in the pictogram level in serum of

horses [70]. Measuring lysergic acid excretion in the urine will also help to determine if feces are the primary route of excretion [71].

5.5 Fecal analysis by LC-MS/MS

Metabolite analyses of the feces by mass spectrometry showed that the same types of products were excreted for both LB and EV. Specifically, *m/z* values that match potential metabolites of oxidation, deamination & oxidation, amide hydrolysis, hydrogenation, or demethylation & oxidation were detected. The only difference was no *m/z* value to indicate hydrogenation or dehydrogenation for LB was detected, only possible metabolites for EV. When both oxidation metabolites for EV and LB, m/z 550 and 702.4 respectively, were compared using a one way ANOVA analysis followed by Bonoferroni's posttest, m/z 550 showed $p<0.0001$ for ANOVA and $p < 0.05$ for post-test of all groups. The LB oxidation metabolite *m/z* 702.4 also showed overall $p < 0.0001$ for one way ANOVA. Both oxidation metabolites showed common fragments *m/z* 196 and 238 for LB, and *m/z* 223 and 208 for EV. A proposed route of formation of *m/z* 702.4 based on ruminant metabolism of 3-methylindole is shown in figure 5.1 and 5.2 [47]. Ergot alkaloids are not commonly associated with the upregulation of glutathione synthesis to help in detoxification, however 3-methylindole is known to form GSH conjugates as a method of detoxification. The role of glutathione in the detoxification of EV and LB should be examined further because of the stability proposed in 3-MI metabolism and the upregulation of glutathione-S- transferase in Tanaree, *et. al.* (2012). Glutathione could offer an explanation to why LB clinical signs are seen and not EV

when both toxins are consumed together. An ion *m/z* 841 was detected and fragmentation showed *m/z* 223, which could be a GSH conjugation of EV[43, 47]. Lehner *et al.* (2004) showed the common fragments of a number of ergot alkaloids and upon examination of current data that MS/MS spectra seen with the fragments *m/z* 223 and 208 are metabolites of ergovaline because the parent masses do not correlate to other known ergot alkaloids or mycotoxins [31, 58].

Peak areas obtained from MRM data was used to determine the linear relationship and Pearson Coefficients between EV and LB. Group I, II, III, and IV showed R^2/Pearson Coefficient values of 0.7641/0.874, 0.7563/0.870, 0.7607/0.872, and 0.7939/0.891 respectively. Not only is EV and LB being consumed at a linear relationship at high concentrations, it is being excreted in the feces with a stronger linear relationship. Since all of the LB metabolites showed fragments of *m/z* 238 or 196 we conclude that metabolism is happening most likely at rings 3, 4, and 5 on the lolitrem B structure in figure 2.2. The MS/MS obtained for the metabolites *m/z* 516, 532, 536, 535, 564, and 566 suggests that metabolism is most likely occurring on rings 5,6, and 7 of the structure shown in figure 2.1. This is shown by the common fragments of 223 and 208 being retained in most cases. This was also proposed in Lehner *et al.* (2004) for a dehydrated metabolite of ergocrystine where the moiety responsible for *m/z* 223, and 208 is retained during metabolism and the hydroxyl group between rings 5 and 6 is removed. [57]

The oxidation and reduction metabolites identified in the feces do correspond with previous findings of ergot alkaloid metabolism which concluded that cytochrome P450 enzymes are up regulated and genes associated with anti-oxidant pathways are

down regulated [43, 72-75] . Several ergot alkaloids used in human medicine are known CYP3A4 substrates and drug-drug interactions can be expected [76]. To our knowledge there is no data relating LB metabolism and upregulation of CYP450 enzymes except for one unpublished experiment conducted by this laboratory which found that genes associated with oxidation and reduction are up-regulated which corresponds to the potential metabolites identified previously. Lanza, *et al.* (2001) did provide a detailed experiment identifying the certain CYP 450 enzymes responsible for the metabolism of 3-MI. Based on this information, figure 5.3 shows a proposed metabolism pathway of LB based on the known CYP 450 metabolism of 3-MI. This proposed pathway again provides more possible routes to form *m/z* 702.4. CYP 2E1 and 3A4 were very selective in the metabolism of 3-MI and was also proposed as a route of detoxification for ergot alkaloids [43, 48]. Interestingly, dehydrogenation has been proposed as a route of metabolism for 3-MI yet we did not find anything that suggested dehydration for LB. Conversely, possible dehydration and dehydrogenation metabolites were identified in EV samples. Since CYP 2F1 and 2F3 were found in Lanza *et al.* (2001) to selectively metabolize 3-MI to the dehydrogenation metabolite, an *in vitro* study comparing the EV and LB metabolism would help understand possible competitive inhibition. This provides a new hypothesis that EV disease symptoms are not seen due to competitive inhibition through CYP2E1, 2F1, 2F3, and/or 3A4.

One detail that should be noted is that the LB metabolites identified also correspond to *m/z* values of other lolitrems [37]. The MS/MS generated fragmentation patterns for these lolitrems were not accessible to verify the identity of the peaks.

However, the statistical differences seen in the LC-MS/MS data matches that of the HPLC-fluorescence feed concentrations of LB and EV. Additionally, the two peaks identified as *m/z* 688.4 showed statistically significant peak areas across all groups and suggests that these ions are metabolites of LB. The ion with *m/z* 688.4 eluted at two different times proposed structures are shown in Appendix E and MS/MS is shown in Appendix A.

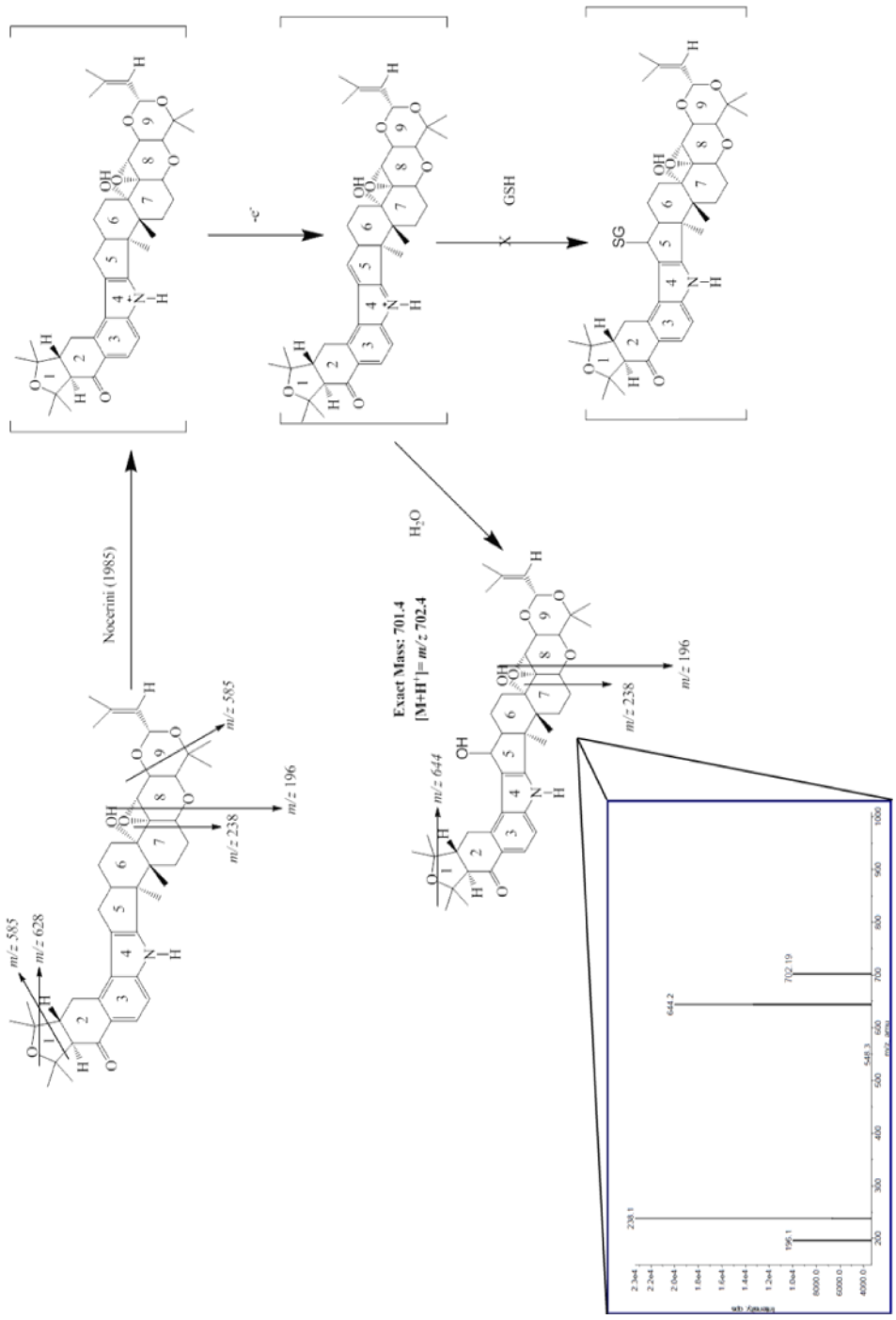


Figure 5.1. Proposed lolitrem B metabolism in bovine species also consuming ergovaline based on the previously reported metabolism of 3-MI in ruminants in Noccerini (1985).

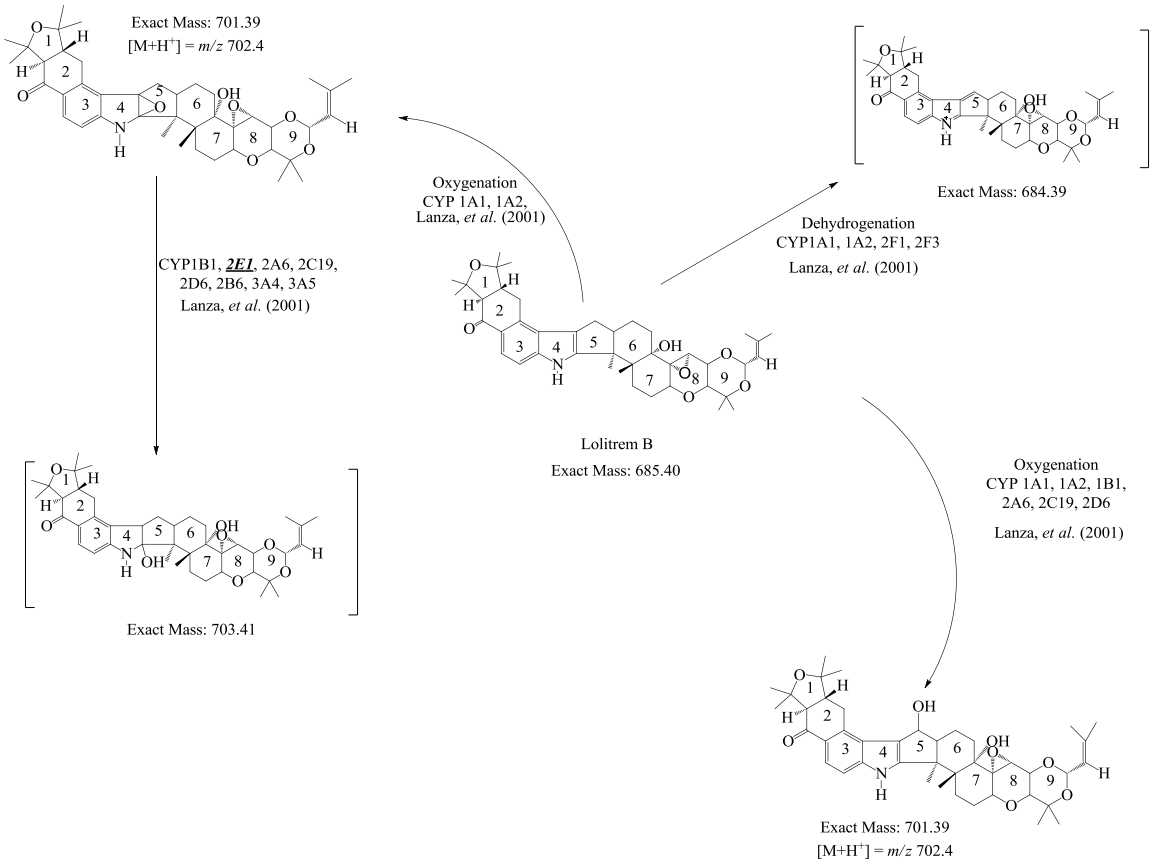


Figure 5.2. Proposed metabolism by CYP 450 enzymes based on the 3-MI metabolites in Lanza, *et al* (2001). Brackets represent molecules that were not detected.

5.6. CONCLUSION

The findings in this study supported the findings in Hovermale *et.al.*,(2001) and also showed that EV and LB are excreted in the feces in a linear relationship. From the LC-MS/MS data we can improve the understanding of LB. Data in this study suggest that LB is most likely metabolized by CYP450 enzymes. The idea that these two toxins are similarly metabolized leads to the need for further studies specifically those using enzymes. Genomic and microsomal assays would help to confirm the up regulation of CYP450 enzymes while metabolomics studies would help understand what anti-oxidant

pathways are being activated. More research is needed to determine the clinical disease impact that results from ingesting these two compounds together. Mixtures of mycotoxins are only beginning to be studied and understanding the synergistic effects will greatly influence how disease from these two toxins are treated in clinical cases.

Additionally, high resolution mass spectrometry would also help to confirm these metabolites.

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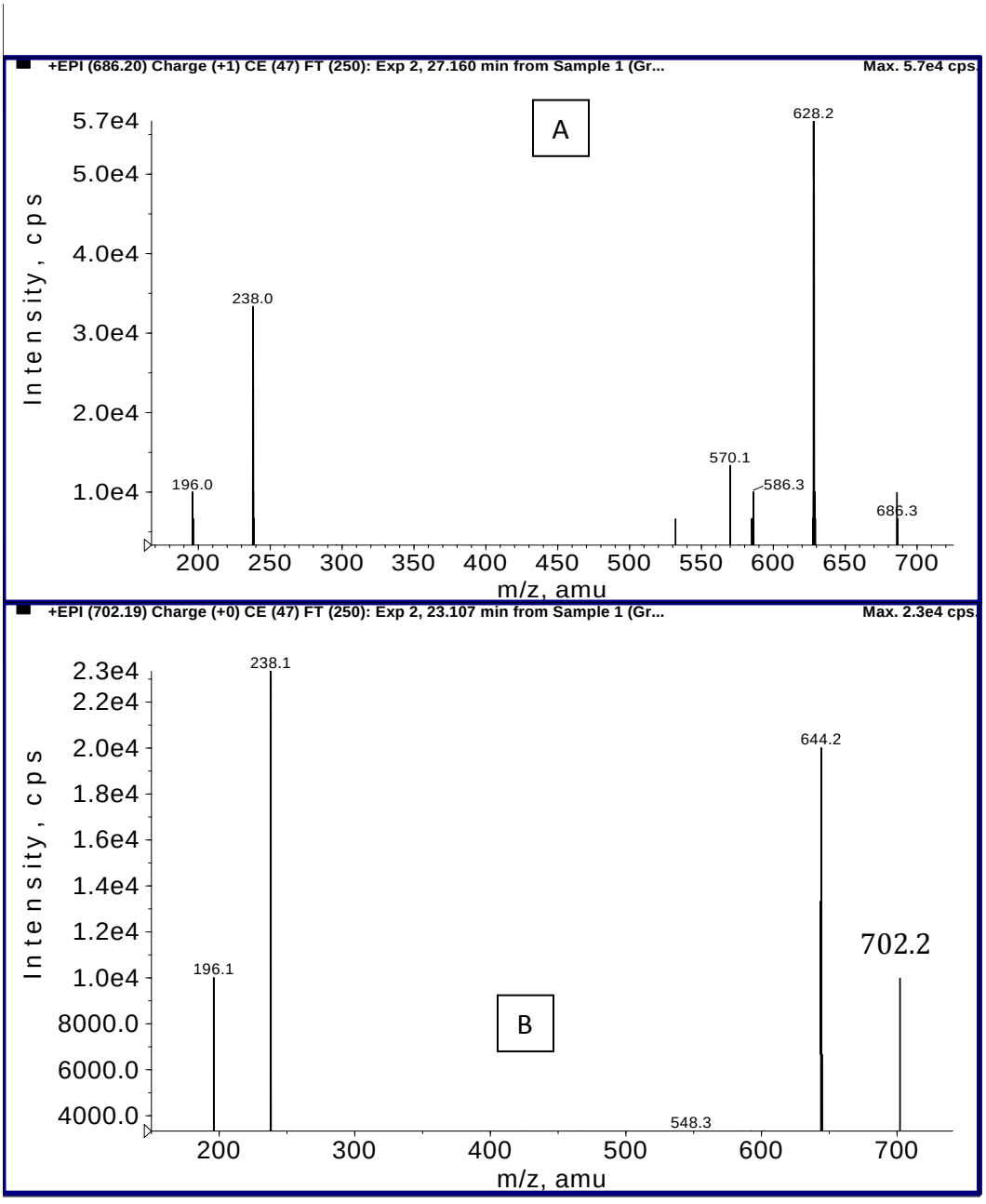
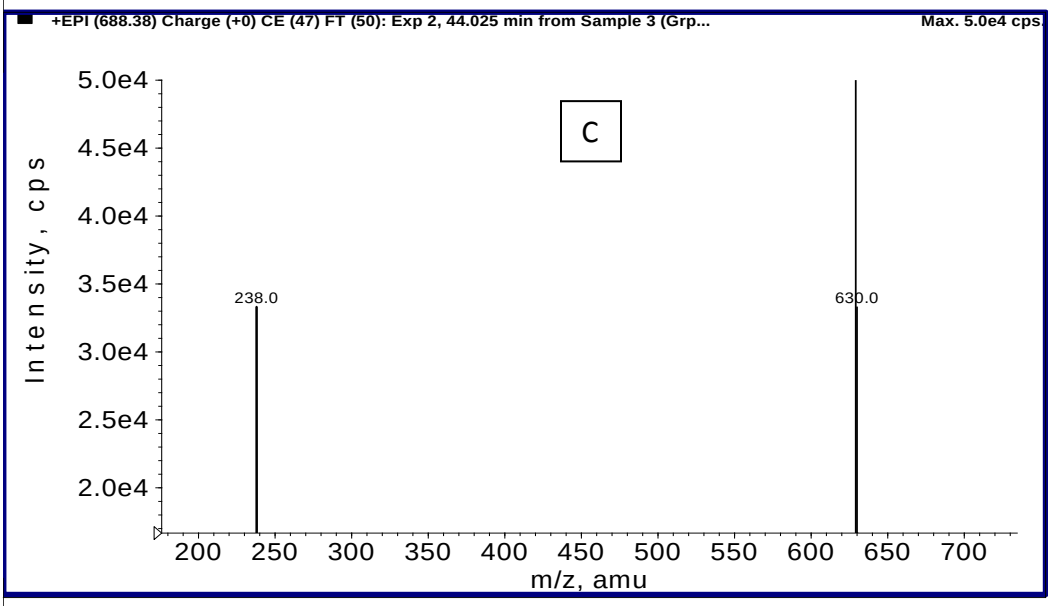
Appendices

Appendix A. **MS/MS of possible lolitrem B metabolites.** MS/MS of potential LB metabolites identified through EMS IDA EPI method using APCI (+).(A) Shows LB identified in feces and (B) Shows possible oxidation metabolite of *m/z* 702.2 (C) Shows the possible hydrogenation metabolite and/or demethylation and oxidation metabolite. (D) Shows the possible deamination and reduction metabolite of *m/z* 688.4.

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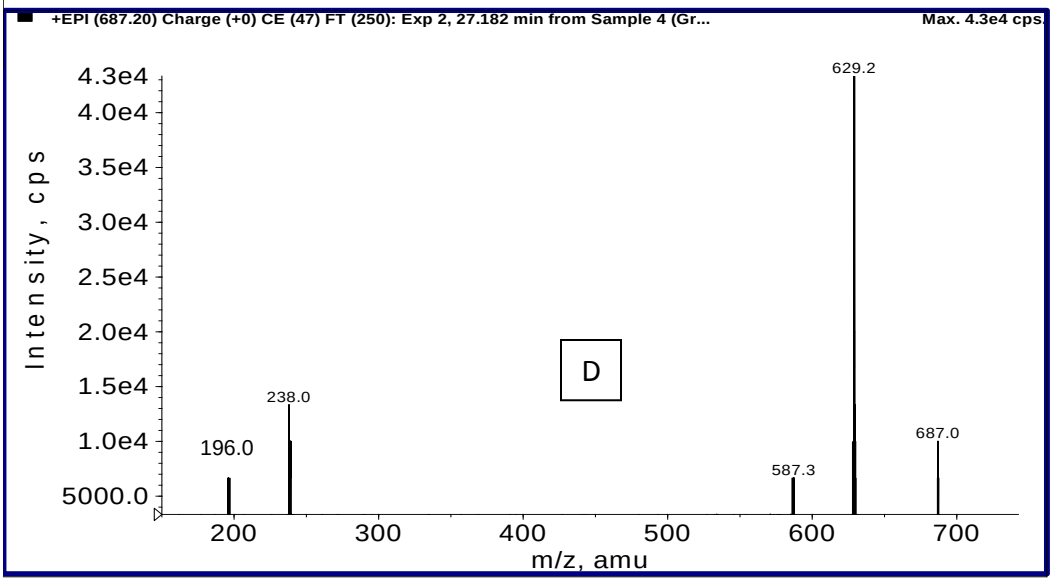
Max. 5.0e4 cps.
Intensity, cps
5.0e4
4.5e4
4.0e4
3.5e4
3.0e4
2.5e4
2.0e4
C
238.0
630.0
200 250 300 350 400 450 500 550 600 650 700
m/z, amu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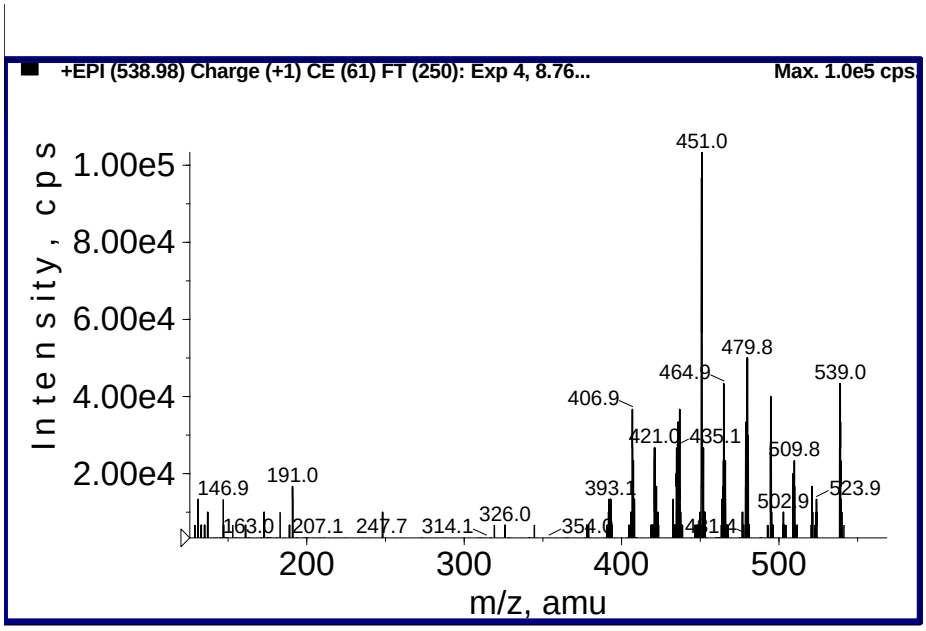
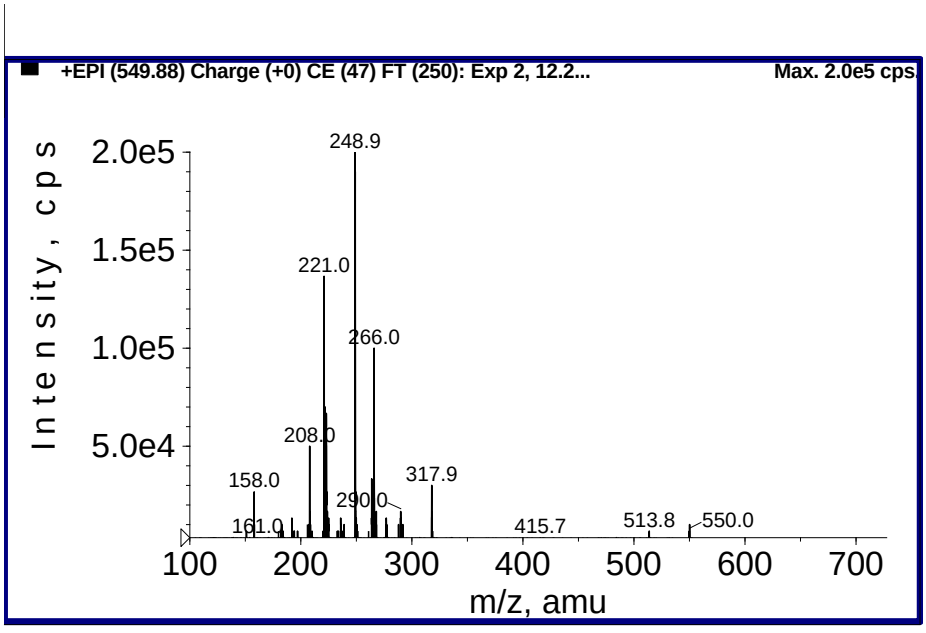
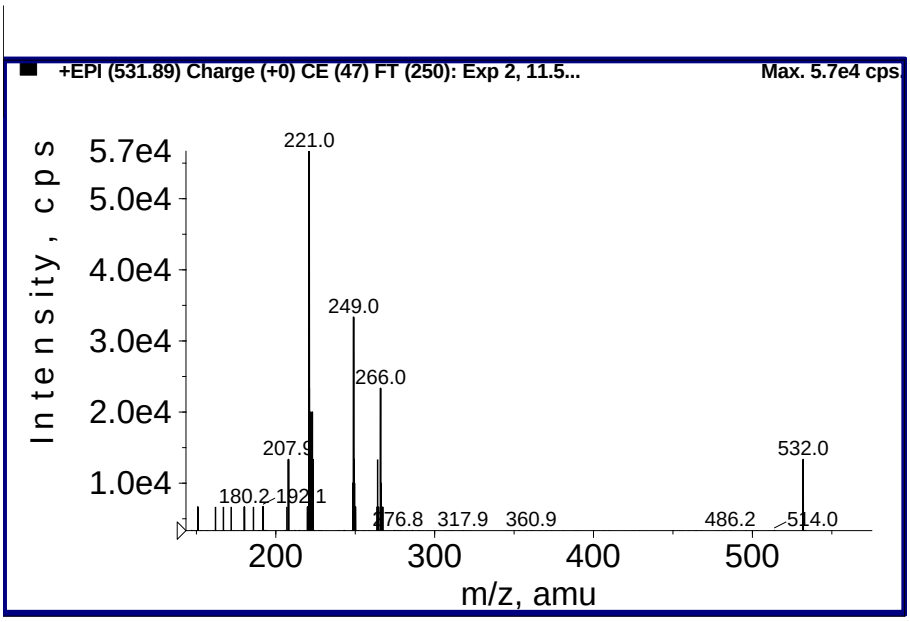
Max. 4.3e4 cps.
Intensity, cps
4.3e4
4.0e4
3.5e4
3.0e4
2.5e4
2.0e4
1.5e4
1.0e4
5000.0
D
629.2
238.0
196.0
687.0
587.3
200 300 400 500 600 700
m/z, amu

Appendix B. **MS/MS of possible ergovaline metabolites.** Shows the MS/MS of potential EV metabolites identified through EMS IDA EPI method using ESI (+).



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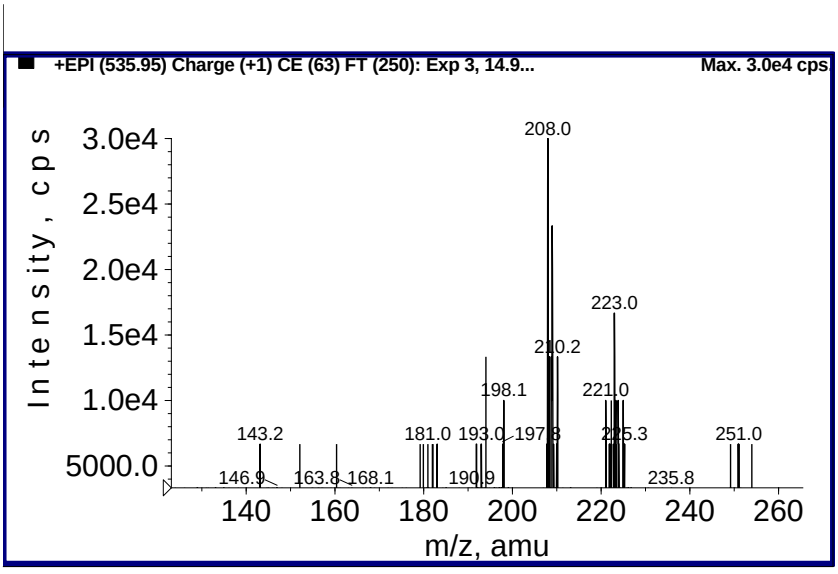
Max. 5.7e4 cps
Intensity, cps
5.7e4
5.0e4
4.0e4
3.0e4
2.0e4
1.0e4
221.0
249.0
266.0
207.9
180.2
192.1
176.8
317.9
360.9
486.2
514.0
532.0
200
300
400
500
m/z, amu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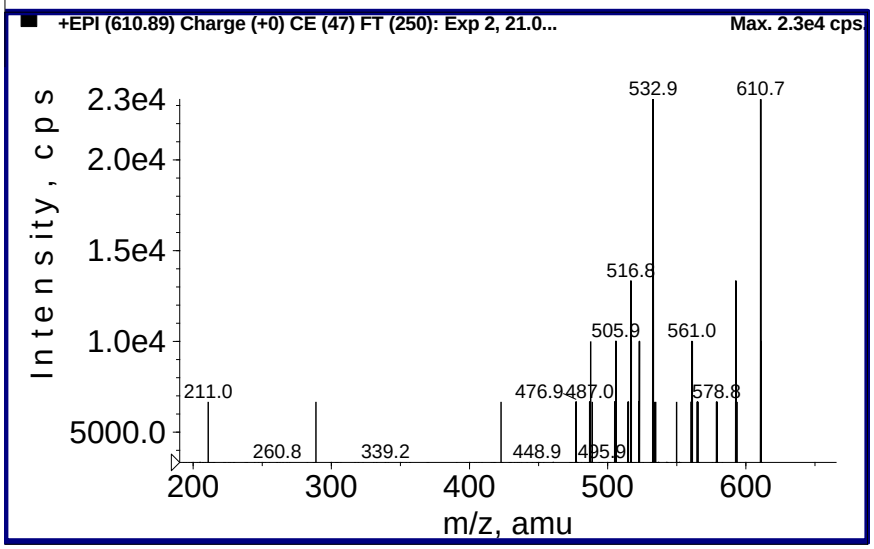
Max. 3.0e4 cps
Intensity, cps
3.0e4
2.5e4
2.0e4
1.5e4
1.0e4
5000.0
208.0
223.0
210.2
198.1
221.0
143.2
181.0
193.0
197.8
225.3
251.0
146.9
163.8
168.1
190.9
235.8
140
160
180
200
220
240
260
m/z, amu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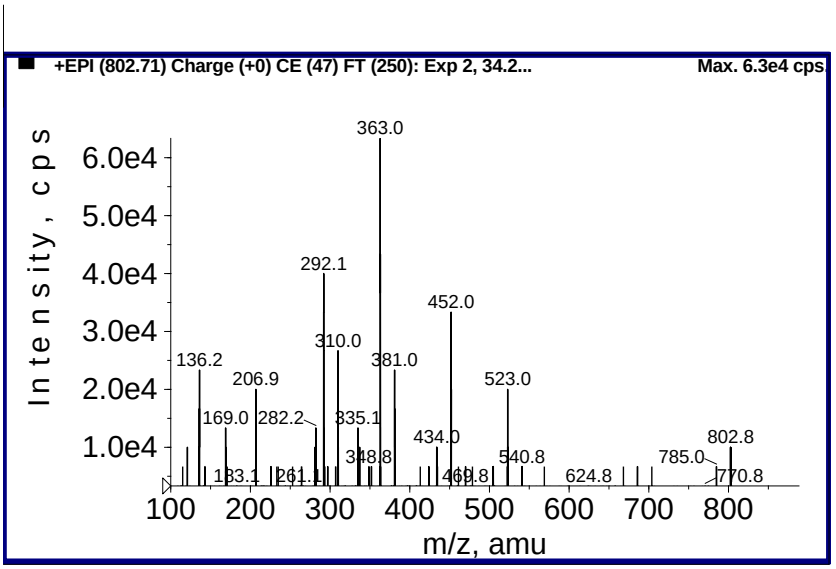
Max. 2.3e4 cps.
Intensity, cps
2.3e4
2.0e4
1.5e4
1.0e4
5000.0
532.9
610.7
516.8
505.9
561.0
211.0
476.9
487.0
578.8
260.8
339.2
448.9
495.9
200
300
400
500
600
m/z, amu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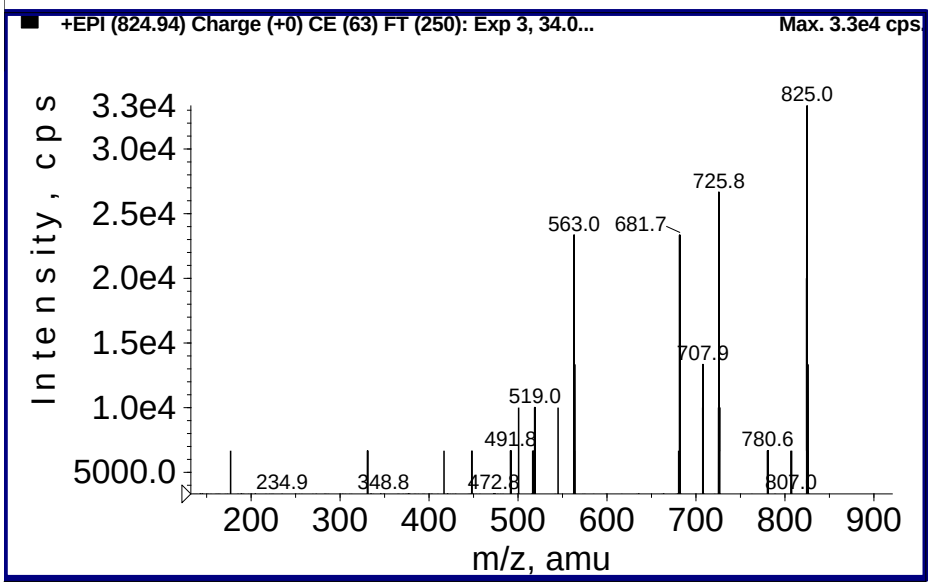
Intensity, cps
6.0e4
5.0e4
4.0e4
3.0e4
2.0e4
1.0e4
363.0
292.1
452.0
310.0
136.2
381.0
206.9
523.0
169.0
282.2
335.1
434.0
802.8
348.8
540.8
785.0
133.1
624.8
770.8
100
200
300
400
500
600
700
800
m/z, amu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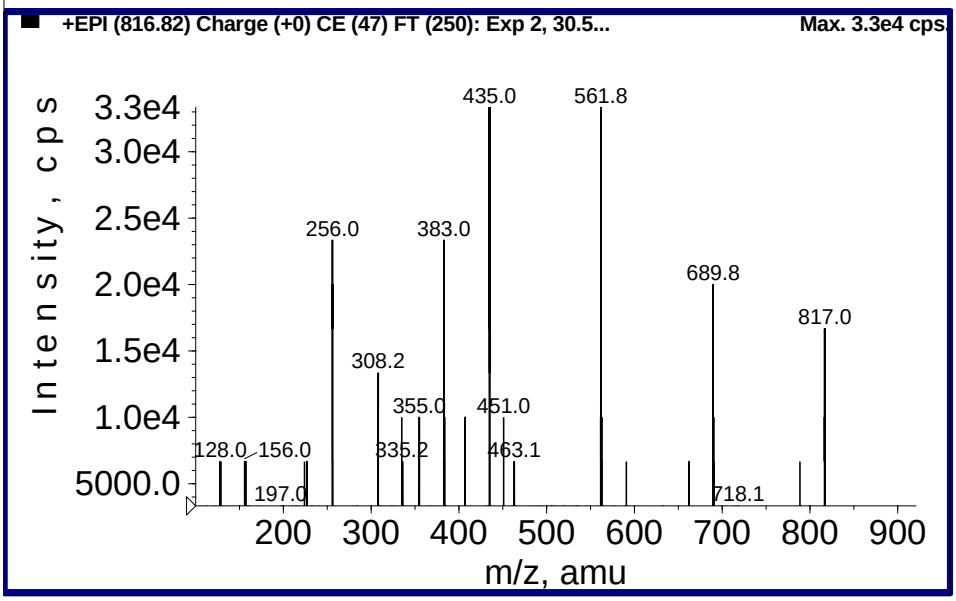
Max. 3.3e4 cps.
Intensity, cps
3.3e4
3.0e4
2.5e4
2.0e4
1.5e4
1.0e4
5000.0
825.0
725.8
563.0
681.7
707.9
519.0
491.8
780.6
234.9
348.8
472.8
807.0
200
300
400
500
600
700
800
900
m/z, amu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Intensity, cps
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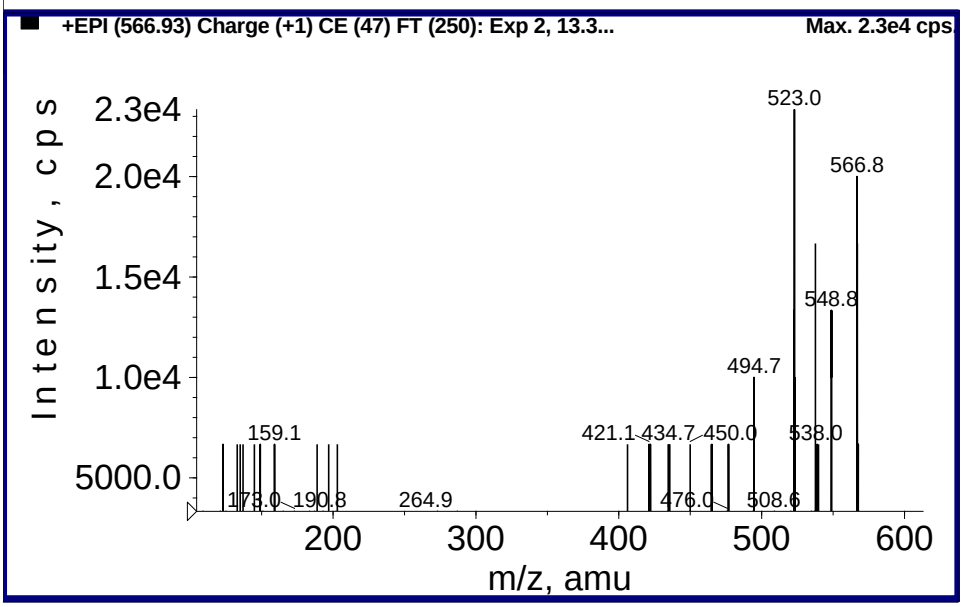
3.0e4
2.5e4
2.0e4
1.5e4
1.0e4
5000.0
435.0
561.8
256.0
383.0
689.8
817.0
308.2
355.0
451.0
128.0
156.0
335.2
463.1
197.0
718.1
200
300
400
500
600
700
800
900
m/z, amu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Intensity, cps
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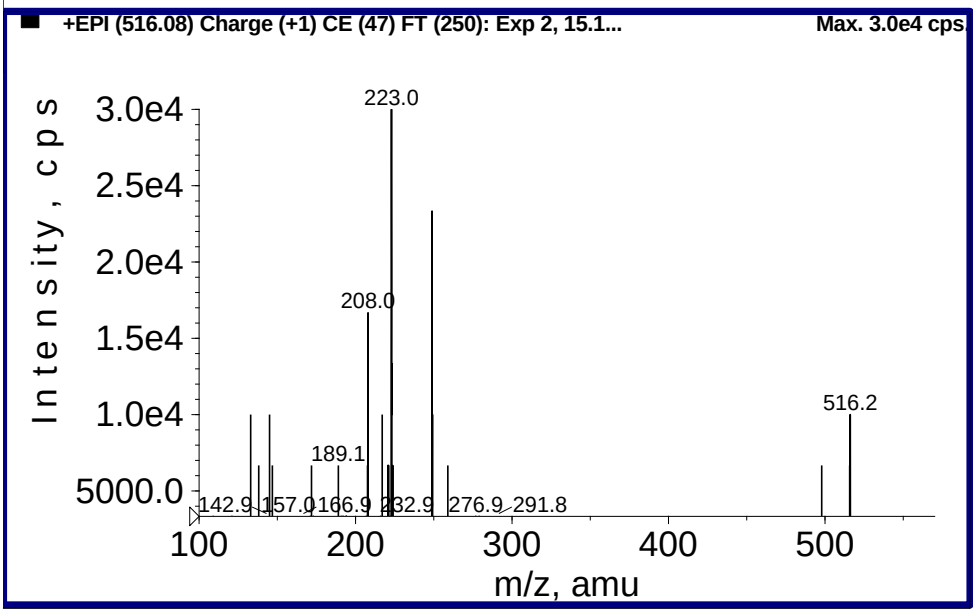
2.0e4
1.5e4
1.0e4
5000.0
523.0
566.8
548.8
494.7
159.1
421.1
434.7
450.0
538.0
173.0
190.8
264.9
476.0
508.6
200
300
400
500
600
m/z, amu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Intensity, cps
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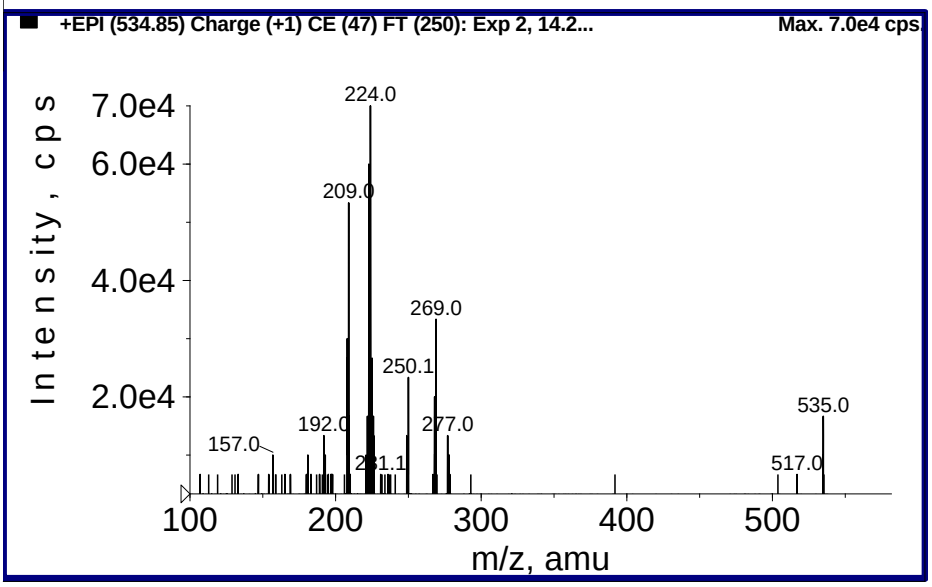
2.5e4
2.0e4
1.5e4
1.0e4
5000.0
223.0
208.0
516.2
189.1
142.9
157.0
166.9
232.9
276.9
291.8
100
200
300
400
500
m/z, amu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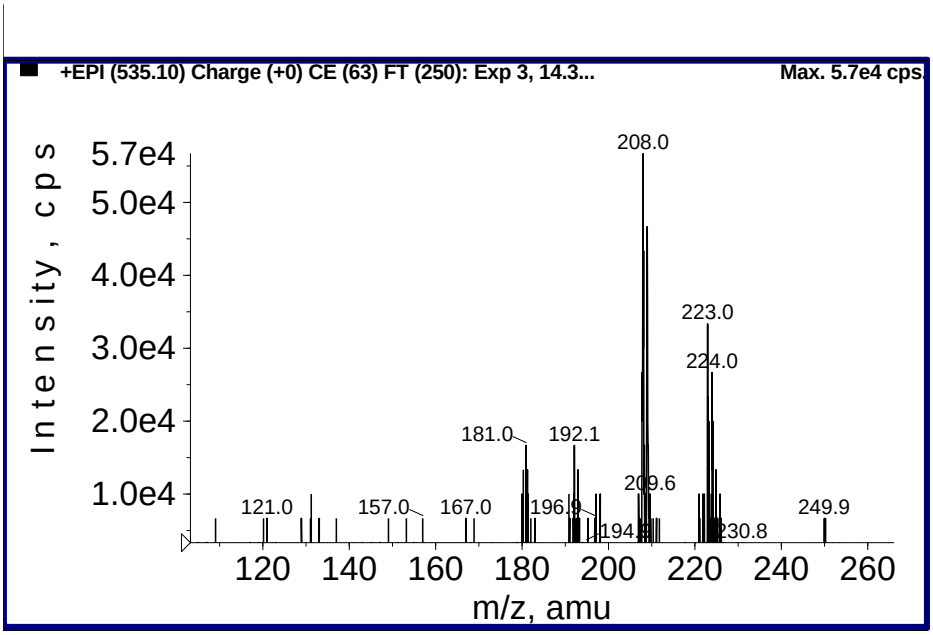
Intensity, cps
7.0e4
6.0e4
4.0e4
2.0e4
224.0
209.0
269.0
250.1
535.0
192.0
277.0
157.0
231.1
517.0
100
200
300
400
500
m/z, amu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Max. 5.7e4 cps.
Intensity, cps
5.7e4
5.0e4
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3.0e4
2.0e4
1.0e4
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224.0
181.0
192.1
209.6
121.0
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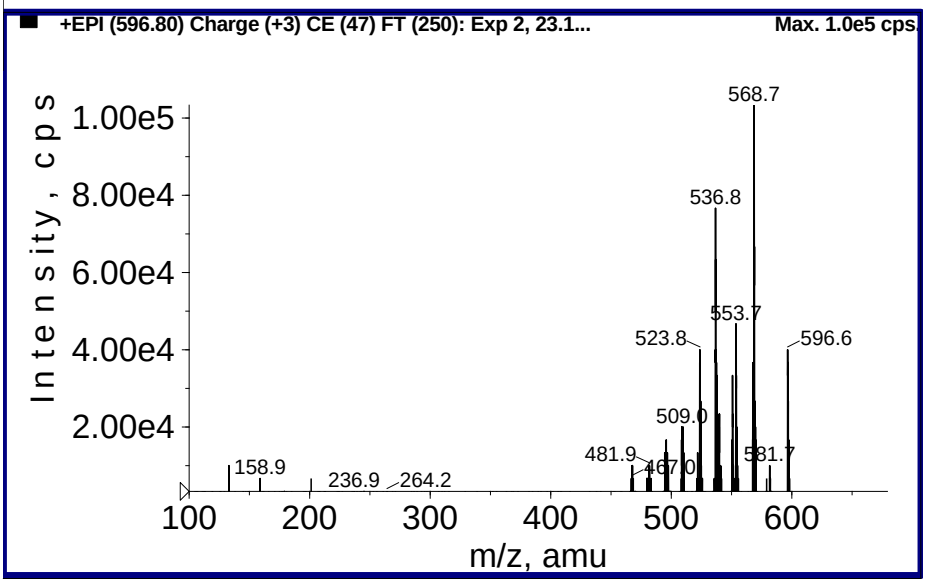
167.0
196.9
249.9
120
140
160
180
200
220
240
260
m/z, amu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Intensity, cps
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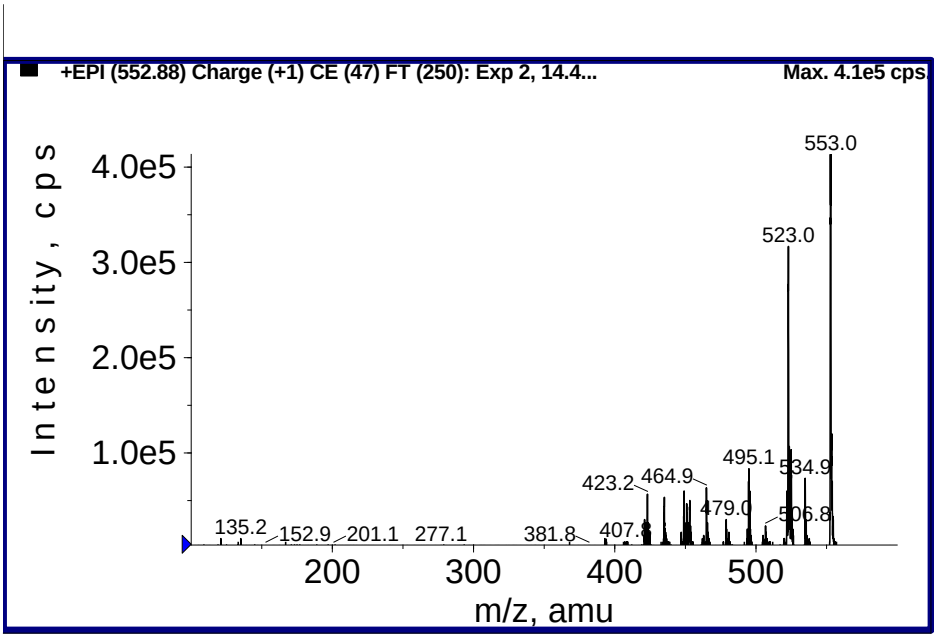
8.00e4
6.00e4
4.00e4
2.00e4
568.7
536.8
553.7
523.8
596.6
509.0
481.9
467.0
581.7
158.9
236.9
264.2
100
200
300
400
500
600
m/z, amu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Intensity, cps
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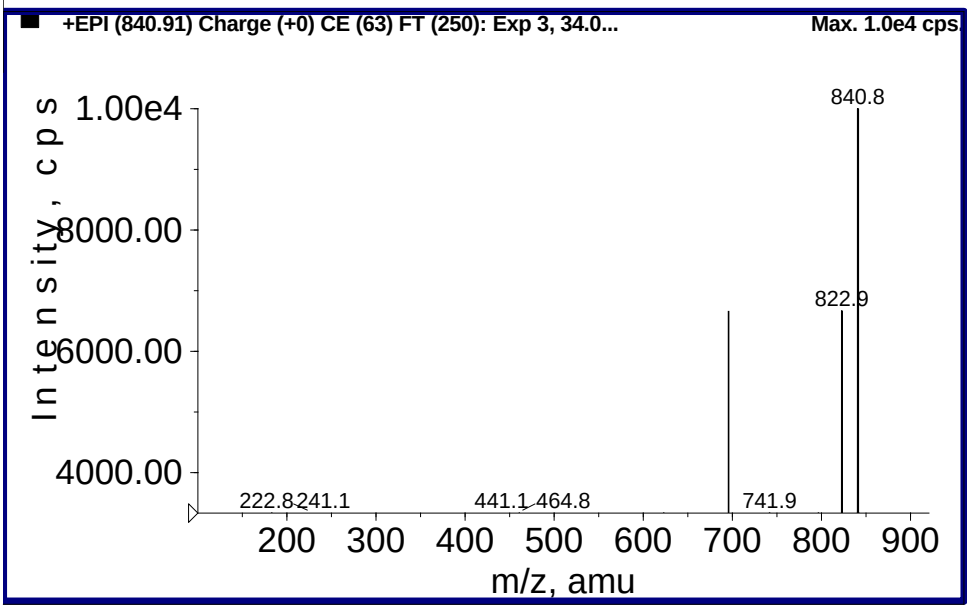
3.0e5
2.0e5
1.0e5
553.0
523.0
495.1
534.9
423.2
464.9
479.0
506.8
135.2
152.9
201.1
277.1
381.8
407.4
200
300
400
500
m/z, amu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Intensity, cps
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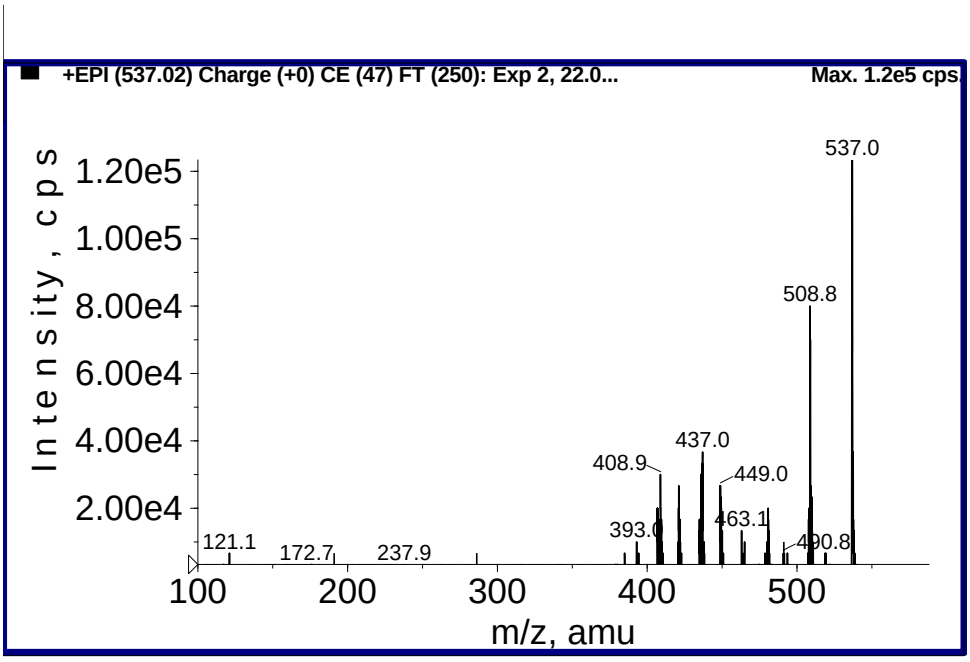
8000.00
6000.00
4000.00
840.8
822.9
222.8
241.1
441.1
464.8
741.9
200
300
400
500
600
700
800
900
m/z, amu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Intensity, cps
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8.00e4
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4.00e4
2.00e4
537.0
508.8
437.0
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237.9
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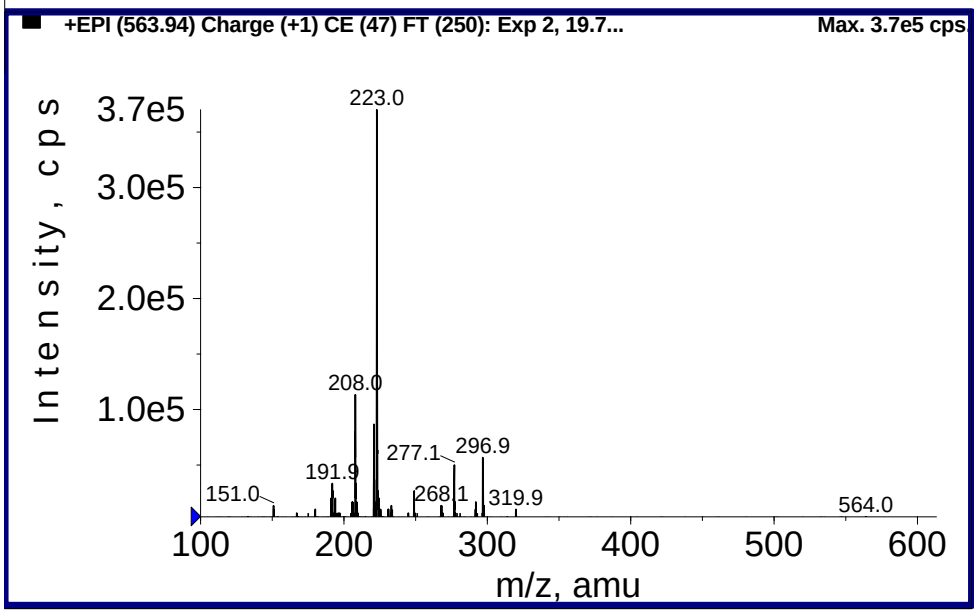
m/z, amu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Intensity, cps
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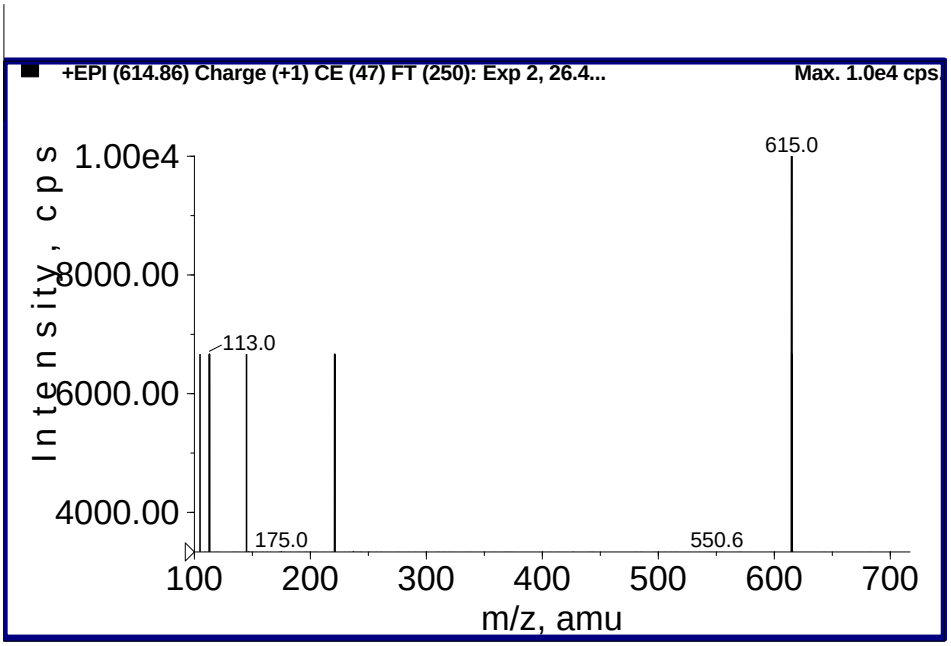
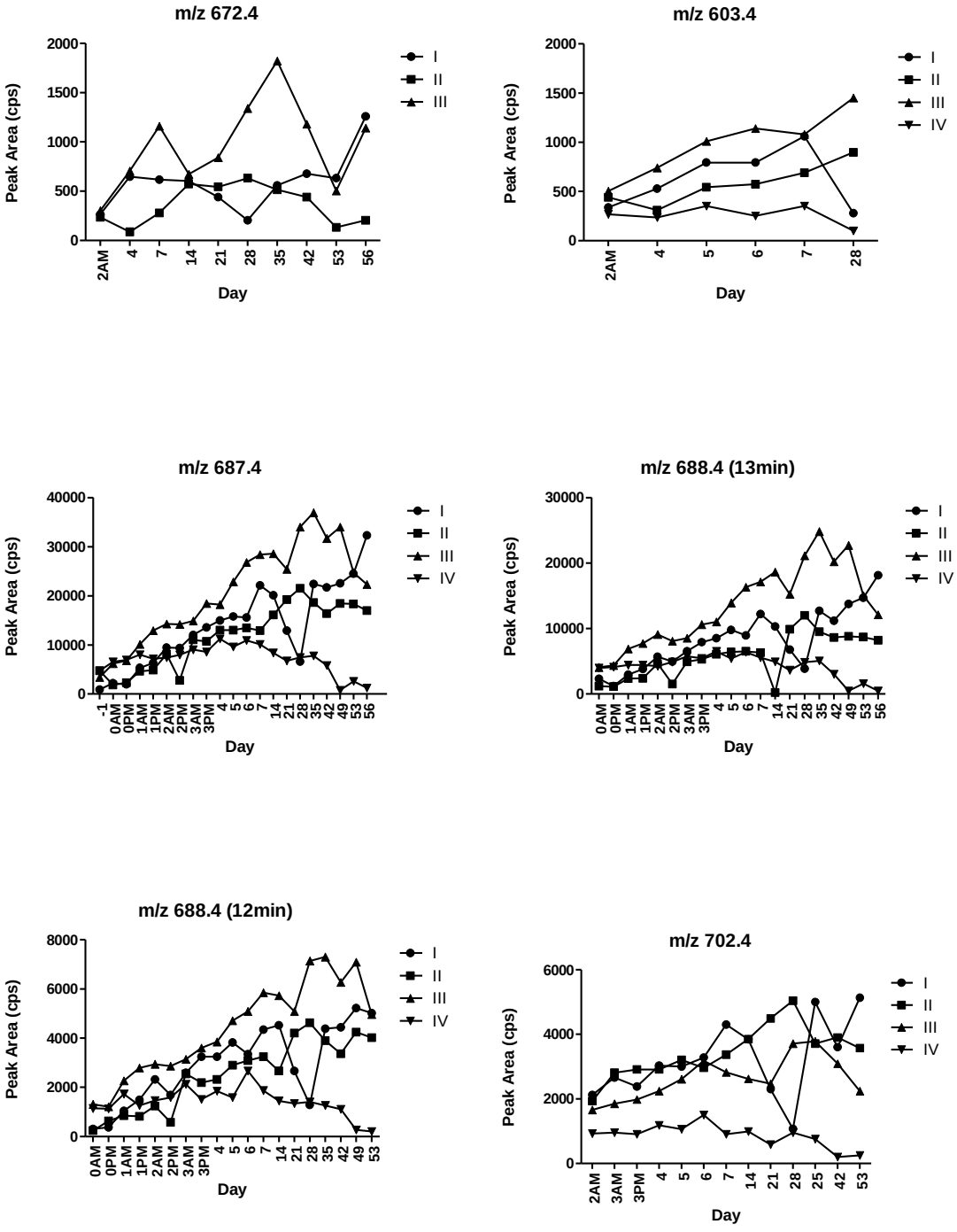
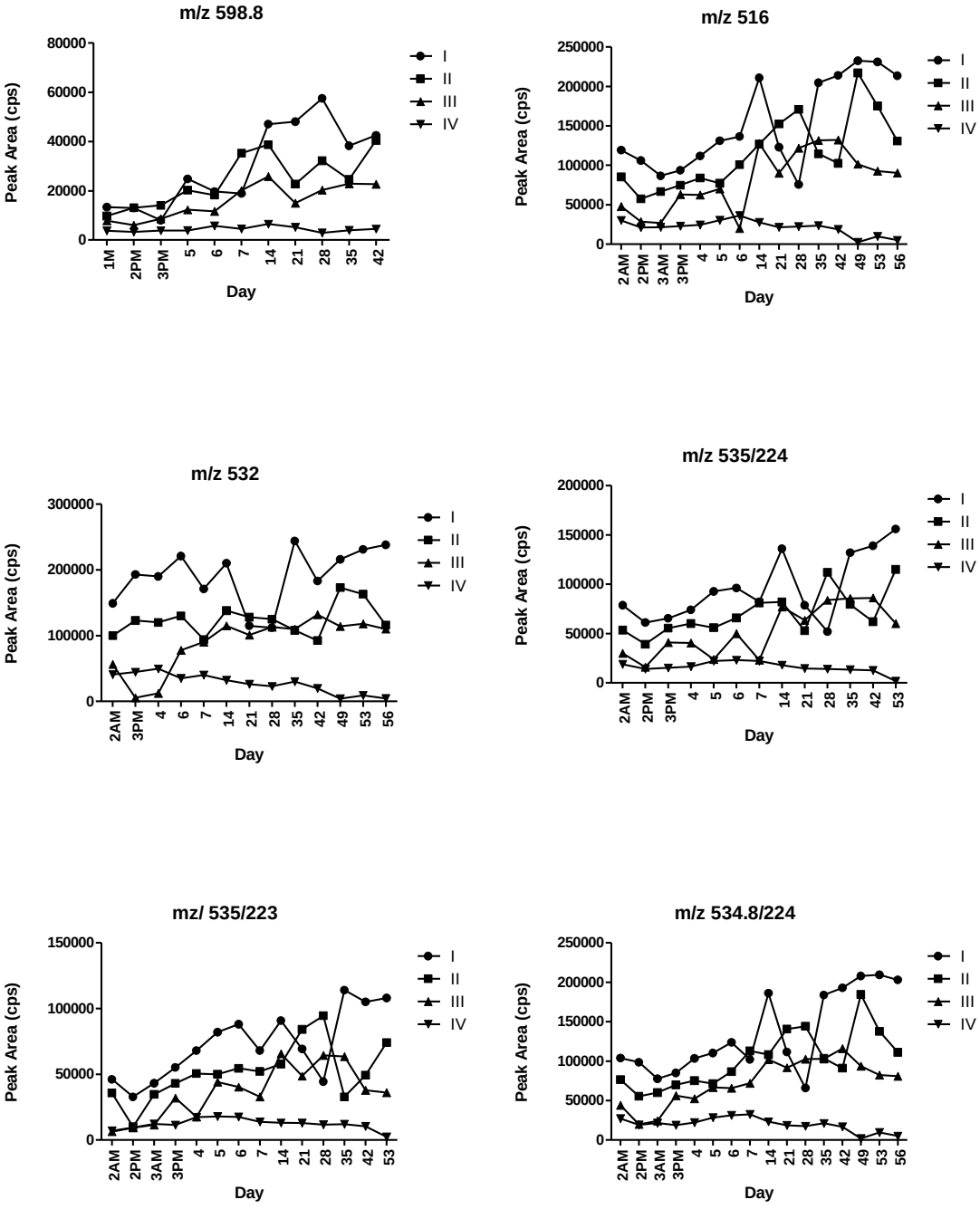
3.0e5
2.0e5
1.0e5
223.0
208.0
277.1
296.9
191.9
151.0
268.1
319.9
564.0
100
200
300
400
500
600
m/z, amu

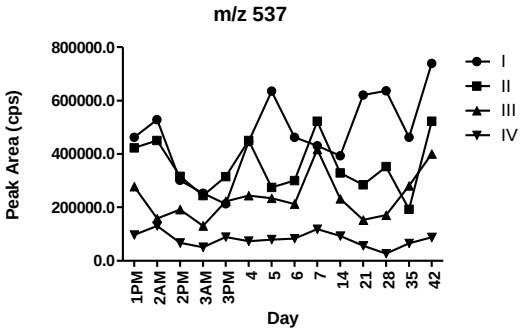

+EPI (614.86) Charge (+1) CE (47) FT (250): Exp 2, 26.4...
Max. 1.0e4 cps
Intensity, cps
1.00e4
8000.00
6000.00
4000.00
615.0
113.0
175.0
550.6
100
200
300
400
500
600
700
m/z, amu

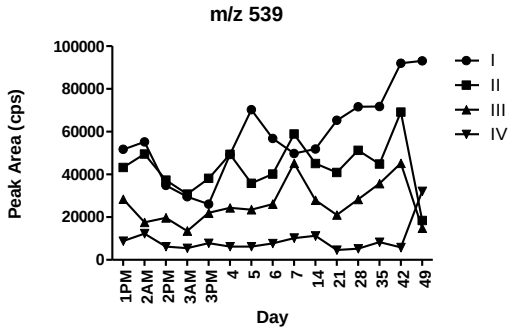
Appendix C. Semi-quantitative graphs of LB metabolites using MRM peak areas and Graphpad Prism

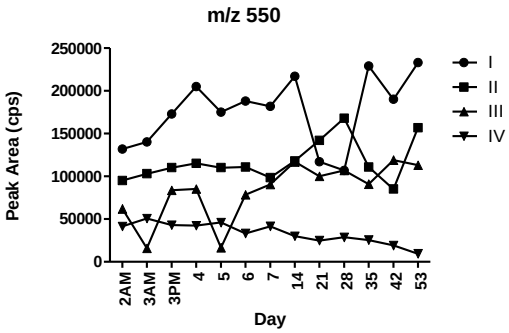


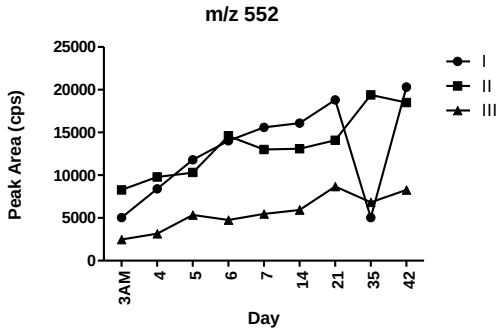
Appendix D. Semi-quantitative graphs of EV metabolites using MRM peak area and Graphpad Prism.

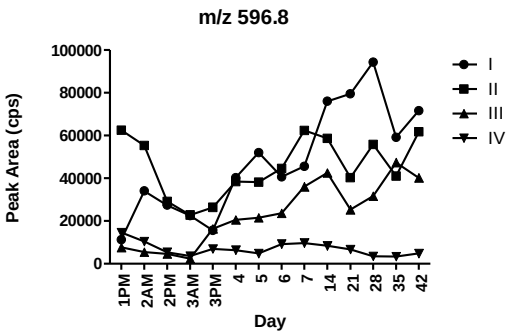


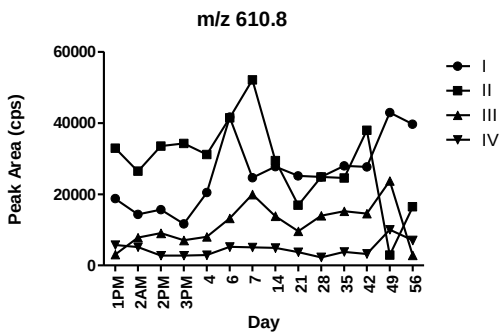

m/z 537
Peak Area (cps)
800000.0
600000.0
400000.0
200000.0
0.0
1PM
2AM
2PM
3AM
3PM
4
5
6
7
14
21
28
35
42
Day
I
II
III
IV

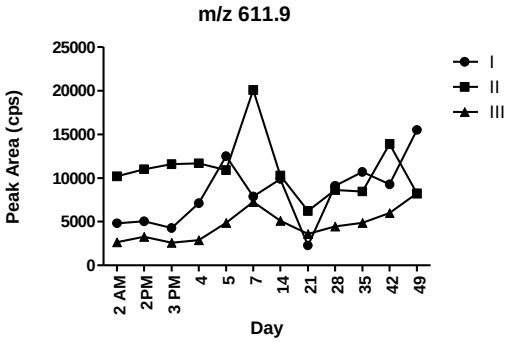

m/z 539
Peak Area (cps)
100000
80000
60000
40000
20000
0
1PM
2AM
2PM
3AM
3PM
4
5
6
7
14
21
28
35
42
49
Day
I
II
III
IV

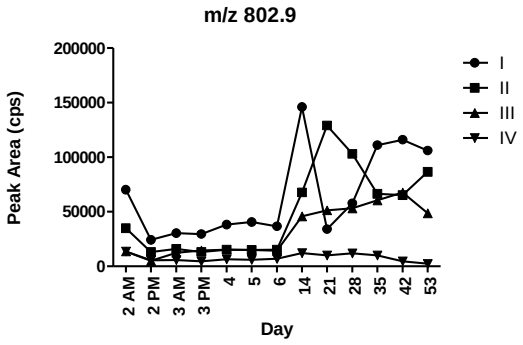

m/z 550
Peak Area (cps)
250000
200000
150000
100000
50000
0
2AM
3AM
3PM
4
5
6
7
14
21
28
35
42
53
Day
I
II
III
IV

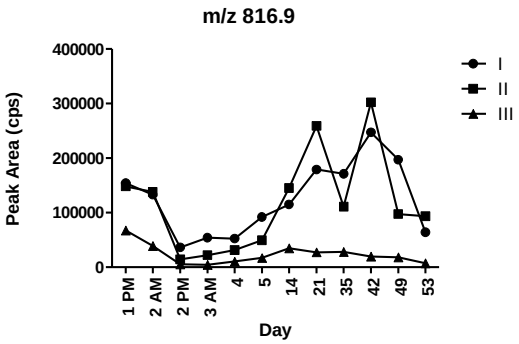

m/z 552
Peak Area (cps)
25000
20000
15000
10000
5000
0
3AM
4
5
6
7
14
21
35
42
Day
I
II
III

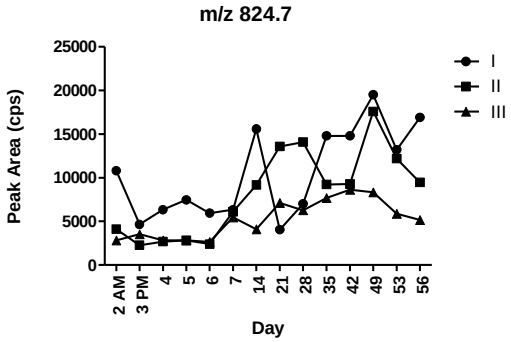

m/z 596.8
Peak Area (cps)
100000
80000
60000
40000
20000
0
1PM
2AM
2PM
3AM
3PM
4
5
6
7
14
21
28
35
42
Day
I
II
III
IV


m/z 610.8
Peak Area (cps)
60000
40000
20000
0
1PM
2AM
2PM
3PM
4
6
7
14
21
28
35
42
49
56
Day
I
II
III
IV

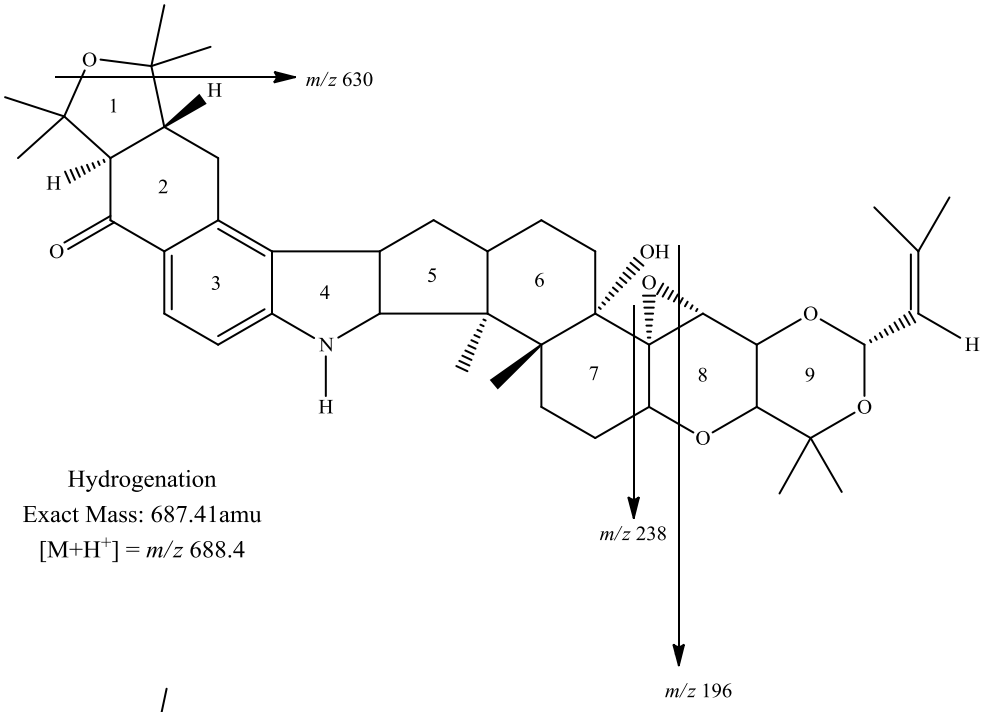

m/z 611.9
Peak Area (cps)
25000
20000
15000
10000
5000
0
2 AM
2PM
3 PM
4
5
7
14
21
28
35
42
49
Day
I
II
III


m/z 802.9
Peak Area (cps)
200000
150000
100000
50000
0
2 AM
2 PM
3 AM
3 PM
4
5
6
14
21
28
35
42
53
Day
I
II
III
IV

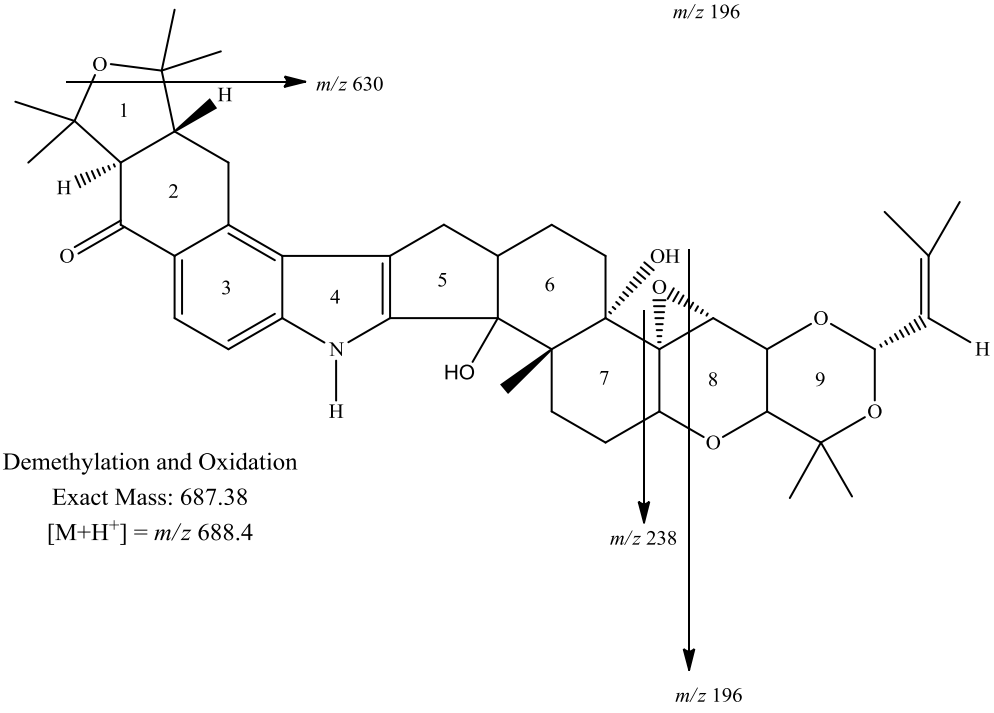

m/z 816.9
Peak Area (cps)
400000
300000
200000
100000
0
1 PM
2 AM
2 PM
3 AM
4
5
14
21
35
42
49
53
Day
I
II
III


m/z 824.7
Peak Area (cps)
25000
20000
15000
10000
5000
0
2 AM
3 PM
4
5
6
7
14
21
28
35
42
49
53
56
Day
I
II
III

Appendix E. Lolitrem B Metabolite Structures. Possible LB metabolite structures based on MS/MS and known fragmentation pattern of LB



Hydrogenation
Exact Mass: 687.41amu
$[M+H^+]$ = *m/z* 688.4



Demethylation and Oxidation
Exact Mass: 687.38
$[M+H^+]$ = *m/z* 688.4