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TALL FESCUE ERGOVALINE CONCENTRATION BASED ON SAMPLE HANDLING AND STORAGE METHOD

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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By

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Lexington, Kentucky

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ABSTRACT OF THESIS

TALL FESCUE ERGOVALINE CONCENTRATION BASED ON SAMPLE HANDLING AND STORAGE METHOD

Ergovaline is produced by the endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams) in tall fescue (*Schedonorus arundinacea* (Schreb.) Dumort. = *Festuca arundinacea* Schreb.) and is blamed for a multitude of costly livestock disorders. Testing of pastures is common in both research and on farm situations. Since ergovaline is known to be unstable and affected by many variables, the objective of this study was to determine the effect of sample handling and storage on the stability of this compound. Homogeneous milled tall fescue sub-samples were analyzed for ergovaline concentration using HPLC after a range of sample handling procedures or storage. Ergovaline was unstable in milled material after 24 hours in storage, regardless of temperature. The decrease in ergovaline after 24 hours ranged from 17 to 60%. These results show that tall fescue sample handling and storage have a significant effect on ergovaline concentrations. In conclusion, accurate laboratory analysis of ergovaline content may require that samples be transported immediately to the laboratory on ice for immediate analysis. Most laboratories are not equipped for same day analysis, therefore researchers and producers should acknowledge that laboratory ergovaline results may be lower than the actual content in the field.

KEYWORDS: tall fescue *Schedonorus arundinacea*, *Neotyphodium coenophialum*, ergot alkaloids and ergovaline, transportation and storage, HPLC with fluorescence detection

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March 4, 2014

TALL FESCUE ERGOVALINE CONCENTRATION BASED ON SAMPLE HANDLING AND STORAGE METHODS

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I dedicate my thesis to my wonderful mom and dad, sister Alex and husband Russell for their constant support of me and my pursuit of understanding. They have endured countless trips to zoos and aquariums, listened to endless factoids concerning every living creature on this planet, and have spent large sums of money on zoo camps, field trips, riding lessons and book and magazine collections. They have wandered the Grand Canyon looking for California Condors, sponsored manatees in Florida, waited hours in line to see a Giant Panda and have welcomed so many homeless creatures into our home (or garage, as in the case of the centipede).

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1.1 Tall Fescue

Tall fescue, *Schedonorus arundinacea* (Schreb.) Dumort. = *Festuca arundinacea* Schreb., is a cool season, perennial, bunch grass native to Europe. It has dark leaves with serrated edges, prominent veins and a panicle seed head (Ball et al., 2007a). In 1931, Dr. E.N. Fergus collected tall fescue plants from the Sutter family farm in Menifee County Kentucky that appeared to be exceptionally well adapted despite the current drought conditions. Dr. Fergus improved on the plants through selective breeding and in 1943 released "Kentucky 31" as an improved cultivar adapted for Kentucky. Kentucky 31 has been widely planted throughout the southeast for pasture, hay production and erosion control on roadsides. Shortly after its commercial release, Kentucky 31 tall fescue was so popular that seed was harvested in the summer and planted in the fall of the same year (Bacon and Siegel, 1988). Kentucky 31 proved to be incredibly well adapted and currently covers an estimated 35 million acres in the Southeastern United States (Ball et al., 2007a). Tall fescue is such a prominent grass species in the transition zone of the eastern United States that this region is often termed the fescue belt. As use of Kentucky 31 became more prevalent, farmers and researchers began to observe that animal performance was below what would be expected based on forage quality analysis. In 1977, Bacon and colleagues announced the discovery of a fungal endophyte, *Epichloe typhina* (Pers.) Tul., in tall fescue plants in pastures where cattle showed poor performance (Bacon et al., 1977).

1.2 About the Endophyte

Most Kentucky 31 tall fescue plants are known to be infected with the fungal endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams, formerly *Ephichloe typhina*); the genotype found in Kentucky 31 is referred to as the "wild type" strain. The relationship between the plant and endophyte is considered a mutualistic one, because both the plant and the endophyte benefit from the relationship. The endophyte lives safely in the intercellular spaces of the plant (Bacon and Siegel, 1988) and derives all of its nutrients

from the plant (Hinton and Bacon, 1984); in turn the endophyte produces beneficial alkaloids to the plant.

Infected stands are more resistant to drought (Arachevaleta et al., 1989), low soil nitrogen (Bacon, 1993), low soil phosphorus (Malinowski et al., 1997), some diseases such as *Rhizoctonia zeae* Voorhees (Gwinn and Gavin, 1992) and some nematodes (Kimmons et al., 1990). Infected tall fescue plants have also been shown to accumulate sugars more rapidly after a stress event than endophyte free plants (Nagabhyru et al., 2013). Forage quality of tall fescue is not affected by the presence of the endophyte (Bush and Burrus Jr., 1988, Hopkins and Alison, 2006, Kallenbach et al., 2003).

Beneficial alkaloids in tall fescue include the pyrrolopyrazine compound peramine and the saturated aminopyrrolizidines (commonly referred to as the "lolines"), which provide primarily insect resistance to tall fescue. Ergot alkaloids such as ergovaline and indolediterpenes such as paxilline and lolitrem B are associated will herbivory deterrence in mammals (Johnson et al., 1984, Porter, 1994, Schardl and Phillips, 1997). Ergovaline has been reported to constitute 80 - 97% of the total ergopeptides found in tall fescue (Belesky et al., 1988, Lyons et al., 1986), therefore the majority of the more recent livestock response studies focus on ergovaline concentrations. Ergopeptides are alkaloids that also contain a tripeptide structure bonded at the C8 position by an amide bond (Smith et al., 2009a).

1.3 Livestock and the Endophyte

When livestock producers first began to notice the symptoms of what is now called fescue toxicity or fescue toxicosis, the causal factor was unclear. Selenium deficiency and high nitrogen applications were initially blamed, however subsequent research showed that selenium injections did not alleviate symptoms (Heimann et al., 1981, Monroe et al., 1988) and fescue toxicity has also occurred in stands with minimal N applications (Taylor et al., 1985). A study in 1991 using pregnant mares on endophyte infected and endophyte free pastures showed that toxicity symptoms were associated with stands where the majority of tall fescue plants were endophyte infected (Putnam et al., 1991). Endophyte infected plants produce a range of ergot and other alkaloids, but ergovaline has been most often implicated as the primary causal agent for livestock toxicity (Foote et al., 2012, Lyons et al., 1986). One major physiological response that is well documented is the vasoconstriction of blood vessels (Klotz et al., 2007, McDowell et al., 2013). The symptoms of fescue toxicosis vary by livestock species and are most well documented in beef cattle and horses.

Cattle

The interaction of ergovaline and cattle has been widely studied and reported, primarily due to the economic impact of fescue toxicity on the beef industry and, to a lesser extent, the dairy industry in the United States. Grazing endophyte infected tall fescue causes fescue foot, fat necrosis and summer slump in cattle. While the first two syndromes are serious issues, the majority of concern in cattle are the results of summer slump, blamed for a combined loss of over $600 million dollars annually in decreased calf numbers and decreased weaning weights alone (Hoveland, 1993).

Fescue foot is a dry gangrene condition of the feet, ears and tail of cattle grazing infected tall fescue. Ergovaline is a known vasoconstrictor, therefore reducing blood flow to the extremities. In cold weather, this can result in the infection and eventual death of tissues at the feet, ears and tail. Additional clinical signs include dull hair coat, scouring, lameness, emaciation and in extreme cases the sloughing of part or all of the hoof, ears or tail (Bush et al., 1979, Garner, 1973).

Fat necrosis is the formation of hard masses of fat, most commonly found in the abdominal cavity, that can compress and obstruct viscous organs (Smith et al., 2004). Observations of fat necrosis in a herd grazing fertilized tall fescue were made as early at 1967 (Williams et al., 1969) and included five deaths. Later research indicated that high nitrogen fertilization rates on tall fescue pastures increased fat necrosis, and that manure and chemical fertilizers had the same effect (Stuedemann et al., 1973). Lyons and colleagues (1986) indicated that nitrogen was not the causal agent, but nitrogen application as a fertilizer or manure stimulate plant growth which in turn stimulates ergot alkaloid production. Etiology of fat necrosis has not been reported in scientific literature.

Summer slump in cattle includes a variety of issues that result in poor animal performance. Normally, blood carries body heat from the animals' core to the periphery including the skin, ears, legs and tail. When blood is close to the surface it is cooled before returning to the body of the animal. The vasoconstrictive nature of ergovaline found in infected tall fescue directly impacts blood flow to the periphery and results in cattle being unable to dissipate body heat under elevated environmental temperatures (Al-Haidary et al., 2013, Aldrich et al., 1993), therefore resulting in increased core body temperatures (Hoveland et al., 1983, Schuenemann et al., 2005b). Average daily gain (ADG) is suppressed on infected pastures (Hopkins and Alison, 2006, Peters et al., 1992, Schuenemann et al., 2005b); which could be due to decreased dry matter intake (DMI), protein digestibility, serum prolactin levels or nitrogen retention (Matthews et al., 2005). While depressed ADG is related to increasing endophyte infection rates in tall fescue stands, it appears other factors are also involved (Crawford Jr. et al., 1989). Similar observations have been seen in dairy cattle (Strahan et al., 1987). Rough hair coats, due to poor shedding of winter hair and continuous growth of summer hair (McClanahan et al., 2008), are often observed in cattle experiencing summer slump (Hoveland et al., 1983) and have been shown to further impact the ability of the cattle to thermoregulate (McClanahan et al., 2008). Agalactia is also observed in cows and often results in decreased weaning weights (Peters et al., 1992).

Fescue toxicity also has reproductive effects in cattle, though not as pronounced as in horses. The uterine environment is apparently not affected in cattle grazing infected tall fescue, but embryo development is retarded (Schuenemann et al., 2005c) and lower conception rates have been observed in cows grazing infected pastures (Tucker et al., 1989). Bulls grazing infected stands have been observed with significantly decreased scrotal temperature and decreased cleavage of resulting embryos, but no difference in testosterone levels, scrotal circumference, motility or morphology (Schuenemann et al., 2005a).

Horses

The negative effects of infected tall fescue in horses are most commonly documented in late term pregnant mares (especially the last 30-60 days) and include prolonged gestation, thick, retained placentas (Monroe et al., 1988), dystocia (Putnam et al., 1991), increased foal and mare mortality (Putnam, Bransby, et al., 1991) and low serum prolactin resulting in agalactia (Kosanke et al., 1987, Monroe et al., 1988, Putnam et al., 1991, Thompson et al., 1986). Barren mares often have prolonged luteal function and decreased breeding efficiency (Brendemuehl et al., 1994) at high ergovaline levels (1171 ppb), but lower levels (867 ppb) appear to have no reproductive effects on these mares (Brendemuehl et al., 1996). Low levels of ergovaline (308 ppb) have also been shown to have little effect on early term mares (Arns et al., 1997), however higher doses (1171 ppb) can result in pregnancy loss (Brendemuehl et al., 1994).

Vasoconstriction appears in all classes of horses grazing infected tall fescue (Abney et al., 1993). However, research results are mixed on the effect of ergovaline on other parameters including ADG in growing horses (Aiken et al., 1993, McCann et al., 1991, Redmond et al., 1991) and recovery post exercise in working horses (Pendergraft et al., 1993, Vivrette et al., 2001). Reportedly, ergovaline has no effect on thyroid function of adult non-pregnant horses (Breuhaus, 2003). Ergovaline ingestion also does not appear to effect stallion sperm motility, number morphology or sperm morphology (Fayrer-Hosken et al., 2008).

Other Animals

Rats being fed endophyte infected seed have shown similar responses as cattle grazing infected tall fescue, including increased core body temperature, decreased feed intake and decreased weight gain (Spiers et al., 2005). Similar to cattle, sheep have exhibited decreased performance due to increased body temperature and decreased forage intake and digestibility (Hannah et al., 1990) and fat necrosis has been observed in goats (Smith et al., 2004).

Establishing Thresholds for Livestock

The question "how much is too much" is often asked when discussing ergovaline in tall fescue. Early feeding studies reported only that pastures, hay or seed were infected or non-infected (Putnam et al., 1991). Later research focused on high and low infected stands, where treatments were applied based on the percent infection rate of a stand or seed lot. These studies often found differences in animal responses, such as when Aiken and colleagues found average daily gain of yearling horses was significantly less on the highly infected pastures (≥75%) compared to pastures with low infection (≤25%) (Aiken et al., 1993). More recent research has focused on the concentration of ergovaline and other alkaloids to answer the question "how much is too much." Table 1.1 contains a brief summary of research where ergovaline concentration and animal responses were reported.

Table 1.1. Reported ergovaline concentrations and animal responses in cattle, horses and sheep.

Ergovaline Conc. (ppb*)	Material Analyzed for Ergovaline	Observations	Reference	Study Duration
Beef Cattle				
154-506	Tall fescue tillers**	decreased body weight and weaning weight	Peters et al. (1992)	120 days repeated
270-340	Tall fescue tillers	decreased average daily gain, scrotal temperature and prolactin	Schuenemann et al. (2005b)	112 days repeated
285	Total diet†	decreased dry matter intake and prolactin; increased rectal temperature with increased environmental temperatures	Aldrich et al. (1993)	34 days
381	Total diet†	decreased prolactin	Aldrich et al. (1993)	20 days
650	Tall fescue tillers	toxicosis elicited	Hill et al. (1994)	60 days
1240	Tall fescue tillers†	winter coat retention; continuous hair growth	McClanahan et al. (2008)	104 days
Dairy Cattle				
782	Total diet†	decreased milk fat, fat yield and milk protein	Kim et al. (2007)	28 days
Early Term Mares				
308	Total diet†	No effect on early term pregnant mares	Arns et al. (1997)	Breeding + 28 days of pregnancy
867	Tall fescue tillers	No lost pregnancies in early term mares	Brendemuehl et al. (1996)	279 days
1171	Tall fescue tillers	prolonged luteal function, increased number of cycles/pregnancy and early embryo death	Brendemuehl et al. (1994)	~120 days

Table 1.1 continued

Ergovaline Conc. (ppb*)	Material Analyzed for ergovaline	Observations	Reference	Study Duration
Late Term Mares				
271	Feed	No loss of late term foals	Youngblood et al. (2004)	21 days
0-700	Range of pasture‡	prolonged gestation, dystocia, agalactia and increased mare and foal mortality	Putnam et al. (1991)	90 days of pregnancy through parturition
Non-pregnant Horses				
190	Hay†	No response in exercising horses	Pendergraft et al. (1993)	106 days
713	Feed†	Vasoconstriction of the distal palmer artery	McDowell et al. (2013)	14 days repeated
2200	Seed	No effect on thyroid function	Breuhaus (2003)	90 days
Sheep				
3000	Total diet†	no effect on total tract digestibility	Hannah et al. (1990)	21 days
6000	Total diet†	decreased total tract digestibility	Hannah et al. (1990)	19 days

*parts per billion

** consumption estimated at 1.1-6.0 mg ergovaline/d

† reported on a Dry Matter basis, all others not reported

‡ reported total ergopeptides

It is likely that the concentration is not the only factor in determining if and how much of a physiological response an animal will show. Clearly the species and class of the animal are factors. Early term mares appear to be less sensitive to ergovaline compared to late term mares. For example, no pregnancies were lost in early term mares at 867 ppb[1] ergovaline (Brendemuehl et al., 1996), however several signs of fescue toxicity including foal and mare mortality were observed in late term mares consuming 0-700 ppb[2] ergovaline (Putnam et al., 1991). Early term mares did show embryo death when consuming 1171 ppb ergovaline (Brendemuehl et al., 1994). Vasoconstriction was observed in non-pregnant horses consuming 713 DM ppb ergovaline (McDowell et al., 2013), however more than double that did not affect the thyroid function of horses (Breuhaus, 2003). Sheep appear to be much more tolerant of ergovaline (Hannah et al., 1990) than other livestock species.

Environment is also likely to play a significant role. Aldrich et al. (1993) observed stronger fescue toxicity symptoms in one group of cattle consuming a slightly lower concentration of ergovaline than for another group consuming a higher concentration. The difference was that the cattle consuming less ergovaline (285 ppb) were also heat stressed. Given that thermoregulation ability in cattle is impacted by ergovaline, it is likely that environmental conditions is an important factor in fescue toxicity.

The presence of other compounds in tall fescue is likely to play a role in physiological responses. It is well documented that ergovaline makes up the majority of ergot alkaloids produced by the endophyte (Belesky et al., 1988) and that it is a strong vasoconstrictor (Klotz et al., 2007); however other compounds, such as lysergic acid, have been shown to be vasoconstrictors as well. Additionally, there was a greater contractile response in extracted vein tissues from ergovaline and lysergic acid together than the responses of either individually, suggesting that there is an additive effect (Klotz et al., 2008). Other

[1] Dry matter/as fed was not reported for any studies unless otherwise noted.
[2] Seasonal ranges only were reported here.

alkaloids have also shown some vasoconstrictive responses, such as ergonovine and ergocristine (Klotz et al., 2009). Recent studies have concluded that the core vasculature (such as the ruminal artery and vein) are more sensitive to the additive effect of all alkaloids than peripheral tissues such as the saphenous vein (Foote et al., 2012).

The genotype of the tall fescue plant will influence the ergovaline production as well as the seed infection rate of the next generation. In Oregon, Welty et al. (1994) found that ergovaline concentrations of 25 KY 31+ plants grown in the same field from the same lot of seed varied from 8 to 1300[3] ppb in 1991 and 0 to 500[1] ppb in 1992 in straw. Ergovaline in seed was reported from 9 to 6064 ppb in 1991 and 3 to 8659 ppb in 1992. Seed produced by infected plants ranged from 1 to 100% infection and 0 to 100% infection in years 1991 and 1992 respectively. Plants that were on the high end of the spectrum in 1991 were also high in 1992 and similarly for low plants in 1991 and 1992.

Given that there are so many factors that potentially affect the threshold for physiological responses in livestock, it can be difficult to answer "how much is too much." The Oregon Cooperative Extension Service has published ranges of tolerance for several species (Table 1.2).

Table 1.2. Threshold ergovaline ranges in total diet for livestock published by the Oregon Cooperative Extension Service*.

Animal	Ergovaline in total diet (ppb**)
Horses†	300-500‡
Cattle	400-750
Sheep	500-800

*(Aldrich-Markham et al., 2003)

**parts per billion

† Except for mares in the last 60 to 90 days of pregnancy, when the threshold is zero.

‡ Dry matter/as fed not reported

[3] These values were not reported but are estimates based on the graphs presented.

Other states and countries have published variations of these thresholds; Australia sets threshold recommendations at 400-800 ppb for cattle (Reed, 1999), Kentucky recommends limits of no more than 200 ppb for late term mares and 800 ppb for early term mares (Smith, Pers. comm., 2013). The Samuel Roberts Noble Foundation (Ardmore, OK) sets threshold limits for all livestock at 150 ppb (Rogers, 2011).

Identification of a specific threshold for late-term mares has been especially challenging to extension personnel. The recommendation of 150 ppb appears to come from a research paper by Stamm et al. in 1994. This experiment used beef steers to observe the influence of ergovaline. They reported a subclinical effect of decreased circulating prolactin at all levels of ergovaline concentration in feed (158, 317 and 475 ppb) compared to the control; however no other effects were observed including changes weight gain and feed efficiency. Implications from this study suggested by the authors made no mention of horses specifically. Several extension publications and other research articles have subsequently cited this research and used 150 ppb ergovaline as a critical threshold level for livestock, such as Kallenbach et al. (2003). Extension publications, including those from the University of Kentucky, have cited Kallenbach et al. (2003) when recommending 150 ppb ergovaline as the critical cut-off for late-term pregnant mares, despite there being no ill effects observed by Youngblood et al. (2004) with 271 ppb ergovaline in the diet. Extremely low recommendations such as 50 ppb ergovaline are often the result of clinical cases reported in veterinary textbooks (Evans, 2012). Most likely, the value of mares, especially in central Kentucky, along with the emotional attachment of owners to horses has led to industry wide acceptance of very low or zero tolerance of ergovaline in late-term mares, despite little evidence in the literature.

1.4 Management of Tall Fescue Pastures to Reduce Livestock Toxicity

Total eradication of tall fescue in the U.S. or on individual farms is not realistic due to the widespread use and persistence of Kentucky 31; therefore producers must learn to manage around tall fescue in their pastures. Extension specialists and researchers have developed several methods of management to reduce the risk of tall fescue toxicity in livestock.

Animal Breed

Producers who are unable to remove tall fescue from their pastures can mitigate symptoms by introducing genetics of *Bos indicus* type cattle such as Brahman into their herds. Studies have found that Brahman or Brahman cross cattle perform better when compared to their *Bos taurus* (such as angus or hereford) relatives on infected tall fescue pastures (Brown et al., 1997, McMurphy et al., 1990). However, *Bos indicus* are not immune to ergovaline; they have a higher tolerance to heat stress than *Bos taurus* and perform well, despite showing sub-clinical signs of fescue toxicosis (Browning, 2003).

Dilution

Dilution is simply reducing the ergovaline in the diet by decreasing the tall fescue and increasing other components of the diet. This is most commonly done in cattle pastures by interseeding clovers and with sufficient dilution, cattle gains can be similar to those on low endophyte pastures (Lusby et al., 1990, McMurphy et al., 1990). Dilution can also be achieved by adding concentrates, such as pelleted soybean hulls (Carter et al., 2010), cracked corn (Forcherio et al., 1995) or creep feeding of a commercial concentrate (Tarr et al., 1994). These studies showed increased average daily gain or body weight in beef steers, beef cows and beef calves respectively compared to those on infected pastures alone. While information is limited in the literature, it is assumed that a similar concept applies to horses.

Pasture and Grazing Management

Careful management of pastures and animals can reduce exposure to toxic tall fescue.

As will be discussed further in the section below ("Ergovaline and Factors Affecting Metabolism in the Plant"), ergovaline is more concentrated in the stem and seedhead of the plant than in the leaves (Greene et al., 2013). Clipping pastures to remove seedheads can reduce the amount of ergovaline that animals are exposed to. Aiken et al. (2012) have recently used the herbicide Chaparral™ (aminopyralid + metsulfuron, produced by Dow AgroSciences) to suppress the emergence of seed heads on tall fescue pastures and have observed higher average daily gain, increased serum prolactin and decreased body

temperatures in cattle compared to non-treated pastures. Forage quality for treated pastures was also greater than non-treated pastures.

Over-grazing of pastures can also expose animals to more ergovaline. As Greene et al. (2013) reported, ergovaline concentrations were highest in the bottom 7.5 cm of infected tall fescue plants. Overgrazing of pastures forces animals to consume this highly toxic material.

Testing

Routine testing of pastures for both endophyte infection percentage and ergot alkaloid concentrations allows managers to identify fields with the greatest risk and move their most sensitive/most valuable animals to pastures with the lowest endophyte or ergot alkaloid concentrations. Several diagnostic laboratories offer endophyte and/or ergot alkaloid testing including University of Kentucky Veterinary Diagnostic Laboratory (University of Kentucky, 2013, Vincelli et al., 2012), University of Missouri Veterinary Medical Diagnostic Laboratory and Oregon State University Endophyte Service Laboratory.

Pharmaceuticals

The use of steroidal implants has been shown to partially alleviate the symptoms of tall fescue toxicity in steers. Increased average daily gains have been observed when using implants in several studies (Coffey et al., 2006, Elizalde et al., 1998). Carter et al. (2010) observed the highest rates of gain when steroid implants were combined with supplementation of pelleted soybean hulls. However Aiken et al. (2006) observed that the benefits of implants decreased as stocking rates increased and implants and supplementation did not alleviate symptoms when administered post grazing (Aiken et al., 2001).

In mares, domperidone has been used to alleviate the symptoms of fescue toxicity. Evans et al. (1999) found domperidone to be safe and effective in preventing prolonged gestations as well as pre- and post-parturition agalactia. Based on the clinical

observations of 1423 mares from 1993-1997, owners and veterinarians found that domperidone was effective treatment for fescue toxicosis in 95% of mares treated (Cross et al., 1999). Domperidone has been shown to be effective in non-pregnant heifers, resulting in increased weight gain and decreased economic losses (Jones et al., 2003). However, daily domperidone treatments are cost prohibitive for cattle producers and some horse owners (estimated at \$15 day^{-1} for an average mare). Thiamin supplementation has also been shown to alleviate toxicosis symptoms in beef cattle during the summer (Lauriault et al., 1990); but Vitamin E appears to be ineffective in dairy cows (Jackson et al., 1997).

Removal

Some producers choose to remove infected tall fescue completely from their pastures to reduce the risk to their livestock. Dr. Bill Witt of the University of Kentucky recommends one of two options for tall fescue removal in cool season grass pastures (Witt, Pers. comm., 2013). Pastures containing more than 50% tall fescue should be killed with glyphosate. In pastures with less than 50% tall fescue and few other weeds, Plateau® (Imazapic, manufactured by BASF) at 700.5-840.6 g ha^{-1} or Cimarron® (metsulfuron methyl, manufactured by DuPont) at 70.05 g ha^{-1} can be applied to remove tall fescue without harming Kentucky bluegrass or orchardgrass in the pasture. Removal by herbicides is an expensive task that requires months of advanced planning; furthermore, infected plants are generally more competitive and will eventually move back into the pasture.

Large scale total removal of the endophyte within tall fescue plants in a field is an impractical goal because fungicides are expensive and show only a temporary response (Williams et al., 1984). However, producers can choose to replace individual pastures with endophyte free (E-) or novel endophyte (NE) tall fescue. Zhuang et al. (2005) indicated that stand replacement was economically beneficial when infection rates were around 74% or higher.

Novel Endophyte Tall Fescue Cultivars

Unlike other endophyte infections of grasses, *Neotyphodium coenophialum* is spread only in seed (Bacon et al., 1977). Storage of infected seed for 1-2 years generally results in death of the endophyte (Ball et al., 2007b, Hoveland, 2009), but seed should be tested before planting. Hill and colleagues (1991) suggested that selective breeding of tall fescue could reduce or eliminate the ergopeptine alkaloids and improve animal health on infected tall fescue. Subsequent studies proved this: breeding tall fescue plants with lower ergot alkaloid concentrations resulted in lower concentrations in offspring plants compared to parent plants in just two generations (Adcock et al., 1997).

Non-ergot alkaloid producing endophytes have been identified and inserted into improved E- tall fescue cultivars to create novel or friendly endophyte tall fescues (Latch and Christensen, 1985). These cultivars benefit from other compounds produced by the endophyte and can have equivalent stand survival and yield compared to endophyte infected (E+) tall fescue (Bouton et al., 2002) while livestock perform equivalently to E- tall fescue (Parish et al., 2003). Ergot alkaloid production in most novel endophyte tall fescue was far below that of the E+ and below the threshold values reported in the literature for all classes of animals. However, some novel endophyte tall fescue cultivars produce levels of ergot alkaloids that may not be safe for the most sensitive classes of livestock. For example, BarOptima Plus E34 has been shown to contain ergovaline concentrations over 200 ppb in the seedhead (Greene et al., 2013), a concentration that some consider toxic to late term pregnant mares.

1.5 Ergovaline and Factors Affecting Metabolism in the Plant

Ergovaline, and its stereoepimer ergovalinine, are peptide alkaloids consisting of a d-lysergic acid linked to a tricyclic peptide functional group by a peptide bond (Berde and Sturmer, 1979), see Figure 1.1. Ergovaline concentrations within a plant have been described to be widely variable due to time of year, weather conditions, management, phenotype and other factors (Belesky et al., 1988, Lyons et al., 1986, Rogers et al., 2011, Rottinghaus et al., 1991). Being a cool season grass, tall fescue grows most vigorously in the spring and fall of the year. This is also generally the time of highest concentrations of

ergovaline (Belesky et al., 1988). Ju et al. (2006) reported that while tall fescue may start growing at 5.2°C, the endophyte will not become active until 10.3°C, therefore sampling of tall fescue for endophyte infection in early spring is not recommended. Extreme stress such as overgrazing, flooding or drought has also been known to cause increases in ergovaline. Some cultivars and genotypes of tall fescue have been noted to have higher than average concentrations (Agee and Hill, 1994). Finally, ergovaline concentrations also vary widely within a single plant; generally the base of the plant contains the highest concentrations, followed by the seed head and stem (Greene et al., 2013). These observations are consistent with the observations that most of the fungal biomass is located in the base of the leaf sheath, but absent or low in the leaf (Hinton and Bacon, 1984). Leaves of the plant have the lowest concentrations, but can still reach toxic levels and can vary greatly within a single leaf (Spiering et al., 2002).

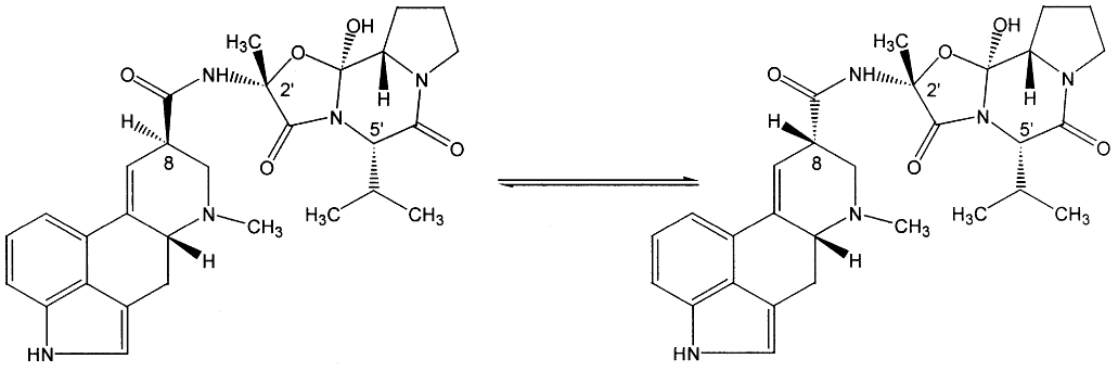

Figure 1.1. Structure of ergovaline and ergovalinine. Epimerization occurs around the 8 carbon. (Smith and Shappell, 2002)

Ergovaline in Seed

Refrigerated or frozen tall fescue seed will not lose significant amounts of ergovaline (Garner et al., 1993). Because of this and the easy of handling, feeding infected seed is the most common way to dose ergovaline for research; however it is not a perfect model. Tall fescue seed in large amounts is often not palatable to animals (Arns et al., 1997) and can be dusty. Additionally, since most livestock encounter ergovaline in tall fescue plants

rather than seed, it is unclear if results from studies feeding seed are comparable to grazing situations.

Ergovaline in Hay

Several studies have looked at the decline of ergovaline concentrations during hay making and storage. Roberts and colleagues (2009) reported a 72% loss in ergovaline in hay and higher losses in ammoniated hay, but the study did not specify the storage time of the hay (Roberts and Kallenbach, 2002). In 2009, a study in Missouri found that most of the initial ergovaline losses happened in the first 2 to 23 days after clipping. Ergovaline continued to decline over the remaining 17 month storage period, however hay made from pastures with high ergovaline concentrations remained in a toxic range even after 18 months. Similar results have been observed in perennial ryegrass (*Lolium perenne* L.) hay and silage, although ergovaline appeared to have a more erratic decline (Fletcher, 2005, Hume et al., 2006). Norman and colleagues (2007) also observed a decrease in ergovaline in stored hay over time, with losses ranging from 27.3% to 79.4% and ergovaline concentrations were reported higher at the surface of the bale when compared to the center of the bale.

Stockpiling of tall fescue is a common alternative to feeding hay in the wintertime in some areas. Kallenbach and colleagues (2003) found that ergovaline decreased over winter by 85% of the original concentration. They suggested that producers feed hay first and then turn animals into stockpiled tall fescue rather than grazing first to reduce the exposure to ergovaline. They also observed a decrease in nutritive value of the stockpiled fescue over time. In related research, alkaloids have been reported as largely absent in standing dead material (Siegrist et al., 2010).

Ergovaline in Pasture

The most common way that most livestock species come into contact with ergovaline is by consuming live tall fescue plants in a pasture. Many research programs take samples of pastures to determine how much ergovaline the animals are ingesting. In addition, some livestock managers evaluate pasture samples regularly for ergovaline concentration

to determine the risk of a specific pasture and help to determine where to graze their most sensitive animals. Very little research has been performed to test the validity of commonly used sample handling and storage procedures. Knowledge of potential losses of ergovaline during transportation and storage of pasture samples is crucial to applying the findings from research studies into on-farm management decisions.

1.6 Ergovaline Analysis

The unstable nature of ergovaline has also hindered progress in the laboratory. Ergopeptine alkaloids are known to be unstable in laboratory settings due to isomerization, hydrolysis and sensitivity to light, air, acid and bases (Garner et al., 1993). Other ergot alkaloid extractions from cereals have been found to be unstable and must be prepared and analyzed the same day or stored below ambient temperature (Krska et al., 2008). Smith and Shappell (2002) found that ergovaline epimerization occurs at 37^{o}C in water, methanol and acetonitrile and equilibria is reached in 1 to 19 hours.

Finding pure ergovaline for standards can be difficult and expensive (Garner et al., 1993). Several methods have been developed for the analysis of ergot alkaloids in tall fescue including Thin-Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and Mass Spectrometry (MS). There are many methods for ergot alkaloid analysis and many more variations of individual methods. This section is designed to be an overview of the most common methods currently in use.

Thin-Layer Chromatography

In 1972, Fowler and colleagues described using thin-layer chromatography (TLC) to analyze lysergide and other ergot alkaloids (Fowler et al., 1972). At the time, TLC was the primary method for analysis of ergot alkaloids. They reported that no one system could completely resolve lysergide from other alkaloids. They recommended a combination of silica-acetone and alumnia-acetone, but this system was not able to identify other individual ergot alkaloids besides lysergide.

High-Performance Liquid Chromatography

In 2002, Spiering published results of a modified method for high-performance liquid chromatography (HPLC) analysis of ergovaline in tall fescue. Current analysis methods require overnight extraction with chloroform and methanol ammonia (75:25:2 v/v) and of at least 50mg of dried ground material. Spiering (2002) was successful in using 2-propanol with lactic acid for one hour extraction using only 20mg of material. This method of HPLC with fluorescence detection was faster than previous methods, required less material and demonstrated a lower limit of quantitation (0.1μg/g).

Mass Spectrometry

Lehner and colleagues (2005) reported a mass spectrometry (MS) method for the analysis of ergot alkaloids in tall fescue. They stated that HPLC with fluorescence or UV detection was primarily a tool for screening samples for common ergot alkaloids, such as ergovaline, and that MS would allow for the identification and quantification of many more ergot alkaloids including lysergic acid, lysergol, ergonovine, ergotamine, ergocornine, ergocryptine and ergocrystine.

1.7 Objectives and Hypothesis

After a review of current literature, it is clear that very little is known about the effect of sample handling and storage on ergovaline content in tall fescue samples. Sample handling procedures are rarely reported in scientific literature nor is the time and temperature of sample storage prior to analysis. In addition to the already complicated task of determining threshold levels in livestock, sample handling and storage could potentially further complicate this task. Many published studies may not report accurate ergovaline concentrations due to varied sample handling and storage methods. Comparing-on farm sample results to research studies is difficult and potentially misleading if sample handling and storage methods are not standard. Comparisons of results from one testing laboratory to another are also difficult due to varied sample preparation, extraction and reporting procedures.

The objective of this study was to evaluate the effects of sample handling and storage on ergovaline concentrations in tall fescue forage. Transportation and short-term storage treatments were designed to mimic the real world scenarios facing producers every day, while longer-term storage conditions were also included to represent likely procedures used in research. The primary hypothesis for this research was that samples transported in a cooler on ice will contain the same concentration of ergovaline as the control, while samples under UV lights and increased heat would show decreased ergovaline concentrations. A secondary hypothesis was that ergovaline would remain stable in samples stored at -20^{o}C or -80^{o}Cwhile samples stored at 22^{o}C or 5^{o}C would show a continual drop in ergovaline over time.

This research was designed to: 1) establish best practices for sample handling and transportation recommendations for research and on farm testing, 2) evaluate the stability of ergovaline in tall fescue tissues stored over time at 22^{o}C, 5^{o}C, -20^{o}C and -80^{o}C and 3) demonstrate the importance of reporting sample handling and storage procedures in future scientific literature.

Chapter Two

Tall Fescue Ergovaline Concentration Based on Sample Handling and Storage Methods

2.1 Introduction

Ergovaline is an ergot alkaloid produced by an endophyte, found in some cultivars of tall fescue, which causes a range of disorders across livestock species. The concentration of ergovaline within an individual tall fescue plant or population can vary by cultivar, management, time of year, location and weather conditions (Mace and Baker, 2012, Smith et al., 2009b). Due to the significant economic impact of fescue toxicity in livestock, many samples are tested every year in research and diagnostic laboratories. Ergovaline is known to be unstable but there is little information in the literature on how ergovaline concentration is affected by sample handling from the pasture to the laboratory or during storage of samples once received at the testing facility.

2.2 Materials and Methods

All samples were gathered from a single pasture on a horse farm located near Lexington, KY on the following dates: 1 May, 2012 (Spring 2012), 21 August, 2012 (Fall 2012) and 11 June, 2013 (Fall 2013). The pasture was approximately 0.4 ha in size and contained primarily cool season grasses including tall fescue, Kentucky bluegrass (*Poa pretensis* L.), and orchardgrass (*Dactylis glomerata* L.), see Figure 2.1. The tall fescue cultivar was assumed to be "Kentucky 31" since there were no records of tall fescue being planted on this farm. Species composition of the pasture was determined by visual estimation of ten, 0.37 m^2 quadrats distributed throughout the field (Table 2.1). This pasture was seeded with orchardgrass and Kentucky bluegrass in the fall of 2011 to increase vegetative cover. Due to the recent overseeding and the excessive distance from the barn, this paddock was ungrazed by horses throughout the entire sampling period, but was routinely mowed to approximately 12.5 cm.

Table 2.1. Species composition of the central Kentucky horse pasture test site at the time of sampling for ergovaline analysis.

	Tall Fescue	KY Bluegrass	Orchardgrass	White Clover	Weeds	Bare Soil
	----------%----------					
April 2012	29	6	27	0	33*	6
August 2012	24	4	63	2	9**	0
May 2013	19	8	46	0	9**	19

* Most common weeds: chickweed (*Stellaria media* L.), Speedwell (*Veronica arvensis* L.)

** Most common weeds: Nimblewill (*Muhlenbergia schreberi* J.F. Gmel), dandelion (*Taraxacum officinale* Weber ex Wiggers)

Endophyte and Ergovaline Status

The pasture had been tested several times for tall fescue ergovaline concentration and endophyte infection from 2007 through 2013 and these analyses consistently showed higher concentrations than surrounding farms (Table 2.2). Endophyte sampling was performed by collecting approximately 20 tillers from 10 locations throughout the field. Tillers were cut at soil level and placed into a plastic bag on ice and transported to the University of Kentucky Regulatory Services Laboratory for testing using immunoblot endophyte test kits from Agrinostics (Athens, GA). The method for ergovaline analysis is described in the section below.

Table 2.2. Historical ergovaline concentration and endophyte infection level of the central Kentucky horse pasture test site.

Date	Ergovaline	Endophyte Infection*
	(ppb)**	(%)
May 2, 2007	280†	81
August 11, 2008	714†	88
August 3, 2009	300†	80
September 30, 2011	2510‡	100
November 8, 2011	461‡	not reported
April 19, 2012	1384‡	100
August 8, 2012	1970‡	not reported
October 6, 2012	608‡	not reported
January 29, 2013	168‡	not reported
April 25, 2013	174‡	not reported
May 21, 2013	1880‡	not reported

* Endophyte infection rate was determined by the sample collection and test procedure described in the Materials and Methods section.

** parts per billion reported as ergovaline plus ergovalinine

†Collection procedure for these samples was the same as described in the text. Analysis was performed by Dr. Lowell Bush at the University of Kentucky using modified HPLC with fluorescence (method developed by Yates and Powell, 1988) and represents ergovaline plus ergovalinine

‡ Collection and analysis determined at the University of Kentucky Veterinary Diagnostic Laboratory by the method described in the Materials and Methods section

Harvest and Sampling for Ergovaline Analysis

On day 0 of each harvest period, approximately 2 kg of fresh tall fescue was harvested using a rice knife at 5-7.5 cm above the soil surface. This material was placed on ice and transported to the laboratory immediately (less than 1 hour). Once at the laboratory, the sample was cut into 2.5-7.5 cm lengths. Milling of material was performed using a Stein Mill (Steinlite Corporation, Atchison, KS). The Stein mill cups have a volume of approximately 500 ml and were filled half full of the cut tall fescue (Figure 2.2). Enough liquid nitrogen was then added to cover most of the material in the cup and allowed to boil until most of the nitrogen had volatilized. This was done to reduce the likelihood that the mill cup would freeze to the mill. The mill cup was then securely placed on the mill

and the frozen forage was milled for approximately two minutes. Material was then taken into the freezer and placed into a bowl (Figure 2.3). The mill cup was rinsed out and the process was repeated until an adequate mass of frozen, milled material was collected in the freezer (about 30 times). The material as then mixed by hand and separated into 42 individual sub-samples containing approximately 5g of material (Figure 2.4). Sub-samples were placed into 120 ml whirl-pak bags and immediately placed into their assigned treatments with three sub-samples assigned to each treatment. Average time from harvest to treatment application was 2.5-4 hours and samples were kept frozen the entire time. Treatments were designed to simulate real world situations.

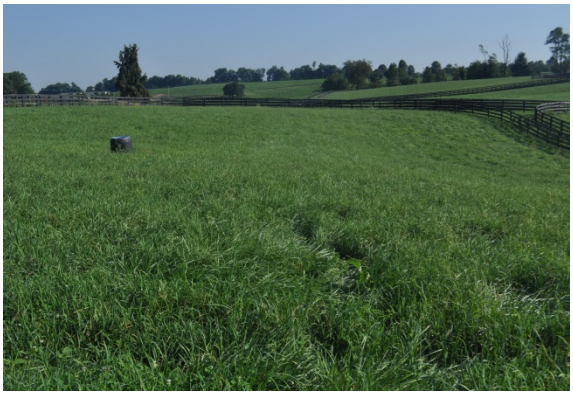

Figure 2.1. Sample field was a 0.4 hectare pasture located on a horse farm near Lexington, KY.

Figure 2.2. Cut lengths of tall fescue in milling cup before milling.

Figure 2.3. Frozen and milled tall fescue before sub-samples are allocated into treatments.

Figure 2.4. Individual sub-samples before being placed into treatments.

Treatments

The Control treatment was placed in the freezer (-20^{o}C) for 2 hours before sample preparation began. The Ice treatment was designed to simulate sample collection by a producer or county agent and being transported to the lab immediately on ice in a cooler. This was achieved by placing the sub-samples into a cooler full of ice for two hours. Similarly, the Light + Heat treatment was designed to simulate the sample being placed inside a vehicle and exposed to high temperatures and UV rays by placing it under full spectrum bulbs (three GE Residential Ecolux 40W bulbs and three Hatch Lighting F40T12/D/DX 40W bulbs) for two hours. Additionally, a 60 watt incandescent light was placed over the samples to increase the heat. The entire apparatus was covered using vinyl and aluminum foil to contain heat. Storage treatments included three treatments of storage at ambient temperature (22^{o}C); these samples were placed uncovered on the lab bench for their respective times. Similarly, refrigerator (5^{o}C) samples were placed in the refrigerator for their allotted time, as were the five freezer (-20^{o}C) samples. For the final harvest, two additional treatments were added: one day in the Ultra-Low freezer (-80^{o}C) and one day in the Ultra-Low freezer then freeze dried before analysis. The Ultra-Low was added to attempt to prevent the 24 hour losses observed in the first two harvests. The Ultra-Low + freeze dried was added to test the effects of the freeze dryer and is the common sample handling procedure for some research laboratories. These various storage locations and times are to simulate storage at a diagnostic laboratory. On the day of analysis, samples were removed from their treatment locations and placed together on the lab bench for less than 10 minutes before sample preparation began. Table 2.3 contains a full list of treatments.

Table 2.3. List of treatments for Sample Handling and Storage

Treatment Abbreviation	Treatment	Time of Treatment	Temperature
C	Control	2 Hours	-20°C
I	Ice	2 Hours	1°C
LH	Light + Heat	2 Hours	38.5°C
A1	Ambient Temp	1 Day	22°C
A2	Ambient Temp	2 Days	22°C
A3	Ambient Temp	3 Days	22°C
R1	Refrigerator	1 Day	5°C
R3	Refrigerator	3 Days	5°C
R6	Refrigerator	6 Days	5°C
F1	Freezer	1 Day	-20°C
F7	Freezer	7 Days	-20°C
F14	Freezer	14 Days	-20°C
F21	Freezer	21 Days	-20°C
F28	Freezer	28 Days	-20°C
UL1*	Ultra-low Freezer	1 Day	-80°C
UL1D*	Ultra-low Freezer	1 Day + Freeze dried	-80°C

* Treatments were added to Spring 2013 harvest only

Extraction and Analysis

The following analysis method was used as adapted from Spiering (2002) by Dr. Lori Smith of the University of Kentucky Veterinary Diagnostic Laboratory for use in diagnostic and research samples of tall fescue as either fresh plants, seed or samples taken from harvested tall fescue hay.

For each sub-sample, 1g ± 0.25g of material was placed in a 15 ml disposable conical centrifuge tube and exact mass was recorded; 4950 µL of internal extraction solution (50% aqueous 2-propanol / 1% (v/v) lactic acid) was added as well as 50 µL internal standard solution (Ergotamine 10 µM). Ergotamine is used as an internal standard because it is structurally and chemically similar to ergovaline, therefore losses of ergotamine through the sample preparation process are assumed to be similar to

ergovaline losses. The sub-sample was then vortexed for ~ 30 seconds. All samples being run that day were then placed on a mixer (Multi Mixer and Rotator (UNICO MTR22), United Products & Instruments, Inc.) for one hour. Finally, samples were centrifuged for 12 minutes at 3000 rpm. The liquid fraction was then removed, filtered using PVDF syringe filers (0.45 um, ~25 mm diameter) and placed into salinized, amber 2-mL autosampler vials with caps (Teflon/rubber septa).

For each day that samples were analyzed, four standards and a blank sample were also created and analyzed. Standards contained 0.1 µM ergotamine and 0, 0.02, 0.10 or 0.25 µM ergovaline in 1ml of total solution. The blank sample containing the internal extraction and standard solutions as described above was mixed, centrifuged and filtered in the same manner as the sub-samples.

Another ~ 1g of material from each sub-sample was weighed, dried for 24 hours at 100^{o}C in a forced-air drying oven (Isotemp Oven, Fisher Scientific) and weighed again to determine moisture content of the original sample. Moisture content of each sub-sample was calculated on the date of analysis to correct for changes in moisture content and was calculated as dry matter = 100 - ((wet-dry)/wet).

Instrumentation

Samples were analyzed using a Dionex U-3000 dual pump high pressure liquid chromatography (HPLC) system equipped with a RF-2000 fluorimeter. The machine was equipped with an Acclaim 120 C18 HPLC Analytical Column (Dionex Corp.; 3 µm particles, 120 Å; 4.6 mm ID X 100 mm) and a Security Guard Column Cartridge System (C18; 4mm X 3.0 mm; Phenomenex). Mobile phases consisted of HPLC grade Acetonitrile and 0.1M Ammonium Acetate; 1:3 ratio for mobile phase A and 3:1 ratio for mobile phase B. The autosampler of the machine was covered to prevent light from reaching the samples and was maintained at 30^{o}C throughout the run. Each sample required 52 minutes to run.

Calculation of Concentration

Peak areas of the four calibrants were charted in relation to their concentration to create a regression line. This line was then used to determine concentration based on the peak areas of ergovaline plus ergovalinine reported for each sample. All regression lines resulted in an R^2 value ≥ 0.99. Concentrations were then adjusted for moisture content and losses based on internal standard; final concentration is reported as ergovaline plus ergovalinine on a dry weight basis. Ergovaline and ergovalinine are stereoisomers that are often present in extract solutions. Degree of epimerization from ergovaline to ergovalinine depends on many variables including type of extraction solvent, amount of light and heat and time in solution (Smith and Shappell, 2002).

Statistics

All results were analyzed in SAS 9.3 using the PROC GLM and lsmeans. Treatments were sliced using the PDIFF statement and significance for mean separation was considered at $P<0.05$.

2.3 Results

Due to a significant date by treatment interaction, ergovaline results are presented by harvest date. All differences were considered significant at $P<0.05$. See Table 2.4 ANOVA for ergovaline concentration after sample storage.

Table 2.4. ANOVA of ergovaline concentration after sample storage

Source	DF	Sum of Squares	Mean of Square	F Value	Pr>F
Model	43	11257077.48	261792.5	139.34	<.0001
Error	88	165339.33	1878.86		
Corrected Total	131	11422416.81			

Spring 2012

See figure 2.5 for a graphical representation of all treatments from Spring 2012 harvest. There was no treatment effect between the control and the ice treatment, but both were significantly higher than the Light + Heat, Ambient Day 1, Refrigerator Day 1 and Freezer Day 1 treatments. Ambient Day 1 was significantly higher than Ambient Day 2

and Ambient Day 3. Refrigerator Day 1 was significantly higher than Refrigerator Day 3 and Refrigerator Day 6. Finally, all freezer treatments did not differ significantly from one another. This harvest illustrates a clear decrease in ergovaline concentration over time for all storage treatments from this harvest.

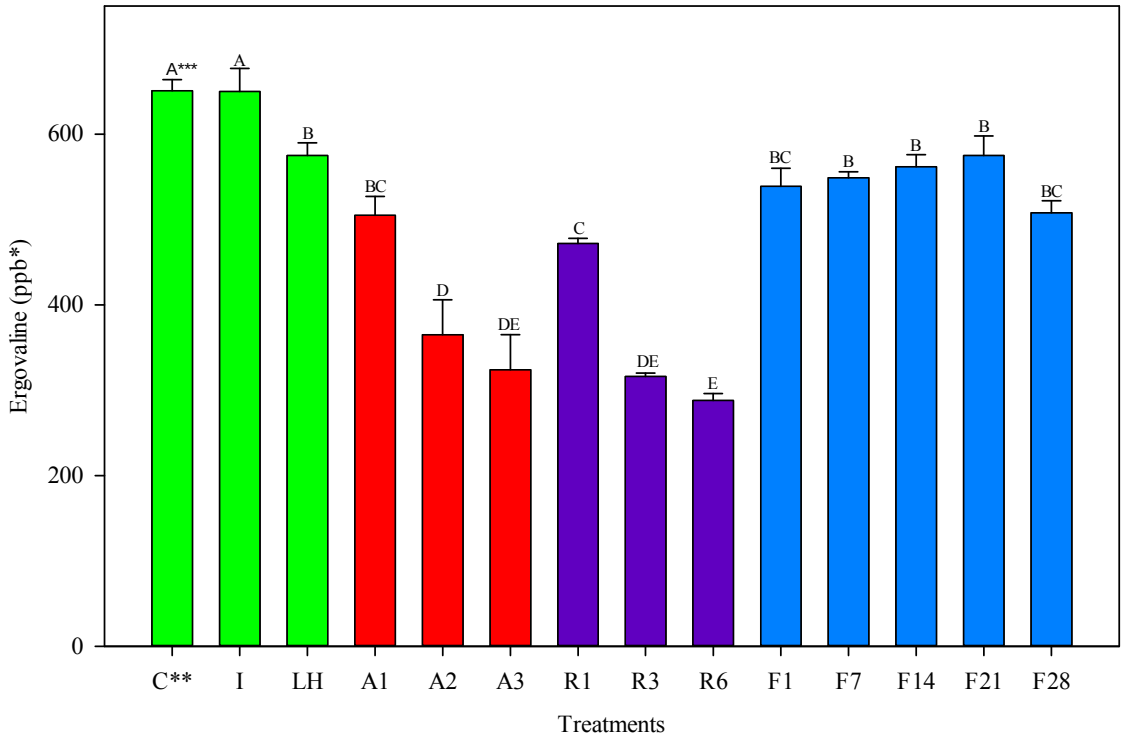

Figure 2.5. Ergovaline concentration of all tall fescue sub-samples in various storage conditions from Spring 2012 harvest.

*parts per billion

**Treatment abbreviations are as follows: Control (C), Ice (I), Light + Heat (LH), Ambient Day 1 (A1), Ambient Day 2 (A2), Ambient Day 3 (A3), Refrigerator Day 1 (R1), Refrigerator Day 3 (R3), Refrigerator Day 6 (R6), Freezer Day 1 (F1), Freezer Day 7 (F7), Freezer Day 14 (F14), Freezer Day 21 (F21) and Freezer Day 28 (F28).

*** Matching letters indicated no statistical difference at a significance level of $P<0.05$

Fall 2012

See figure 2.6 for a graphical representation of all treatments from Fall 2012 harvest. In this harvest, the Control and Ice treatments were not significantly different from each other, but were significantly higher than the Light + Heat and all other treatments. Ambient Day 1 was not different from Ambient Day 2 or Ambient Day 3, but Ambient

Day 2 was significantly higher than Ambient Day 3. Refrigerator Day 1 and Refrigerator Day 6 were not different from each other, but were significantly higher than Refrigerator Day 3. Freezer Day 1, Freezer Day 14 and Freezer Day 28 were not different. Freezer Day 7 and Freezer Day 21 were similar to Freezer Day 14. In Fall 2012 a clear decline of ergovaline over time in the freezer was not observed. This could be due to the high initial concentration of ergovaline.

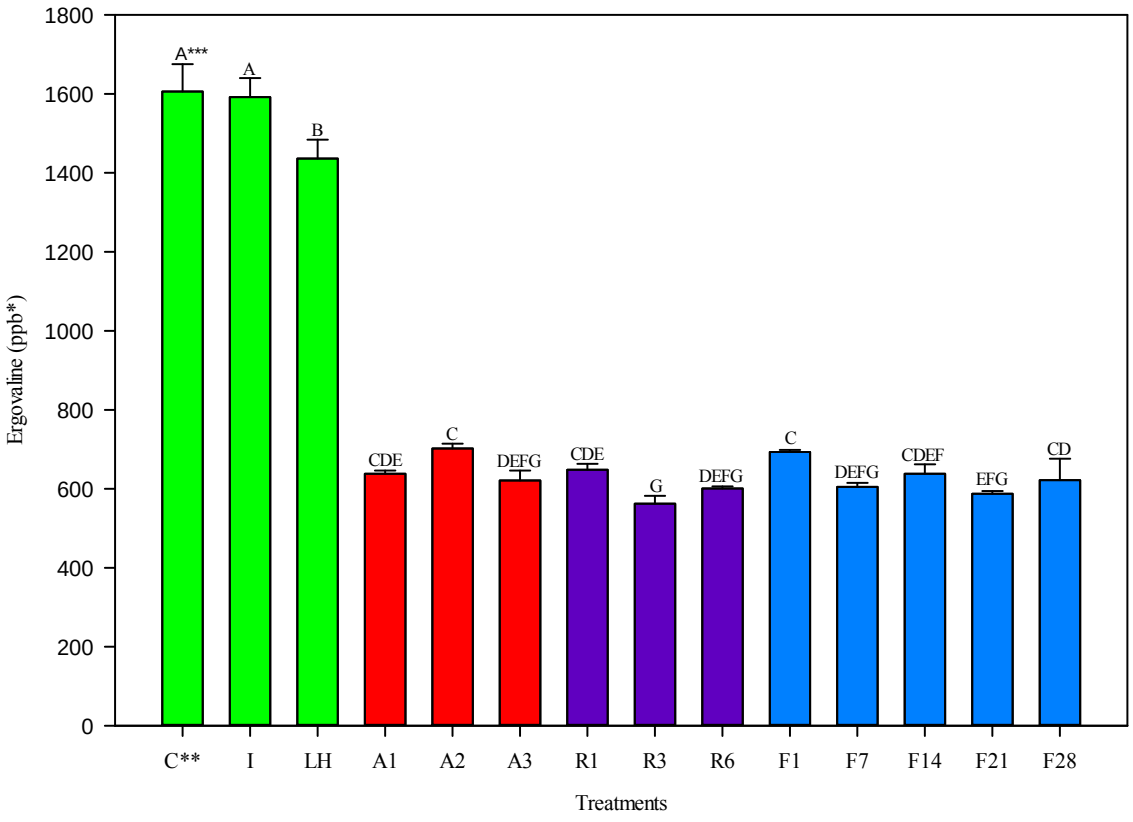

Figure 2.6. Ergovaline concentration of all tall fescue sub-samples in various storage conditions from Fall 2012 harvest.
*parts per billion
**Treatment abbreviations are as follows: Control (C), Ice (I), Light + Heat (LH), Ambient Day 1 (A1), Ambient Day 2 (A2), Ambient Day 3 (A3), Refrigerator Day 1 (R1), Refrigerator Day 3 (R3), Refrigerator Day 6 (R6), Freezer Day 1 (F1), Freezer Day 7 (F7), Freezer Day 14 (F14), Freezer Day 21 (F21) and Freezer Day 28 (F28).
*** Matching letters indicated no statistical difference at a significance level of $P<0.05$

Spring 2013

See figure 2.7 for a graphical representation of all treatments from Spring 2013 harvest. In the Spring 2013 harvest, the Control was significantly higher than all other treatments except the Ice treatment; the Light + Heat treatment was significantly less than Ice treatment. Ambient Day 1 was significantly higher than Ambient Day 2 and Ambient Day 3, which were not significantly different from one another. Refrigerator Day 1 was

significantly higher than Refrigerator Day 3 but not from Refrigerator Day 6. Freezer Day 28 was significantly higher than all other treatments. Freezer Day 1 was not significantly different from Freezer Day 7, Freezer Day 14 and Freezer Day 21. Finally, Ultra Low Freezer Day 1 and Ultra Low Freezer Day 1 + Freeze Dried were not significantly different from each other.

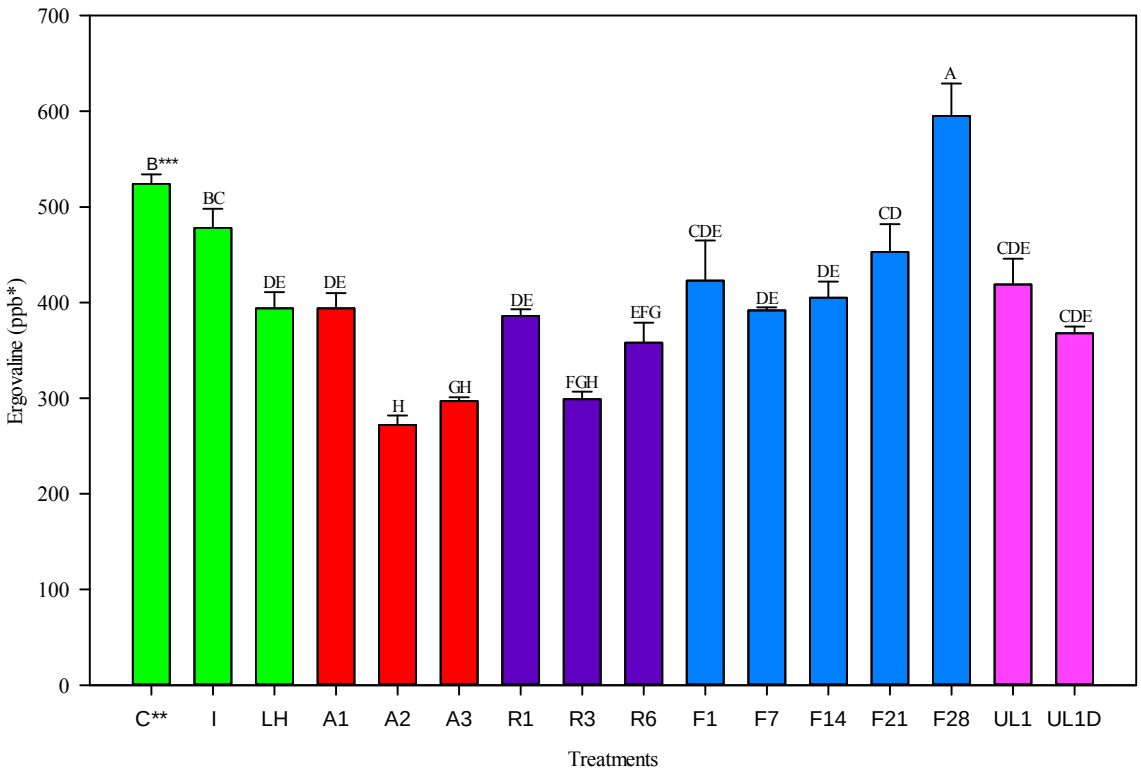

Figure 2.7. Ergovaline concentration of all tall fescue sub-samples in various storage conditions from Spring 2013 harvest.
*parts per billion
**Treatment abbreviations are as follows: Control (C), Ice (I), Light + Heat (LH), Ambient Day 1 (A1), Ambient Day 2 (A2), Ambient Day 3 (A3), Refrigerator Day 1 (R1), Refrigerator Day 3 (R3), Refrigerator Day 6 (R6), Freezer Day 1 (F1), Freezer Day 7 (F7), Freezer Day 14 (F14), Freezer Day 21 (F21), Freezer Day 28 (F28), Ultra-Low Freezer Day 1 (UL1) and Ultra-Low Freezer Day 1 + Freeze Dried (UL1D).
*** Matching letters indicated no statistical difference at a significance level of $P<0.05$.

2.4 Discussion

These results indicated that ergovaline in fresh material, frozen and milled was not consistently stable. Several key observations could be made:

In all three harvests, there was no significant loss of ergovaline in the first two hours when the sub-samples were kept on ice in a cooler, compared to controls. However exposure to heat and UV light resulted in a significant loss after two hours for all three harvests. This suggested that during transportation, sample handling is an important factor. Samples for research and diagnostics should be transported on ice in a cooler to the testing laboratory.

A significant ($P<0.05$) fraction of ergovaline was lost in the first 24 hours regardless of temperature (table 2.5). This indicated that ergovaline was unstable in milled material overnight. Losses ranged from 17-60% across three harvests; however losses within a harvest were the same for all storage conditions. In Spring 2012, 24 hour losses across storage temperatures ranged from 17-27% and in Spring 2013 losses were 19-25%. However losses in the Fall 2012 harvest were higher at 57-60% across storage temperatures. Given that the 24 hour losses in both spring harvests are similar compared to the fall for this experiment, it is possible that ergovaline stability in the plant is dependent on the time of year. However, the concentrations of the control treatments in the spring harvests (651 ppb in 2012, 524 ppb in 2013) were also more similar than the control treatment in the fall (1606 ppb). This suggests that the instability of ergovaline could also be due to the concentration of ergovaline in the plant material at harvest; material with higher concentrations may show a greater percent loss in 24 hours than material with lower concentration at harvest. Norman and colleagues observed wide variations in losses in hay from one location compared to the other (Norman et al., 2007).

Table 2.5. Ergovaline concentrations after 24 hours of storage at various temperatures for three harvests.

	Spring 2012		Fall 2012		Spring 2013	
	----------Ergovaline (ppb*)--------					
Control	651	A†	1606	A	524	A
Ambient (22°C)	505	BC	638	B	394	B
Refrigerator (5°C)	472	C	648	B	386	B
Freezer (-20°C)	539	BC	693	B	423	B
Ultra-low (-80°C)	---		---		419	B

* parts per billion

† Matching letters within a column indicate no statistical difference ($p<0.05$) within a single harvest

From these results, it was observed that for milled plant material stored in a freezer, ergovaline concentrations sometimes trend upward over time. In the Spring 2013 harvest, the Freezer Day 28 treatment had significantly higher ergovaline than all other freezer treatments and the control for that harvest. The raw data and observational notes from sample handling and preparation for this day were reviewed carefully, but there was no indication that these differences were due to lab procedures or analysis errors (9.93% RSD between the three sub-samples).

If additional studies confirm the potential for ergovaline concentrations to increase over time, it could be a major concern to researchers because research samples or low priority diagnostic samples are often stored for weeks before analysis due to high case volume or other lab priorities. A similar phenomenon was observed for the Fall 2012 harvest: an upward trend was evident when Freezer Day 28 was statistically higher than Freezer Day 21. No statistically significant increase was observed in the Spring 2012 with actual values on Freezer Day 1 of 539ppb and Freezer day 21 of 575ppb. This trend was observed in 2011 from a similar study at the University of Kentucky Veterinary Diagnostic Laboratory. Galassie (unpublished work, 2011) observed higher concentrations of ergovaline after 14 days of storage of intact tall fescue plant material; however milled samples with low, high and very high ergovaline concentrations did not show the same increase over time.

2.5 Conclusion

In conclusion, sample handling and storage may have a significant impact on the ergovaline concentrations within the tall fescue plant material. To obtain the most accurate analysis, all samples should be transported on ice to the laboratory immediately after harvest.

Based on the results of this research, ergovaline measurements may be significantly lower after 24 hours of storage, regardless of temperature. Since most testing facilities do not have the capability to perform testing the same day as harvest, researchers, extension personnel and producers should understand that the results they are currently receiving could show tall fescue ergovaline concentrations up to 60% lower than the fresh plant material in the field.

2.6 Implications and Future Research Avenues

This research has raised several new questions in understanding the stability of ergovaline in tall fescue material.

24 Hour Losses

For producers and diagnostic laboratories, the decrease in ergovaline concentration during the first 24 hours has very important on farm management implications. Based on ergovaline results, farm managers and producers may choose to take any number of preventative steps such as administration of domperidone to late-term mares, removal of cow-calf pairs from certain pastures, or reestablishment of a pasture with non-toxic tall fescue or other forages species. These decisions are costly, but doing nothing can result in decreased average daily gains in cattle, lost foals in horses and other costly physiological symptoms. Accurate ergovaline analysis is essential to making these decisions.

Future research should focus on understanding of potential ergovaline losses during plant sampling, transport and storage and develop techniques to prevent these losses. At present this research suggests that diagnostic laboratories should analyze samples the

same day as they are received. This recommendation is unrealistic for most laboratories. Therefore a more appropriate approach may be the development of a prediction equation or adjustment value to calculate concentrations in the field. If the decreases in ergovaline observed in this study had been consistent at the 24 hour mark, then analytical results could simply be increased by that amount. However, these results suggest that the percent ergovaline loss is dependent on either time of year or initial concentration (or both). If time of year is a major factor in ergovaline stability in the first 24 hours, then one solution would be testing samples at harvest and 24 hours later throughout the year to develop a prediction equation to adjust for these losses.

If the initial ergovaline concentration turns out to be the dominate variable, development of an adjustment value may be difficult. A possible solution would be to analyze for other compounds that could be products of ergovaline breakdown. Then a prediction equation could be developed for ergovaline concentration in the field based on the breakdown products after 24 hours of storage. In developing an adjustment value or prediction equation it is essential that they be based on factual data and not simply inserting a constant to account for what cannot be explained. Einstein viewed his cosmological constant, a mathematical fix he inserted to stop the expansion of the universe into his theory of general relativity, as his "greatest mistake" (National Aeronautics and Space Administration, 2012). In this research, the ultimate goal is to understand what happens to ergovaline in the first 24 hours and either account for these losses or prevent them.

Effect of Milling

It is unclear what the effect milling may have on ergovaline before storage. A common practice at this laboratory is to store samples at -20^{o}C as intact plant material (leaves, sheaths, stems and panicles) until the lab can analyze them. The day before analysis, the samples are removed from the freezer, frozen in liquid nitrogen and milled in one afternoon and stored overnight at -20^{o}C and then analyzed the following day.

Controlled studies of the effect of milling would be valuable in the future, but have not been conducted in this lab or by other researchers. In the original sampling procedure for

this study, the tall fescue plant material was to be milled on the day of analysis, but preliminary testing of this procedure showed that an unacceptable level of variability would be added to the results. In the preliminary study, vegetative plant material was collected in the field and whole tillers were randomly allocated into one of six groups. Once approximately 0.5kg of fresh plant material was accumulated per group, each group was placed on ice in a cooler, and transported to the laboratory. All groups were handled in the same manner and analyzed individually using the same method as in the main study (except that these were analyzed in duplicates) on the same day as harvest. As table 2.6 indicates, there was a significant difference between several of these samples even though no treatments had been applied. This suggested that homogeneous subsamples cannot be obtained by separating whole tillers collected from the same plant. This was not surprising given the large variation of ergovaline that can occur throughout the plant and even within a single leaf sheath (Spiering et al., 2002). Mace and colleagues (2012) reported 68% ergovaline variability between plants and 32% within a plant. It is unclear if milling had any effect on the concentrations throughout this study, but milling allowed complete mixing of the plant material before the storage treatments were applied.

Table 2.6. Samples collected by randomly allocating whole tall fescue tillers harvested from a small area of a horse pasture to one of six groups.

Sample	Sub-sample A	Sub-sample B	Average	Relative Standard Deviation**
	-------Ergovaline (ppb)*------			
001	720	673	696 bc†	5
002	895	895	895 a	0
003	785	697	741 b	8
004	550	632	591 c	10
005	664	615	639 bc	5
006	786	683	735 b	10

* parts per billion

** RSD ≤ 10 is considered acceptable

† Matching letters indicate no statistical significance ($P<0.05$)

Dr. Mace and colleagues have observed stratification of samples previously freeze-dried and milled. He has suggested that if some parts of the plant do not grind as efficiently as others, such as pseudostems and stems, then a range of particle sizes would be present in the sample. Smaller particles would separate down to the bottom of the container, leaving the larger particles at the top. When a portion of material was sub-sampled for analysis, it would be possible to collect only the larger particles if proper mixing did not occur just prior to sub-sampling. Because of the uneven distribution of ergovaline within the plant, this could drastically change the analysis results. His lab has also observed differences in the degree of extraction based on particle size. (pers. comm. 2013).

Freeze-Drying

Many researchers currently working in tall fescue alkaloid research routinely freeze dry samples before analysis. Freeze-drying was not initially included in this study because priority at the UK Veterinary Diagnostic Laboratory, where this research was conducted, is rapid turnaround for submitted samples. In the third harvest of this study, two additional treatments were included: ultra-low freezer (-80^{o}C) for one day and ultra-low freezer (-80^{o}C) for one day then freeze dried. The first treatment was included to possibly reduce the 24 hour losses observed in the first two harvests. The second treatment was requested by a fellow researcher whose standard procedure for ergovaline analysis is to freeze harvested plant material in liquid nitrogen, maintain at -80^{o}C overnight and then move to a freeze dryer. There were no additional losses of ergovaline in the freeze dryer compared to one day in the ultra-low freezer. Future studies should focus on better qualifying the effect of freeze drying on ergovaline concentrations.

Increasing Ergovaline in the Freezer over Time

The phenomenon of increasing ergovaline concentrations in the freezer in several of the storage treatments is of great interest to researchers. Research samples are often placed in a freezer for days or weeks before analysis, and increasing ergovaline levels would complicate any interpretation of results. These results cannot be explained by desiccation because all samples were adjusted for dry matter on the day of analysis and the replications in the Spring 2013 were very similar (RSD = 10%). Future research should

determine if this phenomenon is repeatable and then work to understand what is happening to the ergovaline.

Appendix A. Ergovaline concentration from each sub-sample, mean and relative standard deviation of sub-samples and date of analysis for Spring 2012 harvest.

	Sub-samples						Average of sub-samples		RSD*	Date of Analysis
	A		B		C					
Treatments	Ergovaline (ppb**)	Moisture (%)	Ergovaline (ppb)	Moisture (%)	Ergovaline (ppb)	Moisture (%)	Ergovaline (ppb)	Moisture (%)		
Control	626	81.2	667	81.1	661	81.0	651	81.1	3	5/1
Ice	619	81.9	703	80.7	628	81.8	423	81.5	7	5/1
Light + Heat	581	81.1	546	81.5	598	80.8	378	81.1	5	5/1
Ambient Temperature (22°C):										
1 Day	461	82.8	518	81.9	535	82.4	505	82.4	8	5/2
2 Days	445	82.1	340	82.2	310	82.5	252	82.3	19	5/3
3 Days	406	83.5	291	82.9	276	78.2	324	81.5	22	5/4
Refrigerator (5°C):										
1 Day	483	81.1	466	81.3	467	82.0	316	81.5	2	5/2
3 Days	320	82.0	319	82.4	308	81.6	315	82.0	2	5/4
6 Days	283	82.0	277	81.6	304	82.7	206	82.1	5	5/7
Freezer (-20°C):										
1 Day	498	81.1	553	82.2	567	81.6	539	81.6	7	5/2
7 Days	537	81.4	561	81.4	550	81.0	549	81.3	2	5/8
14 Days	540	80.9	589	81.4	558	81.9	562	81.4	4	5/15
21 Days	617	80.9	537	81.5	570	81.3	575	81.2	7	5/22
28 Days	536	81.4	496	81.3	491	81.6	508	81.4	5	5/29

* RSD ≤ 10 is considered acceptable

** parts per billion

Appendix B. Ergovaline concentration from each sub-sample, mean and relative standard deviation of sub-samples and date of analysis for Fall 2012 harvest.

	Sub-samples						Average of sub-samples		RSD*	Date of Analysis
	A		B		C					
Treatments	Ergovaline (ppb**)	Moisture (%)	Ergovaline (ppb)	Moisture (%)	Ergovaline (ppb)	Moisture (%)	Ergovaline (ppb)	Moisture (%)		
Control	1468	78.8	1685	80.0	1664	78.6	1606	79.1	7	8/21
Ice	1612	78.3	1500	79.2	1663	79.1	1592	78.9	5	8/21
Light + Heat	1430	79.7	1356	79.0	1521	79.2	1436	79.3	6	8/21
Ambient Temperature (22°C):										
1 Day	653	80.0	635	80.3	627	80.1	638	80.1	2	8/22
2 Days	719	81.2	679	81.4	709	80.9	702	81.2	3	8/23
3 Days	597	81.0	594	81.3	671	82.2	621	81.5	7	8/24
Refrigerator (5°C):										
1 Day	652	79.6	671	79.4	620	79.7	648	79.6	4	8/22
3 Days	567	78.8	594	80.0	526	79.2	562	79.3	6	8/24
6 Days	608	79.5	591	79.4	605	79.7	601	79.5	1	8/27
Freezer (-20°C):										
1 Day	702	78.8	693	78.9	685	79.1	693	78.9	1	8/22
7 Days	599	80.3	625	76.7	592	79.5	605	78.8	3	8/28
14 Days	626	77.7	605	78.8	684	78.9	638	78.5	6	9/4
21 Days	574	80.4	594	78.9	593	78.3	587	79.2	2	9/11
28 Days	687	77.7	559	79.1	740	78.8	662	78.5	14	9/19

* RSD ≤ 10 is considered acceptable

** parts per billion

Appendix C. Ergovaline concentration from each sub-sample, mean and relative standard deviation of sub-samples and date of analysis for Spring 2013 harvest.

	Sub-samples						Average of sub-samples		RSD*	Date of Analysis
	A		B		C					
Treatments	Ergovaline (ppb**)	Moisture (%)	Ergovaline (ppb)	Moisture (%)	Ergovaline (ppb)	Moisture (%)	Ergovaline (ppb)	Moisture (%)		
Control	506	79	542	80.1	523	79.5	524	79.5	3	6/11
Ice	440	79.4	486	80.9	509	79.7	478	80.0	7	6/11
Light + Heat	405	79.4	361	79	415	79.6	394	79.3	7	6/11
Ambient Temperature (22°C):										
1 Day	362	80.8	415	80.5	405	81.7	394	81.0	7	6/12
2 Days	285	80.8	252	80.9	278	80.8	272	80.8	6	6/13
3 Days	292	82.3	305	80.8	294	81.3	297	81.5	2	6/14
Refrigerator (5°C):										
1 Day	391	79.2	395	79.9	372	80.3	386	79.8	3	6/12
3 Days	308	81.3	284	80.3	305	80.9	299	80.8	4	6/14
6 Days	316	80.5	379	81.3	380	80.6	358	80.8	10	6/17
Freezer (-20°C):										
1 Day	500	79.7	355	79.3	414	79.5	423	79.5	17	6/12
7 Days	389	80.3	390	81.9	397	79.8	392	80.7	1	6/18
14 Days	394	78.9	439	80	382	79.9	405	79.6	7	6/25
21 Days	434	80.9	510	80.2	415	79.8	453	80.3	11	7/2
28 Days	613	80	643	80.8	529	79.7	595	80.2	10	7/9
Ultra-Low Freezer (-80°C):										
1 Day	442	79.6	450	79.6	366	80	419	79.7	11	6/12
1 Day + Freeze Dried	355	4.4	380	1.3	369	3.3	368	3.0	3	6/25

* RSD ≤ 10 is considered acceptable

** parts per billion

Appendix D. Number of calibrants used and R^2 value for calibration curve on each day of analysis for all harvests.

Harvest	Date	# Calibrants*	Calibration Curve R^{2**}
Spring 2012			
Day 0	5/1	3	0.9915
Day 1	5/2	3	0.9998
Day 2	5/3	3	0.9974
Day 3	5/4	3	0.9994
Day 6	5/7	3	0.9993
Day 7	5/8	3	0.9987
Day 14	5/15	3	0.9949
Day 21	5/22	3	0.9916
Day 28	5/29	4	0.9975
Fall 2012			
Day 0	8/21	3	0.9998
Day 1	8/22	4	0.9997
Day 2	8/23	4	0.9992
Day 3	8/24	4	0.9988
Day 6	8/27	3	0.9988
Day 7	8/28	3	0.9998
Day 14	9/4	4	0.9987
Day 21	9/11	3	0.9992
Day 28	9/19	3	1.000
Spring 2013			
Day 0	6/11	4	0.9986
Day 1	6/12	4	0.9994
Day 2	6/13	4	0.9998
Day 3	6/14	4	0.9968
Day 6	6/17	3	1.000
Day 7	6/18	3	0.9999
Day 14	6/25	4	0.9967
Day 21	7/2	4	0.9953
Day 28	7/9	4	0.9988

* four calibrants were made fresh each day. Outlier calibrants were sometimes excluded from calculations to strengthen calibration curve.

** 0.9900 or greater was considered acceptable

Appendix E. Ergovaline Concentration in tall fescue samples in response to various transportation and storage conditions for all harvests.

Treatments	Spring 2012		Fall 2012		Spring 2013	
	---------- Ergovaline (ppb)* ----------					
Control	651	A**	1606	A	524	B
Ice	650	A	1592	A	478	BC
Light + Heat	575	B	1436	B	394	DE
Ambient Temperature (22°C):						
1 Day	505	BC	638	CDE	394	DE
2 Days	365	D	702	CDE	272	H
3 Days	324	DE	621	DEFG	297	GH
Refrigerator (5°C):						
1 Day	472	C	648	CDE	386	DE
3 Days	316	DE	562	G	299	FGH
6 Days	288	E	601	DEFC	358	EFG
Freezer (-20°C):						
1 Day	539	BC	693	C	423	CDE
7 Days	549	B	605	DEFG	392	DE
14 Days	562	B	638	CDEF	405	DE
21 Days	575	B	587	EFG	453	CD
28 Days	508	BC	622	CD	595	A
Ultra-Low Freezer (-80°C):						
1 Day	-		-		419	CDE
1 Day + Freeze Dried	-		-		368	CDE

* parts per billion

** Matching letters represent no statistical difference ($P<0.05$) within harvest date

Appendix F

Inter-Laboratory Comparison of Ergovaline Concentration in Tall Fescue

Introduction

Analysis of tall fescue samples from livestock pastures is common throughout the United States to evaluate the risk of fescue toxicity to animals on pasture. These results are then used to alter management of pasture and/or animals to reduce potential problems. While there are many variables that affect the amount of ergovaline in tall fescue samples, it is unknown how much additional variation is introduced due to varied sample handling and storage conditions, sample preparation and extraction of samples across laboratories. The goal of the main thesis addresses variation associated with handling and storage conditions. The work in this appendix focuses on variation that can occur between laboratories due to differences in sample preparation, extraction, and analytical methods. The objective of this research is to determine the degree of variation seen among several prominent testing laboratories.

Materials and Methods

Material used in this study was originally collected as part of the UK Horse Pasture Evaluation Program in May of 2012 and analyzed by the University of Kentucky Veterinary Diagnostic Laboratory (UK VDL). In June, several client samples were identified that contained high ergovaline + ergovalinine (>1000 ppb) concentrations and moved to ultra-low freezer (-80^{o}C) storage. Each sample contained some milled material and some whole plant material left over from the original sampling. In July of 2013, these samples were taken to the UK VDL and stored at -20^{o}C. The remaining whole plant material was frozen in liquid nitrogen, milled and mixed with the existing milled material. Each sample was then mixed well and analyzed for ergovaline + ergovalinine concentration. Samples containing high concentrations and which had a large volume of material were then freeze dried using a bulk tray dryer (Labconco, Kansas City, MO) and analyzed for ergovaline concentration. After freeze drying, 3 samples still contained high (>1000 ppb) ergovaline concentrations (testing performed at the UK VDL). These samples were then mixed together and divided into 7 sub-samples. Each sub-sample was

then tested again for ergovaline + ergovalinine concentration to ensure that all samples were identical. Results from this are shown in Table 1 and illustrate that there was very little variation between the sub-samples.

Table 1. Ergovaline concentration of 7 sub-samples used for inter-laboratory comparison.

Sample	Ergovaline ppb*
A	1131
B	1166
C	1064
D	1121
E	1040
F	1001
X	1071
SD	57
RSD	5

* Represents ergovaline + ergovalinine parts per billion

Sub-samples were shipped overnight or hand-delivered to their respective laboratories in padded envelopes on July 23, 2013. Local recipients were instructed to leave the envelope on their lab bench over night before analysis. All recipients were instructed to report results back within 2 weeks. Participating laboratories are listed in table 2 and were instructed to handle their sub-sample just as they would handle routine diagnostic case samples.

Table 2. Participating Laboratories.

Laboratory	Location
Agrinostics Ltd.	Watkinsville, GA
Iowa State University Veterinary Diagnostic Laboratory	Ames, IA
Oregon State University Endophyte Service Laboratory	Corvallis, OR
University of Kentucky Lowell Bush Research Laboratory	Lexington, KY
University of Kentucky Veterinary Diagnostic Laboratory	Lexington, KY
University of Missouri Veterinary Medical Diagnostic Laboratory	Columbia, MO

Results

Table 3 contains the reported analysis from the 6 participating laboratories. The identity of individual laboratories is not connected to reported analysis to protect the confidentiality of each lab. All samples were analyzed using some variation of HPLC except sample F, which was analyzed using ELISA. Laboratories A and C reported ergovaline only, while laboratories E and X reported a combined ergovaline/ergovalinine and laboratory F reported total ergot alkaloids. Samples X and D were analyzed in July, samples A and C in August and samples E and F in November and December respectively. Because of the variations in analytes reported and the dates of analysis, statistical comparisons of these results are not beneficial.

Table 3. Reported methods and results for sub-samples used in inter-laboratory comparison.

Sample	Date of Analysis	Method	Reported Analyte
A	8/8/2013	HPLC with fluorescence detection	541 ppb ergovaline
C	8/8/2013	HPLC with fluorescence detection	545 ppb ergovaline
D	7/30/2013	HPLC with fluorescence detection	1320 ppb ergovaline
E	11/17/2013	HPLC with fluorescence detection	755 ppb ergovaline + ergovalinine
F*	12/15/2013	ELISA	2482 ppb total ergot alkaloids
X	7/24/2013	Reversed-phase chromatography using UPLC with fluorescence detection	1022 ppb ergovaline + ergovalinine

* Sample F was shipped on 10-8-13 because the original sample, B, was lost

Conclusions

We can conclude that producers will get different analysis results based on what laboratory analyzes the sample. These differences are likely due to differences in a number of variables including: sample preparation and extraction method, standards used and analysis method and reported analytes. Researchers, extension personnel and veterinarians should keep this in mind when comparing cases from one area of the country to another, across testing facilities, when interpreting data from literature or developing guidelines.

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Vita

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