MITIGATION OF CONDENSED TANNINS FOUND IN SERICEA LESPEDZA (*LESPEDZA CUNEATA)*

by

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M.S., Colorado State University, 2009

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Abstract

Sericea lespedeza (**SL**; *Lespedeza cuneata*) is classified as an invasive plant throughout the Great Plains. It infests over 600,000 acres in Kansas. Increasing grazing pressure on SL may reduce seed production and slow the spread of the plant; however, intake of SL by grazing beef cattle is poor, due to the presence of tannins in the plant. Condensed tannins reduce protein digestion by ruminants and may also decrease plant palatability. Detailed study of the appetite-suppressing effects of SL under controlled conditions is essential in order to develop appropriate strategies to increase grazing pressure on this plant. Such information could lead to a degree of biological control of this noxious weed using domestic herbivores.

We compared intakes of tallgrass prairie hay by beef cows when hay was either uncontaminated or heavily contaminated by SL. Beef cows fed contaminated hay exhibited a profound aversion to compared to similar uncontaminated hay. Furthermore, differences in voluntary DMI between contaminated and uncontaminated hays of similar chemical composition were manifested rapidly after introduction of contaminated hay into beef cow diets.

Supplementation with corn steep liquor (CSL) increased tolerance of beef cows for SL. It ameliorated the negative consequences of tannin consumption in a dose-dependent manner when fed to beef cows in confinement. The beef cows in our study had only limited opportunity to selectively avoid SL because it was offered in chopped form and in a mixture with other forage species. It was unknown if beef cattle supplemented with CSL would readily consume forage contaminated by SL when uncontaminated forage was available simultaneously. Therefore, we examined the effects of CSL fed to beef cows on voluntary selection of tallgrass prairie hay contaminated by SL when uncontaminated tallgrass prairie hay was also available. Supplemental CSL (0.6 kg DM/d) increased both acceptance of and tolerance for SL by beef cows. It ameliorated some of the negative consequences of tannin consumption on digestible DM intake. In addition, voluntary consumption of SL-contaminated forage increased by 25% in supplemented vs. unsupplemented beef cows. It is unknown if supplemental CSL can promote voluntary selection of actively-growing SL by beef cattle grazing native rangeland in the Kansas Flint Hills.

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Major Professor
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Dedication

I am dedicating this work to my parents, Allen and Marla, my fiancée, Nichole and to God. Through Him all things are possible.

CHAPTER 1 - REVIEW OF LITERATURE

Microhistological Analysis of Grazed Diets

The botanical composition of diets grazed by herbivores has been evaluated through direct observation of bite counts, analysis of stomach contents from dead or sacrificed animals, analysis of ruminal or esophageal digesta collected from fistulated animals, and analysis of fecal material (Holechek et al, 1982). Microhistological identification of plant fragments in ruminal or esophageal digesta is generally regarded as the method least prone to estimation error; however, analysis of fecal material has become the most widely used method for quantifying the botanical composition of herbivore diets. The popularity of fecal microhistological analysis over that of esophageal or ruminal digesta stems from the fact that it is less intrusive, requires no surgical modification of the animal, does not disrupt normal grazing behavior, sample collection is not constrained by the availability of fistulated animals, and it can be used to characterize diets of non-domesticated species (Leslie et al., 1983; Soder et al., 2009).

Microhistological characterization of the botanical composition of grazing-herbivore diets using any digesta component relies upon the accurate identification of the distinguishing epidermal characteristics of various forage plants when viewed under magnification (Anthony and Smith, 1974). An assumption must be made that the epidermis of all ingested plants survives digestion in proportion to its appearance in the diet and is recognizable in the digesta component of choice. Since each plant has a unique cuticle, which is generally undigested in the gut, their use makes identifying plant species convenient and reasonably sure.

Anthony and Smith (1974) and McInnis et al. (1983) directly compared microhistological evaluations of fecal material and ruminal masticate to characterize grazing herbivore diets. Sampling of ruminal contents required that an animal be surgically fistulated. Such animals required more care and management than intact animals and required also that animals be restrained for 30 to 90 minutes to facilitate ruminal evacuation prior to sample collection. To make an accurate comparison between fecal and ruminal characterization techniques required three things: 1) that the same analytical procedures are used for both sets of samples; 2) that correct sample population sizes are used to achieve a predetermined rate of variability control;

and 3) that the majority of plant material be readily identifiable under magnification (Chapuis et al., 2001). Anthony and Smith (1974) reported that to effectively characterize diets using ruminal samples, the sample population must be at least 2-5 times greater than the population required for fecal characterization. Sparks & Malechek (1968) reported a 1:1 ratio between relative frequency of plant fragments in fecal material and actual dry weight percentages in the diet.

Limitations of microhistogical characterization

Microhistological characterization of grazed diets has analytical limitations. Regardless of sampling technique, digestibility and retention times of forages must be taken into account. The ability to detect minor dietary components in fecal samples becomes more difficult as the extent of digestion increases and retention time increases (Wydeven and Dahlgren, 1982). The major limitation of fecal microhistological analysis is that it tends to over-estimate shrub and grass components in herbivore diets, while at the same time under-estimating the forb component when compared to ruminal analysis (Stewart, 1970; Slater and Jones, 1971; Anthony and Smith, 1974; Vavra et al., 1978; McInnis et al., 1983; Lewis, 1994). Anthony and Smith (1974) reported that this bias may be due to the greater relative digestibility of forbs compared to shrub leaves and grasses. Interestingly, these researchers also reported that ruminal samples only represented what had been consumed within the 2 most recent feeding periods, while fecal samples tended to represent 4 to 8 feeding periods. Other factors that affect the accuracy of the microhistological technique included: woody materials present in the diet (Holechek and Valdez, 1985a; 1985b), observer errors (Holechek et al., 1982), calculation procedures used to estimate diet botanical composition (Holechek and Gross, 1982), sample-preparation techniques (Vavra and Holechek, 1980; Holechek et al., 1982), and presence of unidentifiable plant fragments in fecal material (Slater & Jones, 1971).

Chapuis et al. (2001) reported that diets must not be characterized during only a single season of the year and represented as an average of annual consumption; inter-seasonal variation in the forage selection patterns of herbivores can be significant.

A general drawback to using microhistological techniques to characterize the botanical composition of grazing-herbivore diets is the loss of sensitivity that occurs when attempting to

differentiate plant fragments that originate from closely-related plant species (Carriere, 2000). Under these circumstances, it may only be possible to categorize individual plant fragments into family or genus groups.

Lespedeza cuneata

Lespedeza cuneata, commonly referred to as sericea lespedeza (**SL**), is a perennial herbaceous legume native to eastern Asia (Bradley & Masters, 2007). Sericea lespedeza was imported to the United States from Japan in 1896 as a potential warm-season, perennial, drought-resistant forage crop (Altom et al., 1992; Koger et al., 2002; Dudley & Fick, 2003). It later became a popular crop in areas where soil fertility was poor (e.g., the southeastern United States; Lynford et al., 1967; Kalburtji & Mosjidis, 1992; Koger et al., 2002). Sericea lespedeza has been used to prevent topsoil loss on highly-erodable land and along roadsides (Stitt, 1939; Koger et al., 2002; Mantz, 2007). Following its introduction into the Tallgrass Prairie biome during the 1980s, SL became a serious ecological threat to the region (Eddy et al., 2003).

Initiation of this problem began with the enactment of the 1985 Farm Bill. A provision within the bill, the Conservation Reserve Program (CRP), mandated reestablishment of native grasses; however, most of the native-grass seed harvested for this purpose was contaminated with sericea lespedeza and it was unwittingly propagated via the CRP program. Sericea lespedeza is a highly invasive species that is a prolific seed producer. It generates an estimated 670 kg of seed / hectare annually when grown in pure culture (Wang et al., 2008). Aggressive growth enables SL to out-compete native forage species, reducing native grass production by 92%; it also harms native insect populations and decreases habitat availability for native birds (Eddy et al., 2003). Sericea lespedeza has spread rapidly throughout Kansas since its unintended introduction and can now be found in regions of Colorado, Missouri, Nebraska, Oklahoma, and Texas. It infests approximately 600,000 acres in Kansas alone (Eddy et al., 2003). Colorado and Kansas have declared SL a noxious weed and Missouri is considering declaring SL a noxious weed at the time of this writing (Bradley and Masters, 2007; Mantz, 2007). Spread of SL in the Tallgrass Prairie biome has caused concern for its ecological integrity (Eddy et al., 2003).

Intake of SL by most classes of grazing livestock is negligible due to the presence of tannins in the plant (Terrill et al., 1989; Mantz et al., 2009). Condensed tannins significantly

reduce dietary protein and DM digestion by ruminants (Jones and Mangan, 1977; Terrill et al., 1989). These circumstances create negative post-ingestive consequences which deter consumption (Mantz et al., 2009). Poor intake of sericea lespedeza translates to negligible grazing pressure, which ensures that sericea lespedeza will be able to produce seed and continue to proliferate.

In areas of Kansas where sericea lespedeza is prevalent (i.e., the Flint Hills), growing transient stocker cattle on native tallgrass range is a major agricultural avocation (Mantz, 2007). Much of the grazing in the Flint Hills region has been managed over the past 3 decades under a management system known as intensive early stocking (Mantz, 2007). Intensive early stocking (IES) dictates that an entire year's grazable forage allocation be consumed by transient stocker cattle in 90 to 120 days, usually from mid-April or early May to mid-July or late August. In contrast, the yearly allocation of grazable forage is usually removed through herbivory over 150 to 210 days (between mid-April and late October) in conventional season-long grazing management. In order to achieve similar stocking rates (animal units / acre / year) in both season-long and IES systems, stocking densities (animal units / acre) under IES management are typically double those for season-long management (Smith and Owensby, 1978). The popularity of IES stems from the fact that cattle performance increases per unit of land area, compared with season-long management (Mantz, 2007; Smith and Owensby, 1978). The spread of SL throughout the Flint Hills has damaged the viability of IES stocker enterprises.

Sericea lespedeza is a poor forage crop in native range ecosystems because of high concentrations of condensed tannins found within its leaves, coupled with high amounts of lignin in stems (Lynford et al., 1967; Petersen and Hill, 1991). Stocker cattle tend to graze SL only when it is immature and tannin concentrations are relatively small. Grazing of SL ceases as tannins increase with plant maturity (Stitt, 1939; Altom et al., 1992; Wang et al., 2008). When grown in monoculture or when phenologically immature SL is still only marginally palatable even in low-tannin varieties (Wehtje et al., 1999).

Control of Lespedeza cuneata

Two general methods of SL control have been evaluated by scientists: treatment with herbicides at different stages of plant growth and implementation of biological controls via

introduction of natural plant predators. Control of sericea lespedeza with chemicals has been effective when applied early in the infestation (Eddy et al., 2003). Herbicides applied at ground level will retard the spread of sericea lespedeza; however, they are difficult to apply properly under dense plant canopies and on steep or rocky terrain. Aerial broadcast application of herbicides can alleviate terrain issues but does not penetrate the dense plant canopies that characterize the Tallgrass Prairie (Eddy et al., 2003).

Bradley and Masters (2007) reported that application of triclopyr [(3,5,6-trichloro-2-pyridinyl)oxy acetic acid] was effective when applied to vegitative SL in June or July. In addition, control was achieved with metsulfuron [2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]cabonyl]amino]sulfonyl]benzoic acid] application during early bud to bloom growth stages (which typically occur in September and October). Koger et al. (2002) reported that the most effective times to apply herbicides were during the branched-stem and flowering growth stages for metsulfuron and during the simple-stem and branch-stem growth stages for triclopyr and fluroxypyr [1-methylheptyl ((4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy)acetate]. Bradley and Masters (2007) reported also that prepackaged combinations of triclopyr and fluroxypyr provided equal or better single-season stem-density reduction than triclopyr or metsulfuron alone.

Use of herbicides in combination or alone may result in only a 7 to 9% single-season reduction of the pretreatment stem density (Koger et al., 2002). Annual use of herbicides may be necessary for long-term control of SL. Likewise, use of any herbicides may also destroy broad-leaf native vegetation (i.e., forbs) that are an integral part of Tallgrass Prairie plant communities (Eddy et al., 2003).

Eddy et al. (2003) examined the possibility of using insects as a form of biological control to stop SL from spreading. The 3-cornered alfalfa hopper, (*Spissistilu festinus* Say, Family Membracidae) preyed upon SL during April and May and the grasshopper (*Schistocerca americana* Drury, Family Locustidae) preyed upon SL during the latter poritons of the growing season. Presence of SL did not alter populations or growth of either insect; however, only grasshopper herbivory reduced seed production. .

The lespedeza webworm (*Tetralopha scortealis* Lederer, Family Pyralidae), which has been studied in Virginia, North Carolina, and Georgia, generally occupies the same ecosystem niches as grasshoppers. Lespedeza webworms damage SL by defoliating the plant and encasing it within a matrix of silk threads. Lespedeza webworms reduced seed production of SL from 644 seeds / plant to 5.7 seeds / plant (Eddy et al., 2003). This reduction in seed production ultimately reduced the capability of SL to replenish itself following herbicide treatments. Unfortunately, lespedeza webworms had a low survival rate in Kansas, presumably due to dry conditions during summer and the cold of winter. Therefore, lespedeza webworms must be re-established annually for effective SL control.

Ruminants are generally more resistant to the effects of tannins than are non-ruminants (Robbins et al., 1991). Certain species of ruminants have remarkable tolerances for the tannin and palatability issues associated with SL. Goats are more resistant to the effects of tannins than are cattle and sheep (Robbins et al., 1991). In fact, goats may develop a strong preference for SL once they have become accustomed to the plant (Hart, 2001); however, Provenza et al. (1990) noted that this preference may become less pronounced as tannin concentrations increase with SL maturation during the growing season.

Hart (2000) reported the effects of goat grazing on population dynamics of SL in native range ecosystems. Goats preferentially browsed on seedling stems, thereby reducing the spread and perpetuation of SL. During an initial control attempt, the number of goats required to control sericea lespedeza was relatively large (6 - 8 goats / infested acre); however, fewer goats were required in subsequent growing seasons. Only 25% of SL was removed in the first year of grazing by goats; however, the surviving plants were weakened significantly and exhibited depressed growth the following year. After 4 years of goat grazing, little SL remained. Once this level of control was achieved, a minimum of 3 goats/infested acre were recommended to prevent SL reinfestation.

Little specific attention has been given to the use of beef cattle as a potential biological control for SL. Unlike herbicides and insects, using livestock as a biological control mechanism for SL presents landowners with an opportunity to recover their investment in SL control. Goats would serve in this capacity; however, few Flint Hills pastures are adequately fenced to contain

goats, predation on goats would be difficult to limit, and the Midwestern market for goat meat is underdeveloped. Beef cattle, the most economically relevant herbivore in the Flint Hills, would be much more convenient to use as a biological control agent. The obvious problem with this approach is relatively poor tannin tolerance of beef cattle. An understanding of tannin chemistry may provide insight into possible mechanisms to enhance tannin tolerance of that species.

Biological Activity of Tannins

Biosynthesis of tannins

Tannins are polymers of flavan-3-ol units, which are derived from both the pheyipropanoid and malonate-CoA pathways (Figure 1; Aerts et al., 1999). Synthesis of tannins is similar to that of the formation of anthocyanin pigments (Aerts et al., 1999). The shared first step of the pathways is catalyzed by chalcone synthase and the final shared step is catalyzed by dihydroflavonol 4-reductase (Aerts et al., 1999). To be committed to forming tannins, leucoanthocyanidins must be reduced by NADPH to form catechins by leucoanthocyanidin reductase (Aerts et al., 1999). Tannins, which are chemically similar to lignins, are synthesized by the phenylpropanoid biosynthetic pathway (Figure 2; Aerts et al., 1999). Positive correlations between high concentrations of lignin and tannin occur particularly when growth and nutrient quality of the soil are poor (Aerts et al., 1999). Tannins condense via polymerization as plant tissues mature (Aerts et al., 1999).

Negative effects of tannins

Tannins are functionally defined by their ability to bind proteins and are found naturally in 2 forms: condensed tannins, which are the most widely-occurring secondary compounds in vascular plants, and hydrolysable tannins which are found only in dicotyledons (Aerts et al., 1999; Bergvall and Leimar, 2005; DaCosta et al., 2008). Tannins are primarily stored in plant vacuoles, the epidermis, and trichomes of leaves (Aerts et al., 1999). Nutritional consequences are multifaceted and differ between tannin types. Tannin concentrations vary among plant species, plant component, and phonological maturity. Individual plant components may contain up to 40% tannin by weight depending on maturity and plant type (Bergvall \and Leimar, 2005).

Tannins spontaneously complex with macromolecules and metal ions via the activity of phenolic nuclei (Aerts et al., 1999). Tannin complexes decrease digestibility of plant nutrients, particularly protein, at concentrations exceeding 6% of dietary dry matter (McAllister et al., 2005). Severity of this effect depends upon characteristics of the tannin consumed and animal adaptation to tannins (Robbins et al., 1991). Tannins may also exert allelochemical effects through toxicosis and have negative effects on palatability (Waterman et al., 1980). These effects are likely to occur when the concentrations of condensed tannins relative to hydrolysable tannins are high (Bergvall and Leimar, 2005; DaCosta et al., 2008).

Presence of plant secondary compounds, like condensed tannins, deters grazing (Bergvall and Leimar, 2005). Many herbivores self-regulate consumption of secondary compounds and the plants that contain them in ways that minimize negative effects of the secondary compounds (Bergvall and Leimar, 2005; Lyman et al., 2008). Grazing ruminants dilute intake of potentially-toxic secondary compounds with non-toxic plant materials to meet nutrient needs while still consuming a modicum of plant secondary compounds like condensed tannins (Bergvall and Leimar, 2005).

Condensed tannins are polyphenolic compounds with massive molecular weights that bind strongly to proteins, forming insoluble complexes within the lumen of the gut (DaCosta et al., 2008). Condensed tannins are the predominant form of plant secondary compound found in SL and are in a class of polyphenols that are common to many plant species.

Condensed tannins found in SL form semi-irreversible complexes with proteins found in feedstuffs (Jones and Mangan, 1977; Veresegyhazy and Fekete, 1990; Hagerman et al., 1992; Agudelo et al., 1997; Barahona et al., 1997; Vasta et al., 2009). Significant protein-tannin complexes form when tannin concentrations are as little as 4% of dietary dry matter (Agudelo et al., 1997; Martinez et al., 2005; McAllister et al., 2005). These compounds can be oxidized under aerobic conditions; however, under anaerobic conditions polymers formed with condensed tannins are stable (Hagerman et al., 1992).

Tannin-protein complexes are a function of environmental pH and involve hydrophobic effects and hydrogen bonds (Aerts et al., 1999). Protein precipitation is greatest when pH is near the isoelectric point of the proteins. When pH values are above the pKa of tannin phenolic

groups (i.e., pH 8) condensed tannins will not precipitate proteins. Condensed tannins preferentially bind to large proteins, conformationally-open proteins, and proline-rich proteins (Hagerman et al., 1992). Effects of CT are most likely associated with inhibition of proteases or by protecting proteins from protease activity (Aerts et al., 1999).

The extent of condensed-tannin activity suggests that other factors in digestion may also be affected. In addition to binding proteins, tannins decrease digestion of carbohydrates, fatty acids, and overall dry matter. Microbial activity, microbial populations, and dry matter intake are also negatively affected (Singh and Roy, 1980; Hanely and Robbins, 1992; Barahona et al., 1997). Condensed tannins are also suspected of indirect negative effects on intestinal, liver, and renal function (Robbins et al., 1991). Condensed tannins alter the membranes of intestinal cells, thereby decreasing enzyme secretion or inactivating functional enzymes (Waterman et al., 1980).

Decreased enzyme activity ultimately decreases the ability to digest feedstuffs into readily-absorbable components within the intestine. In particular, α-amylase and enterokinase activity are inhibited by condensed tannins (Blytt et al., 1988). Barry and Manley (1986) reported that this fact accounted for decreased carbohydrate digestion in high-tannin diets. Condensed tannins also decrease digestion of cellulose (McArthur, 1988) and hemicellulose (Robbins et al., 1987).

Condensed tannins reduce intestinal retention rates in ruminants fed forage-based diets (Robbins et al., 1987). Apparently, this does not occur in ruminants fed concentrate-based diets (Barry and Manley, 1986; Vasta et al., 2009). Vasta et al. (2009) reported that DMI by ruminants decreased in forage diets which contained condensed tannins but increased in concentrate diets containing condensed tannins. These researchers noted that there were minimal changes in digestibility of concentrate diets with condensed tannins but profound decreases in digestibility of forage diets with condensed tannins.

Both condensed and hydrolysable tannins exert negative effects on microbial growth (Gamble et al., 1996). Condensed tannins exert their negative effects by inhibiting ruminal microbial processes (Salawu et al., 1999). Condensed tannins reduce ruminal protozoal counts; this effect is coincident with decreased ruminal fiber degradation (Salawu et al., 1999; Acamovic and Brooker, 2005).

Benefits of tannins

Some properties of condensed tannins may be beneficial to ruminant animals. These properties include: control of bloat, anthelmintic activity, and reduced methane production. In addition, small daily intakes of condensed tannins may improve gross measures of animal productivity. Frothy bloat incidence is decreased when small amounts of condensed tannins are introduced into ruminant diets (Aerts et al., 1999; Rochfort et al., 2008. The mechanisms behind reduction of bloat involve the formation of tannin protein complexes in the rumen. Proteins, particularly those that are highly soluble, have been implicated as the primary causative agent in bloat. If these proteins can be bound by condensed tannins in the rumen, the likelihood of froth formation is reduced. Interseeding pastures that contain bloat provocative forages (e.g., alfalfa) with species of plants that contain condensed tannins may reduce the occurrence of bloat (Aerts et al., 1999; Rochfort et al., 2008).

Condensed tannins have anthelmintic properties in ruminants. Incidence of resistance to traditional anthelmintic compounds has focused attention on the use of condensed tannins for the control of intestinal nematodes. Inclusion of birdsfoot trefoil and chicory in diverse pastures can reduce nematode infestations in ruminants (Aerts et al., 1999; Rochfort et al., 2008).

Grazing ruminants are responsible for the majority of the animal-origin methane produced. Acetate, a voliatile fatty acid produced by ruminal fermentation, is produced abundantly during fermentation of fibrous diets. When actetate production is high, there is an excess of free hydrogen within the rumen. Excess hydrogen is disposed of through methane formation which is subsequently eructated by the animal. Alteration of fermentation products to decrease acetate production relative to other volatile fatty acids reduces the amount of methane produced. Condensed tannins decreased methane production in ruminants when incorporated into diets at a rate of 2 to 4% of diet DM (Williams et al., 2009).

Condensed tannins are able to increase production capabilities of certain livestock species. Min et al. (1999) demonstrated that presence of low levels of condensed tannins in diets of sheep improved reproductive performance and wool production. In addition, Rochfort et al. (2008) noted that milk yield by ewes fed diets with small amounts of condensed tannins was greater than that by ewes fed tannin-free diets.

Small intakes of condensed tannins have been associated with greater post-ruminal protein availability (Hanely and Robbins, 1992; Barahona et al., 1997). Condensed tannins readily bind proteins in the rumen, rendering them impervious to microbial attack. When ruminal protein degradation is minimized by low dietary inclusion of condensed tannins, tannin-protein complexes pass to the abomasum where they are exposed to hydrochloric acid. A limited degree of hydrolysis of the tannin-protein complex may occur under these conditions (Haring et al., 2007). Altering the site of degradation for amino acids that are poorly represented in microbial cell protein may lead to improved animal performance in certain circumstances.

Polyethylene Glycol

Polyethylene glycol (**PEG**) binds to condensed tannins and prevents them from interacting with proteins (Mantz, 2007). In the absence of PEG, tannins bind strongly to proteins resulting in formation of insoluble tannin-protein complexes (Villalba and Provenza, 2002). When bound to PEG, condensed tannins lose their affinity for dietary proteins (Waghorn et al., 1987). Polyethylene glycol has been proposed to interrupt H bonding between proteins and phenolic hydroxyl groups of condensed tannins (Mantz, 2007). Supplementation of ruminant diets with PEG increased intake or digestibility of feeds containing tannins in some circumstances (Priolo et al., 2000; Landau et al., 2002; Villalba et al., 2002; Mantz et al., 2009).

Feeding PEG in conjunction with high-tannin forage diets has not been widely adopted in the U.S. for two reasons: 1) feeding PEG at the rates necessary to increase intake of high-tannin forages is cost prohibitive and 2) feeding PEG at the rates necessary to increase intake of high-tannin forages is disallowed from a regulatory standpoint (AAFCO, 2008). Therefore, it is advantageous to identify substances that are generally regarded as safe (GRAS; FDA, 2011), cost effective, and that mitigate the consequences of consuming a diet high in tannins. Compounds that share the chemical characteristics of PEG (i.e., those that contain long hydrocarbon chains) may also interact with tannins and prevent formation of tannin-protein complexes.

Corn Steep Liquor

Corn steep liquor (**CSL**) is one of several byproducts produced during the wet milling of corn (Liggett and Koffler, 1948; Talpada et al., 1987; Hull et al., 1996). The volume of CSL

produced in the United States is in excess of 650,000 tons annually (Hull et al., 1996; CRA, 2006). Corn steep liquor is a sweet-smelling, viscous slurry that may range in color from light to dark brown (Talpada et al., 1987). The dry matter percentage of CSL ranges from 45 – 55 % (Liggett and Koffler, 1948; Talpada et al., 1987; CRA, 2006). Production methods for CSL vary somewhat between production facilities; therefore, chemical composition may be slightly different from source to source. In addition, small variations in chemical composition are common between production runs within a single plant (Liggett and Koffler, 1948).

Advances in the steeping process have enabled production facilities to decrease product variability (CRA, 2006). Not all variation can be controlled. Liggett and Koffler (1948) reported that there was a slight variability in between seasons, which was attributed to changes in the microbial flora present during the steeping process. As a feed for livestock, CSL is a good source of protein, free amino acids, energy, vitamins, and minerals (Wagner et al., 1983; Talpada et al., 1987; CRA, 2006). In addition, CSL has an effective storage life that is much longer than most other byproduct feed (Scott et al., 1997).

Corn steep liquor in animal diets

Corn steep liquor has been approved by the American Association of Feed Control Officials as a *Generally Regarded as Safe* compound for use in food animal diets (AAFCO, 2008). It has been used to manufacture feeds for companion animals, fish, ruminant livestock, monogastric livestock, poultry, and honeybees (Talpada et al., 1987; CRA, 2006).

Use of CSL in animal diets has increased in part because of the cost of molasses. Wagner et al. (1983) reported that supplemental CSL resulted in greater weight gains by cows and heifers than supplementation with other compounds of similar chemical composition. Supplementation with CSL reduced ruminal concentrations of acetate while increasing butyrate and isovalerate concentrations, compared with other supplements. Wagner et al. (1983) concluded that CSL was an effective protein source for cattle consuming low quality forages.

Corn steep liquor has also been used to improve the quality of pelleted feeds. Defrain et al. (2003) also noted that the protein from CSL tended to compliment the fiber in typical pellet binders like soybean hulls. Increasing the amount of CSL in pelleted feeds tends to increase

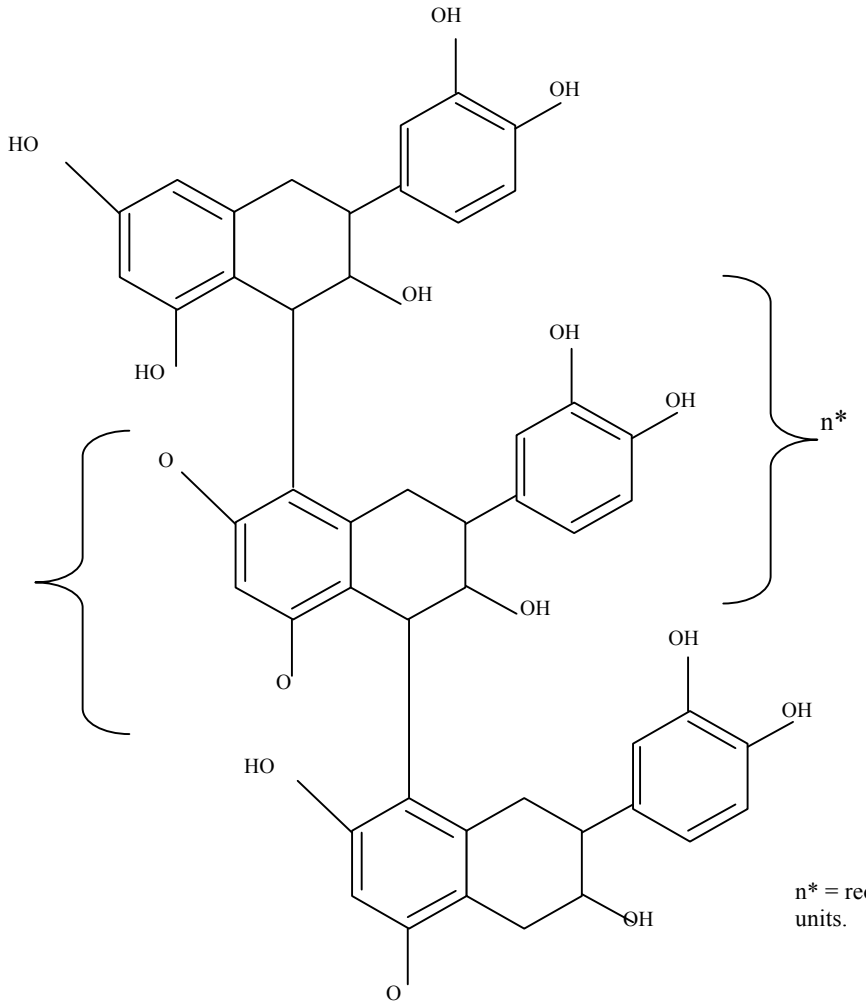
pellet durability index. When combined, CSL and soybean hulls are a good source of methionine and can replace a portion of the corn in cattle diets at a lesser cost.

Corn steep liquor in industrial applications

Corn steep liquor has long been used in the production of certain pharmaceuticals (Lawford and Rousseau, 1997). It was first used in the 1940s as a growth medium in the production of penicillin (Saha and Racine, 2010). Today, CSL is widely used in industrial fermentation, production of vitamins, production of peptides, and production of mannitol (Lawford and Rousseau, 1997; Obayori et al., 2010; Saha and Racine, 2010). Traditional methods for mannitol production require use of expensive media components to sustain the life of bacteria which produce mannitol. Substituting CSL for these media provides inexpensively the vital nutrients and growth factors that mannitol-producing bacteria require. Variations in CSL pH can alter the growth and metabolism of the bacteria; therefore, it is imperative that when substituting CSL into bacterial growth media that pH is controlled (Saha and Racine, 2010). Corn steep liquor has also been an important alternative in the growth media of bacteria that consume pollutants. Obayori et al. (2010) reported that growth media fortified with CSL increased bacterial degradation of pyrenes, a common industrial pollutant. This effect was attributed to increased hydrocarbon availability to bacteria.

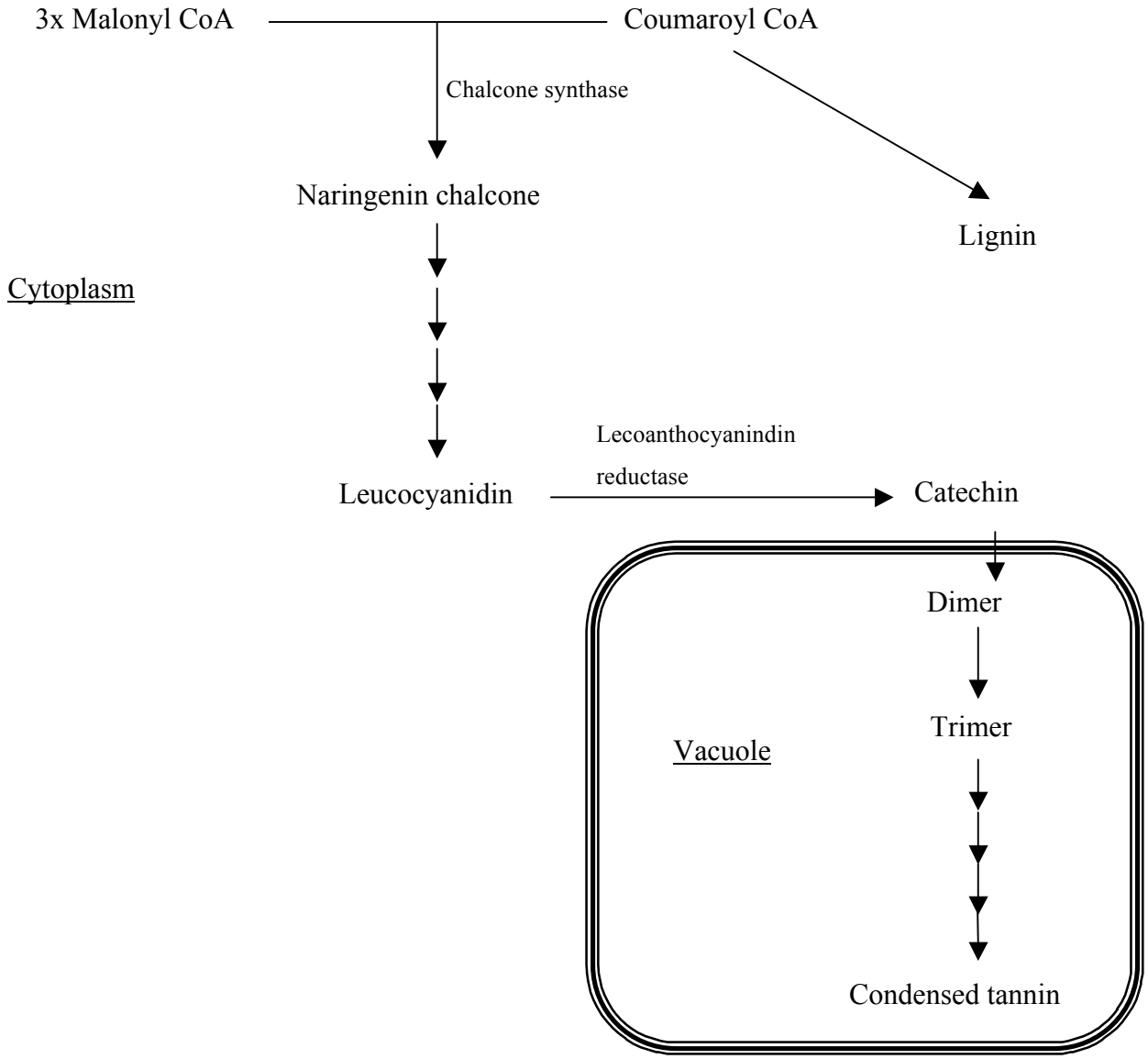
Tables and Figures

Figure 1.1: Structure of a condensed tannin subunit adapted from Hagerman et al., 1992.



n* = reoccurring subunit ranging from 4-5 units.

Figure 1.2: Proanthocyandin biosynthesis adapted from Aerts et al., 1999.



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CHAPTER 2 - MICROHISTOLOGICAL CHARACTERIZATION OF THE BOTANICAL COMPOSITION OF DIETS GRAZED BY BEEF COWS IN THE KANSAS FLINT HILLS DURING WINTER

Abstract

A study was conducted on native tallgrass range in the Kansas Flint Hills to establish the validity of using microhistological analysis of cattle feces to quantify the botanical composition of mature cow diets grazed during winter. Standard microscope slides were prepared of cross-sectional leaf and stem particles from 10 predominant range plants (*Andropogon gerardii* (AG); *Schizachyrium scoparium*; *Bouteloua curtipendula*; *Bouteloua gracilis* (BG); *Panicum virgatum*, *Sorghastrum nutans* (SN), *Amorpha canescens* (AC), *Symphyotricum ericoides*, *Liatris punctata*, and *Dalea purpurea* (DP)). Mature beef cows (n = 10) were allowed to graze a single native tallgrass pasture for 30 d. Subsequently, 1000 g of wet fecal material was collected from each cow. Fecal material was dried and ground to pass a 1 mm screen; subsamples were affixed to slides (5 slides / cow) and compared to standards. Each slide was divided into twenty fields. Range-plant particulate counts were analyzed by animal; plant prevalence in fecal material was assumed to be equivalent to diet composition on a DM basis. Diets were composed of 66% grass and 34% forbs. Predominant grass species included AG (9%), BG (16%), and SN (13%). Predominant forbs were AC (8%), and DP (14%). This subgroup of plants represented 54% of the diets selected by cows in our study. Nine percent of grass-plant fragments could not be identified with any of the grasses for which standards were prepared: moreover, 4% of forb fragments could not be identified with any of the forbs for which standards were prepared. Unknown plant fragments composed 6% of all plant fragments in our slide fields. In conclusion, methods for measuring diet composition of herbivores from microhistological examination of fecal samples were successfully adapted to cattle grazing native range in the Kansas Flint Hills during winter. Results of this study may allow for the management of rangeland for the propagation of forages that are more heavily consumed by grazing ungulates.

Introduction

Microhistological analysis has been widely used for determining diet composition and habitat usage by wild and domestic herbivores (Holechek et al., 1982). Validation of the methods used to analyze botanical composition of grazed forages from fecal material has been conducted by Sparks and Malechek (1968) and Holechek and Gross (1982). Variation was noted in fecal recovery of certain forages based upon differences between animal species (Leslie et al., 1983). Previous studies have concluded that fecal microhistology is an effective method for determining dietary composition when there is an inability to monitor grazing behavior or when collection of masticate samples in not practical (Anthony and Smith, 1974; Holechek, 1982; Lewis, 1994) . Comparisons between fecal and masticate sample analysis have demonstrated that fecal analysis is an acceptable method for estimating dietary composition of grazing ungulates (Anthony and Smith, 1974; Wydeven et al., 1982; Lewis, 1994; Chapuis et al., 2001).

There have been questions about whether plant phenology can mask microhistological differences between forage species. Holechek et al. (1982) examined the effects of plant growth stage on microhistological characteristics and concluded that plant maturity had little influence on the ability of observers to differentiate between forage species.

Primarily, fecal samples have been used to estimate the botanical composition of diets selected by grazing ruminants. In our study, the prevalence of 10 predominant grasses and forbs native to the Kansas Flint Hills were examined in the diets of grazing mature beef cows. Our objective was to establish the validity of using microhistological analysis of cattle feces to quantify the botanical composition of diets grazed by mature beef cows during winter.

Materials and Methods

Sample Collection

Mature, non-pregnant beef cows (n = 10; average initial weight = 530 ± 26 kg) were maintained on a single, dormant, native Tallgrass pasture at the Kansas State University Commercial Cow-Calf Unit. Approximately 95% of above-ground biomass on these pastures was composed of the following forage species: Big Bluestem (*Andropogon geradii*; **AG**), Little Bluestem (*Schizachyrium scoparium*; **SS**), Sideoats Grama (*Bouteloua curtipendula*; **BC**), Blue

Grama (*Bouteloua gracilis*; **BG**), Switch Grass (*Panicum virgatum*; **PV**), Indian Grass (Sorghastrum *nutans*; **SN**), Lead Plant (*Amorpha canescens;* **AC**), Heath Aster (*Symphyotricum ericoides*; **SE**), Dotted Gayfeather (*Liatris punctata*; **LP**), and Purple Prairie Clover (*Dalea purpurea*; **DP;** Haddock, 2005). Cattle were allowed to adapt to the pasture for 30 d. On d 31 of the experiment, animals were temporarily moved to a corral. Approximately 1000 g of wet fecal material was collected from each cow using the rectal grab technique. Wet fecal samples were sealed in plastic containers. Samples were subsequently hand-mixed and a 40-g subsample was retained for analysis.

Standard Preparation

Standard slides of each forage type were prepared approximately 14 d prior to the study. Each standard was derived from a pure sample of each forage type. Slides were prepared using the methods described by Holechek (1982). Training of observers was required to obtain an acceptable accuracy of identification of plant fragments. Training methods were described by Holecheck and Gross (1982). Briefly, observers viewed standard slides to become familiar with individual differences between forage species. Observers then viewed slides at random until they could identify each species based on anatomical differences. Plant fragments that appeared in cattle diets that were not one of the 10 predominant range plants for which standards were prepared were classified as either an unknown grass or an unknown forb. In our experiment, the proportion of all grass fragments that were classified in this manner was 9%; moreover, the proportion of all forb fragments that were classified as unknown was 4%.

Sample Preparation

Samples were prepared using methods described by Holechek (1982). Fecal samples were soaked in a 50% (v/v) ethanol solution overnight. After soaking, samples were homogenized in a blender, rinsed with de-ionized water, and filtered through a No. 200 US-standard testing sieve to remove contaminants. Excess water was drained and the samples were placed in a drying oven at 55°C for 96 h. Finally, samples were ground (#4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) through a 1-mm screen and stored in plastic containers for subsequent slide preparation (Bennett et al., 1999).

Slide Preparation

Slides were prepared using methods described by Holechek (1982). Dried fecal samples (1.5 – 2 g) were placed in beakers and soaked in distilled water for 1 h. Sodium hydroxide (0.05 M) was then added to each beaker and samples were soaked for an additional 20 min to destroy plant pigments. Sodium hydroxide was removed by washing the samples with de-ionized water over a No. 200 US-standard testing sieve. After NaOH was removed, the samples were placed in a blender with approximately 25 ml of distilled water and homogenized for 1 min.

One to 2 drops of Hertwig's solution was placed on each microscope slide immediately before samples were mounted to allow samples to be easily positioned on the slide. Once samples were positioned on the slides, they were held over a propane flame until the Hertwig's solution evaporated. Hoyer's solution was added to slides and they were again held over a propane flame in order to fix the samples in place. Prepared slides were dried in a 55°C oven for 96 h before viewing.

Dried slides were read on a compound microscope at 10x magnification. The microscope was equipped with a digital camera; each slide field was photographed for comparison with standard slides. Twenty fields per slide were selected randomly from the entire slide view and were used to measure the frequency with which plant fragments appeared. Plant fragment prevalence in slide fields was assumed to be equivalent to prevalence in fecal samples and in grazed diets on a DM basis.

Statistics

All data were analyzed as a completely random design using the mixed procedure of SAS (SAS Inst. Inc., Cary, NC). Class variables included animal, slide, and field within slide. The model included terms for forage species, animal, slide, and field. Type-3 error rates were used to report differences between forage species. A simple t-test was used to test for differences between means. Means were separated using the method of Least Significant Difference and reported with a pooled standard error. Means were considered to be different when $P \leq 0.05$.

Results and Discussion

Although the cattle used in our study were of the same class (i.e., mature cows) and age (i.e., 4 y) and were grazed as a single cohort, there were differences ($P < 0.01$) between animals in the proportions of various range plants that were grazed. Table 2.1 summarizes mean values and SEM for 10 predominant grass and forb species in the diets of beef cows in our trial. Overall, grasses composed 65.74% of the diet: 8.91% was AG, 8.07% was SS, 15.06% was BC, 8.88% was BG, 8.14% was PV, and 12.95% was SN. Nine percent of grass-plant fragments could not be identified with 1 of the 6 grasses for which standards were prepared. A preference for BC and SN was apparent within this particular group of cattle.

A significant proportion of cow diets (34.26%) was composed of forbs, in spite of the fact that the experiment was conducted on dormant winter range and most forbs were difficult to locate visually. Forb composition in the diets of grazing cows was 7.89% AC, 7.00% SE, 4.18% LP, and 13.26% DP; 4% of forb fragments could not be identified with the 4 forb species for which standards were prepared. Altogether, plant fragments that could not be assigned a positive species identification composed 6% of the diet. We interpreted these data to indicate that a relatively minor proportion of all plant fragments in fecal samples could not be classified according to plant species.

Prior research indicated that there were differences in botanical composition of diets when estimates were made from fecal samples compared with masticate samples. Differences were due in part to variations in plant digestibility and time of plant consumption (Anthony and Smith, 1974; Wydeven and Dahlgren, 1982; Lewis, 1994). These studies indicated that fecal analysis tended to overestimate dietary consumption of shrubs and grasses compared to analysis of ruminal contents (Lewis, 1994; Chapuis et al., 2001). Similarly, Lewis (1994) noted that microhistological analysis of fecal matter tended to underestimate the forb component of the diet compared to microhistological analysis of ruminal contents.

Carrière (2002) noted that it was occasionally difficult for researchers using the microhistological technique to differentiate between closely-related plant species. In our study, this was true of AG and SS. In instances where technicians identified individual plant fragments

as either AG or SS but were unable to discern any species-specific characteristics, the observations were classified as either an unknown grass or an unknown forb.

Questions have arisen regarding the proper sample size when assessing treatment differences in botanical composition of grazed diets using the fecal microhistological technique. Anthony and Smith (1974) calculated that fecal microhistological analysis of diet composition required a minimum of 15 animals per treatment. Under the conditions of our study, a minimum sample size of 13 mature cows (range = 3.1 cows for DG to 12.4 cows for BB) was calculated to be necessary to detect a difference in mean species preference of 2% with 95% confidence, across the 10 predominant range plants in the Tallgrass Prairie region (Sokal and Rohlf, 1969).

Results from our study were interpreted to indicate that the methods derived and verified by Holechek et al. (1982), Sparks and Malechek (1968), and Bennett et al. (1999) were viable means for estimating the botanical composition of mature cow diets grazed during the winter in the Tallgrass Prairie of Kansas.

Tables and Figures

Table 2.1: Botanical composition of diets selected by beef cows grazing native range in the Kansas Flint Hills during winter.

Species	Botanical Composition (% of diet DM)	Minimum	Maximum	SE	CV (%)
Grasses					
Andropogon geradii	8.91	0	10	0.100	7.01
Schizachyrium scoparium	8.07	0	6	0.127	6.97
Bouteloua curtipendula	15.06	0	10	0.128	3.76
Bouteloua gracilis	8.88	0	8	0.107	5.34
Panicum virgatum	8.14	0	9	0.129	7.24
Sorghastrum nutans	12.95	0	10	0.135	8.54
Unidentified grasses	9.00	0	20	0.161	1.46
Forbs					
Amorpha canescens	7.89	0	10	0.111	6.04
Symphyotricum ericoides	7.00	0	8	0.095	3.25
Liatris punctata	4.18	0	5	0.085	9.01
Dalea purpurea	13.26	0	10	0.124	4.14
Unidentified forbs	4.00	0	20	0.144	1.81

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CHAPTER 3 - EFFECTS OF SUN-CURING AND HARVEST MATURITY ON CONCENTRATION AND PROTEIN-BINDING CAPACITY OF CONDENSED TANNINS IN SERICEA LESPEDEZA (*LESPEDEZA CUNEATA*)

Abstract

A study was conducted to evaluate the effects of sun-curing and harvest maturity on concentrations of condensed tannins (**CT**) and protein-precipitable phenolics (**PPP**) in sericea lespedeza (SL). Samples of SL (n = 200 plants/sample) were collected from a single native tallgrass pasture at 1- to 4-wk intervals from June 24 to October 11 that corresponded to single-stem, branched-stem, budding, flowering, and senescent stages of plant phenology. Samples were divided randomly into 2 equal portions that were either dried via sun-curing or were frozen immediately after harvest and later freeze dried. Total phenolics were extracted from dried, ground SL samples using a modified methanol-extraction technique and were analyzed for CT and PPP. Concentrations of CT in sun-cured SL were less (main effect of treatment - $P < 0.01$) than that in fresh SL. Concentration of CT in SL responded cubically ($P < 0.01$) over time; CT was least during June and October and peaked during August. Peak CT concentration corresponded to the flowering stage of the SL life cycle. Concentrations of PPP in SL also changed over time but the magnitude of the effect was influenced by treatment (treatment × period – $P < 0.01$). Concentrations of PPP in sun-cured SL responded cubically ($P < 0.01$) as the growing season advanced; PPP was least during June and October and peaked during August. In contrast, PPP in fresh SL responded quadratically ($P < 0.01$) over time, indicating that significant concentrations of PPP remained in SL late into the growing season. Concentration of CT and PPP in SL decreased dramatically during drying and storage. These data may explain why sharp avoidance of SL by grazing livestock is not observed when SL is fed in the form of sun-cured hay. Understanding how drying and plant growth stage influence tannins in SL could lead to more effective research models for the study of SL intake by ruminants.

Introduction

Sericea lespedeza (*Lespedeza cuneata*) is a noxious weed that infests approximately 600,000 acres of native Tallgrass range in Kansas (Eddy et al., 2003). Intake of sericea lespedeza by grazing livestock is poor, due presumably to the presence of tannins in the plant (Terrill et al., 1989; Mantz et al., 2009). Condensed tannins reduce protein digestion by ruminants (Jones and Mangan, 1977); condensed tannins may also decrease plant palatability.

Prolific seed production, in combination with little or no grazing pressure, has contributed to the rapid spread of sericea lespedeza on Kansas rangelands (Eddy et al., 2003). Increasing grazing pressure on sericea lespedeza may reduce seed production and slow its advance; however, development of appropriate research models to study sericea lespedeza intake by ruminants has been slow. Tannin concentration in sericea lespedeza changes dramatically during drying and storage (Terrill et al., 1989, 1990, and 1994). Therefore, sharp avoidance of sericea lespedeza by grazing livestock is not generally observed when sericea lespedeza is fed to livestock in the form of sun-cured hay (Terrill et al., 1989; Mantz et al., 2009). Little is known about how harvest maturity and sun-curing influence the concentration of condensed tannins in sericea lespedeza or the degree of protein-binding by condensed tannins over the course of an entire growing season. Such information could lead to more effective research models for the study of sericea lespedeza intake by ruminant livestock. Therefore, the objective of our study was to examine changes in condensed-tannin concentrations and in protein-binding capacity of condensed tannins throughout the growing season in both sun-cured and fresh sericea lespedeza.

Materials and Methods

Sample Collection and Preparation

Samples were collected during the summer and fall of 2009, from a single 65-ha pasture in Greenwood County, Kansas. Plant-species composition on the study site was estimated using the modified step-point technique described by Owensby (1973); sericea lespedeza comprised 19.3% of all plants encountered during the procedure. Above-ground biomass of sericea lespedeza averaged 0.1 kg/m^2.

Individual sericea lespedeza plants were collected from the study site at 1 to 4-wk intervals from June 24 to October 11 (n = 200 plants / sampling date) that corresponded to

single-stem, branched-stem, budding, flowering, and senescent stages of the plant (Koger et al., 2002). Plants were clipped approximately 1 cm above the soil surface.

At the time of collection, samples were either allowed to sun cure in burlap bags or were flash-frozen as described by Terrill et al. (1990). Frozen samples were later freeze dried. All dried samples were ground with dry ice (0.5-mm screen; #4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) to preserve tannin structure and bioactivity (Makkar, 2003).

Extraction of Condensed Tannins

Extraction of condensed tannins was adapted from methods described by Makkar (2003). Each ground sericea lespedeza sample was thoroughly mixed and a 200 mg subsample was collected. Ten ml of 50% Methanol (v/v) was added to each sample in a 50-ml beaker and the mixture was stirred. Samples were agitated in an ultrasonicator (Blackstone Ultrasonics, Sheffield, PA) for 2 × 10-min periods. Samples were allowed to stand for a period of 5 min between agitations. The resulting solution was transferred to 15-ml polyethylene tubes and centrifuged at 3,000 × *g* (4°C) for 15 min. The supernatant was decanted into a clean 50-ml beaker and chilled; the pellet was washed 2 × with 5 ml of 50% Methanol (v/v). The centrifugation step was repeated after each wash and supernatant was decanted. All supernatant from a single sample was combined for tannin analysis.

Measurement of Condensed Tannins

Methods used to measure the amount of condensed tannins in harvested forages were adapted from Makkar (2003). A 100-µl aliquot of supernatant from each sample was placed into individual 1.5-ml Eppendorf tubes; 600 µl of Butanol-HCl and 20 µl of ferric reagent, which was used to increase the sensitivity and reproducibility of this assay (Makkar, 2003), were added to each tube. Samples were incubated for 60 min in a 100°C water bath. Samples were allowed to cool and then placed in a 96-well microplate. Sample absorbance at 550 nm was measured using a UV spectrophotometer equipped with Gen5 software (Biotech Inc., Winooski, VT). Absorbance was adjusted to condensed-tannin concentration using leucocyandin as a standard (Makkar, 2003).

Measurement of Protein-Precipitable Phenolics

Standards (200 µl) containing 0, 50, 100, and 150 µl tannic acid in 50% Methanol (v/v) were prepared from a standard solution (0.5 mg tannic acid/ml). These mixtures were added to 400 µl of bovine serum albumin (BSA). Aliquots of extracted tannins (20 ml) were mixed with 400 µl of BSA solution (100 mg BSA in 100 ml acetate buffer) and a complementary amount of 50% Methanol (v/v) [e.g. 25 µl extract: 175 µl methanol] and mixed thoroughly. This dilution allowed for measurement of tannic acid in the tannin-protein complexes of sericea-lespedeza leaf. Samples and standards were allowed to stand at 4°C for 16 h and then centrifuged for 10 min at 3000 × *g* (4°C). The supernatant was discarded and the pellet was dissolved using 300 µl of 1% sodium dodecyl sulfate (w/v) solution. A 200 µl aliquot was removed from each sample and added to 600 µl of sodium dodecyl sulfate-triethylamine solution and 200 µl of Ferric-chloride reagent. Iron in the form of Ferric-chloride reacted with tannin phenolics to express a pink chromatophore that was measurable spectrophotometrically (Makkar, 2003). The resulting solution was allowed to stand at room temperature for 30 min before being placed into a 96-well microplate together with standards.

Absorbance was measured at 510 nm using a UV spectrophotometer equipped with Gen5 software (Biotech Inc., Winooski, VT). Concentrations of tannic acid in the tannin-protein complexes were determined using a standard curve. Values were multiplied by 1.5 (each sample was dissolved in 1.5 ml of 1% sodium dodecyl sulfate solution) to calculate the amount of tannin in the tannin-protein complex (Makkar, 2003).

Statistical Analysis

Data were subject to 2-way ANOVA using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Class variables included harvest date and treatment. The model included terms for harvest date, treatment, and harvest date × treatment. F-tests were constructed using the type-3 error mean squares. Treatment × harvest date interactions were detected; therefore, treatment effects were reported by harvest dates. Within harvest dates, treatment means were separated using the method of Least Significant Difference. Least Squares Means were considered to be different when $P \leq 0.05$. Trends in concentrations of condensed tannins and in protein-binding

capacity of condensed tannins over time were characterized using orthogonal polynomial contrasts.

Results and Discussion

Condensed tannins

Condensed tannin concentrations in sun-cured forage were 105.69, 107.04, 155.09, 154.39, 172.09, and 81.26 ± 3.06 g/kg (DM basis) for samples harvested on 6/24, 7/3, 7/25, 8/24, 9/15, and 10/11, respectively (Figure 3.1). In contrast, condensed-tannin concentrations in sericea lespedeza samples that were frozen immediately after harvest were 123.04, 198.99, 228.04, 217.87, 222.48, and 158.13 ± 3.06 g/kg (DM basis) for samples harvested on 6/24, 7/3, 7/25, 8/24, 9/15, and 10/11, respectively (Figure 3.1). Allowing forage to sun cure substantially decreased detectable condensed tannins at all stages of sericea lespedeza maturity (main effect of treatment - $P < 0.01$). Terrill et al. (1989) suggested that such differences were due to reduced solubility of tannins, caused by polymerization reactions between tannins and other compounds in the forage as the plant dries. Alternatively, wilting and maceration of the forage may have disrupted the 3-dimensional structure of condensed tannins.

Concentration of condensed tannins was different (main effect of harvest date - $P < 0.05$) at each successive growth stage of sericea lespedeza. Concentration of condensed tannins in fresh and sun-cured samples responded cubically ($P < 0.01$) over time; it was least during June and October and peaked during mid August. Peak concentrations corresponded to the flowering stage of the sericea lespedeza life cycle. Previous reports indicated condensed-tannin concentrations were maximal immediately prior to seed dispersal (Cope et al., 1971; Cope and Burns, 1974). This was the case in our study as well.

Protein-binding capacity

Protein-binding capacity of condensed tannins in sericea lespedeza was estimated from the concentration of protein-precipitable phenolic compounds in purified samples of condensed tannins prepared from each of our samples. Concentrations of protein-precipitable phenolics in sun-cured forage were 12.2, 20.0, 18.0, 22.0, 37.5, and 10.0 ± 0.002 µg/200µg of condensed tannin, respectively (Figure 3.2). Concentrations of protein-precipitable phenolics in sericea

lespedeza samples that were frozen immediately after harvest were 12.5, 40.0, 43.0, 43.5, 41.0, and 15.0 ± 0.002 μg / 200 μg of condensed tannin, respectively (Figure 3.2).

Terrill et al. (1989) indicated that the concentration of protein-precipitable phenolics in sun-cured sericea lespedeza may be underestimated because of polymerization between condensed tannins and certain other plant compounds during drying. A similar underestimation may also occur in fresh sericea lespedeza because of maceration and any wilting while sampling.

Allowing forage to sun cure appeared to decrease the protein-binding capacity of condensed tannins in sericea lespedeza (Figure 3.2). Protein-binding capacity of condensed tannins was different ($P < 0.01$) at each successive growth stage. Concentrations of protein-precipitable phenolics changed over time but the magnitude of the effect was influenced by treatment (treatment × period – $P < 0.01$; Figure 3.2). Concentrations of protein-precipitable phenolics in sun-cured sericea lespedeza responded cubically ($P < 0.01$) as the growing season advanced; protein-precipitable phenolics were least during June and October and peaked during August. In contrast, protein-precipitable phenolics in fresh sericea lespedeza responded quadratically ($P < 0.01$) over time, indicating that condensed tannins in sericea lespedeza retained significant protein-binding capacity late into the growing season.

Implications

Results from this study suggest that allowing sericea lespedeza to sun cure after harvest decreased dramatically the amount of extractable condensed tannins and the capability of condensed tannins to bind proteins; moreover, condensed tannin concentration and protein-binding capability peaked near the flowering stage of sericea lespedeza. We believe these data explain why sharp avoidance of sericea lespedeza exhibited by grazing livestock is difficult to replicate in a laboratory setting when the plant is offered to livestock in the form of sun-cured hay. Understanding how drying and plant growth stage influence condensed tannin concentrations and protein-binding capacity of sericea lespedeza could lead to more effective research models for the study of sericea lespedeza intake by ruminant livestock. Our adaptations to established procedures for extracting and isolating condensed tannins and protein-precipitable phenolics reduced generation of hazardous byproducts by approximately 80%.

Tables and Figures

Figure 3.1: Effects of sun-curing and harvest date on concentration of condensed tannins in sericea lespedeza

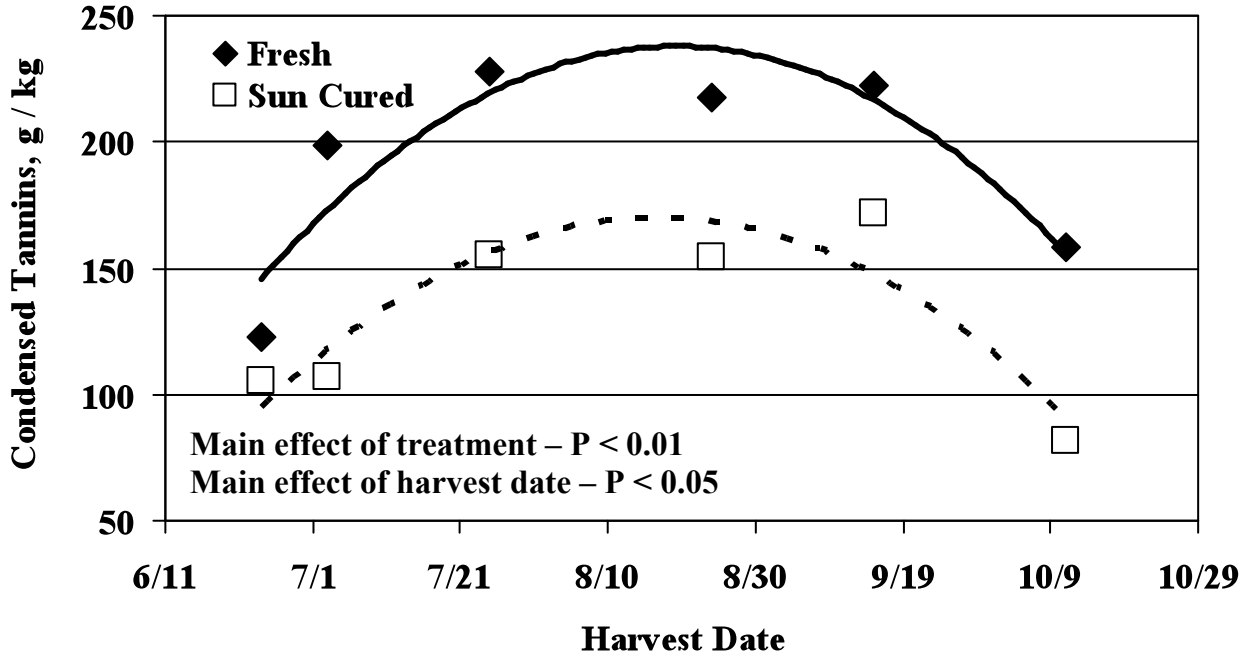
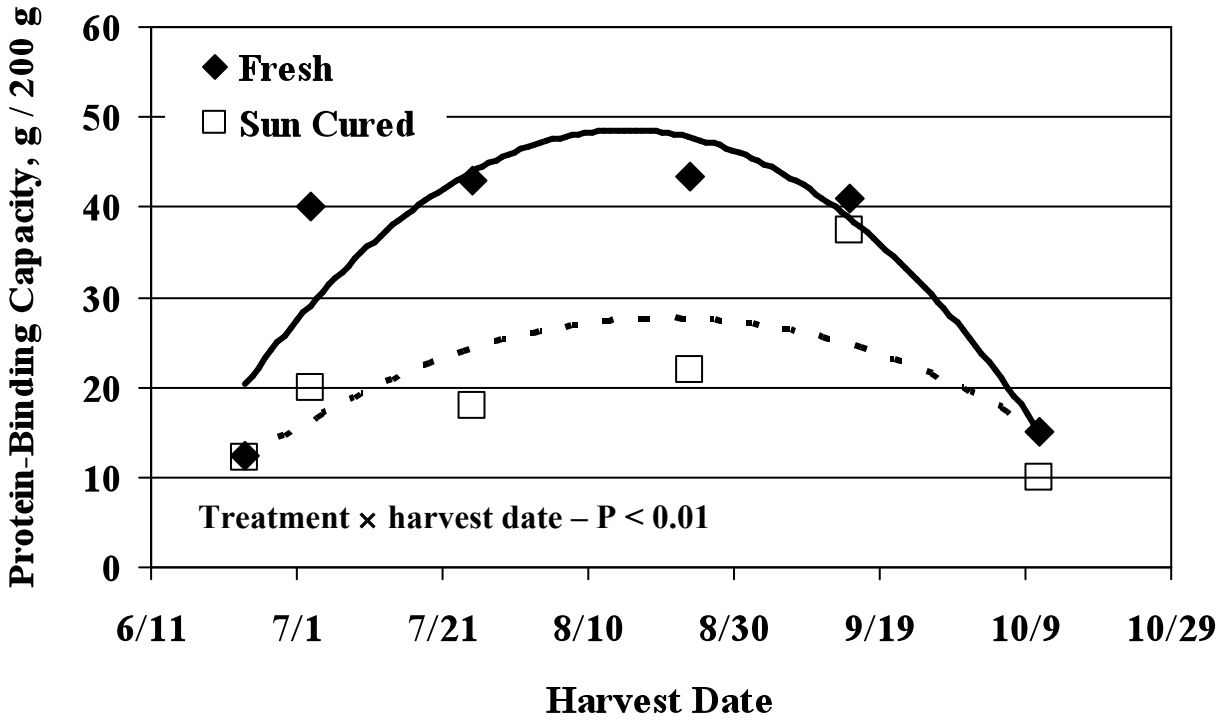


Figure 3.2: Effects of sun-curing and harvest maturity on protein-binding capacity of condensed tannins in sericea lespedeza



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CHAPTER 4 - HIGH-TANNIN FORAGE UTILIZATION BY BEEF COWS I. INTAKE AND DIGESTION OF TALLGRASS PRAIRIE HAY CONTAMINATED WITH SERICEA LESPEDEZA (*LESPEDEZA CUNEATA*)

Abstract

Mature, non-pregnant, non-lactating beef cows (n = 24; initial BW = 463 ± 69 kg) were used to assess voluntary DMI of tallgrass prairie hay contaminated with sericea lespedeza in a 30-d trial. Cows were assigned randomly to 1 of 2 dietary treatments: uncontaminated tallgrass prairie hay (**UC**; 5.4% CP, 40% ADF) or tallgrass prairie hay contaminated with sericea lespedeza (**C**; 5.5% CP, 41% ADF). These forages were similar in botanical composition, except for the presence of sericea lespedeza. Sericea lespedeza constituted 19.3% of C by weight (DM basis); condensed tannin concentration in sericea lespedeza plants selected from C ranged from 200 to 250 g/kg forage DM. All cows were individually fed UC for *ad libitum* intake using a Calan-gate feeding system for 20 d. Voluntary forage DMI was not different ($P = 0.32$) between treatments during that time and averaged 113 ± 3.0 g/kg $BW^{0.75}$. On d 21, hay contaminated with sericea lespedeza was abruptly substituted for uncontaminated hay in the diets of cows assigned to C. Voluntary forage DMI was monitored for an additional 10 d. Voluntary forage DMI by cows assigned to UC remained relatively stable (112 ± 2.8 g/kg $BW^{0.75}$) during that time, while voluntary forage DMI by cows assigned to C decreased (treatment × time, $P < 0.01$) sharply and averaged 61 ± 8.9 g/kg $BW^{0.75}$. Nutrient digestion was assessed during the last 6 d of the trial using ADIA as an internal marker. Total-tract DM, CP, and NDF digestibilities were not different ($P \geq 0.29$) between C and UC. In contrast, total digestible DMI by cows fed UC was more than 2-fold greater ($P < 0.01$) than that by cows fed C (64 vs. 29 ± 6.2 g/kg $BW^{0.75}$ for UC and C, respectively). Our results were interpreted to indicate that tallgrass prairie hay contaminated with sericea lespedeza may be a useful model for the study of the appetite-suppressing effects of that plant. Furthermore, differences in voluntary DMI between

contaminated and uncontaminated hays of similar chemical composition were manifested rapidly after introduction of C into beef cow diets.

Introduction

Sericea lespedeza (*Lespedeza cuneata*) is classified as an invasive plant throughout the Great Plains, the eastern United States, and eastern Canada (USDA, 2010). It infests approximately 600,000 acres of native Tallgrass range in the Kansas Flint Hills (Eddy et al., 2003). Prolific seed production has contributed to the spread of sericea lespedeza on rangelands (Wang et al., 2008). The aggressive nature of the plant reduces native grass production by up to 92% (Eddy et al., 2003). Herbicides retard the spread of sericea lespedeza but application is difficult and expensive under the best of circumstances and impossible in steep or rocky terrain (Eddy et al., 2003).

Intake of sericea lespedeza by grazing beef cattle is poor, due presumably to the presence of tannins in the plant (Terrill et al., 1989; Mantz et al., 2009). Condensed tannins reduce protein digestion by ruminants (Jones and Mangan, 1977) and may also decrease plant palatability.

Increasing grazing pressure on sericea lespedeza may reduce seed production and slow the spread of the plant; however, the difficulties associated with measurement of intake by grazing beef cattle have hampered development of workable research models. Detailed study of the appetite-suppressing effects of sericea lespedeza under controlled conditions is essential in order to develop appropriate strategies to increase grazing pressure on this plant. Such information could lead to a degree of biological control of this noxious weed using the most economically-important grazer (i.e., beef cattle) in the Kansas Flint Hills.

Feeding sericea lespedeza as sun-cured hay to confined beef cattle would be a convenient way to study the intake-limiting properties of this plant. This approach has not been attempted to date because previous research indicated that allowing sericea lespedeza to sun cure after harvest sharply decreased the amount of extractable condensed tannins in the plant and the capability of condensed tannins to bind proteins (Terrill et al., 1989, 1990, and 1994; Eckerle et al., 2010). Based on these reports, it was doubtful whether sun-cured prairie hay containing sericea lespedeza would produce the aversion in confined beef cattle that is commonly observed

in free-ranging beef cattle exposed to the fresh plant. Therefore, the objective of our study was to compare intakes of tallgrass prairie hay by beef cows when hay was either uncontaminated or heavily contaminated by sericea lespedeza.

Materials and Methods

All procedures used in the care, handling, and sampling of animals in our study were reviewed and approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol # 2650.5).

Tallgrass prairie hay contaminated by sericea lespedeza was harvested from a single pasture in Greenwood County, Kansas, cut, sun-cured, packaged into 0.75 × 0.5 × 0.5 m bales (approximately 35 kg), and stored at the Kansas State University Commercial Cow-Calf Unit. Forage was harvested in late July, which corresponded to the budding stage of sericea lespedeza and maximal concentration of extractable condensed tannins (Eckerle et al., 2010).

Plant-species composition within the study area was estimated using a modified step-point technique (Owensby, 1973); sericea lespedeza comprised 19.3% of all plants encountered during the procedure. Above-ground biomass of sericea lespedeza averaged 1,001 kg/acre. Concentrations of extractable condensed tannins in individual sericea lespedeza plants ranged from 200 to 250 g/kg forage DM.

Uncontaminated tallgrass prairie hay was harvested in Pottawatomie County, KS also late in July. Uncontaminated hay was verified to be free of sericea lespedeza, cut, sun-cured, packaged into 1.5 × 1.5 m cylindrical bales (approximately 450 kg), and stored at the Kansas State University Commercial Cow-Calf Unit. Species compositions of contaminated and uncontaminated forages were similar in all respects, except for the presence of sericea lespedeza.

Bales of each forage type were sampled to determine CP and ADF concentration and were paired based on similarity in those values. Average CP and ADF concentrations of contaminated and uncontaminated hay are shown in Table 4.1. Purposeful selection for similarity in protein and fiber concentrations between forage types was intended to prevent confounding forage quality with effects on intake. Bales of contaminated and uncontaminated hay selected for the study were ground separately to a 10-cm particle size.

Mature, non-pregnant, non-lactating beef cows (n = 24; initial BW = 463 ± 69 kg; initial BCS 4.2 ± 0.76) were housed in a single pen (40 × 80 m) equipped with a Calan-gate feeding system. Gated feed bunks were covered but the remaining area of the pen was open to ambient air and wind. Cows were fitted with a single transponder capable of opening only 1 gated feed bunk and trained to use the Calan-gate feeding system over a period of 30 d. During this time, cows were fed uncontaminated prairie hay for *ad libitum* intake. Cows were offered forage twice daily at 0600 and 1800 (130% of rolling 5-d average DMI). Daily forage refusals were collected and weighed at 0530. Daily voluntary DM intakes were determined by subtracting daily refusals from the total amount of hay offered. Intakes were expressed in g/kg $BW^{0.75}$. Once forage DMI stabilized at approximately 2.5% BW, the trial was initiated.

Cows were stratified by age and BCS and assigned randomly to 1 of 2 dietary treatments: uncontaminated tallgrass prairie hay (**UC**) or tallgrass prairie hay contaminated with sericea lespedeza (**C**). All cows were individually fed UC hay for *ad libitum* intake for an additional 20 d to initiate the trial. Voluntary forage DMI was not different ($P = 0.32$; data not shown) between treatments during that time and averaged 113 ± 3.0 g/kg $BW^{0.75}$. On d 21, hay contaminated with sericea lespedeza was abruptly substituted for uncontaminated hay in the diets of cows assigned to C. Voluntary forage DMI was monitored for an additional 10 d.

Total-tract diet digestion was assessed from d 26 to 30 according to Olson et al. (2008). Forage samples were collected from d 25 to 29. Fecal grab samples were collected every 4 h on d 26 to 30. The collection interval was staggered 2 h each day to account for diurnal variation in fecal output and composition.

Daily forage, ort, and fecal samples were dried in a forced air-over (96 h; 50°C), weighed, and ground (#4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) to pass a 1-mm screen. Forage samples were composited within forage type; fecal and ort samples were composited across days within animal.

Forage, orts, and feces were analyzed for DM (16 h; 105°C), OM (8 h; 450°C), and N (FP-528, LECO, St. Josephs, Michigan). These samples were also analyzed for NDF, ADF, and acid detergent insoluble ash (ADIA) using procedures described by Van Soest et al. (1991).

Total tract nutrient digestion coefficients were calculated using ADIA as an internal marker (Cochran and Galyean, 1994; Olson et al., 2008).

Intake data were analyzed as a completely randomized design with repeated measures (PROC MIXED; SAS Inst. Inc., Cary, NC). The model included terms for treatment, day, and treatment × day. Fixed effects were tested using type-3 error rates. Treatment × day effects were detected ($P < 0.01$); therefore, interaction Least Squares means were reported with pooled standard errors.

Digestibility data were analyzed as a completely randomized design (PROC GLM; SAS Inst. Inc., Cary, NC). Class variables included animal and treatment. The model included a term for treatment only. Fixed effects were tested using type-3 error rates. Least Squares means were separated by the method of Least Significant Difference and reported with pooled standard errors. Means were considered to be different when $P \leq 0.05$.

Results and Discussion

Voluntary forage DMI by cows assigned to UC remained relatively stable (112 ± 2.8 g/kg $BW^{0.75}$) during the last 10 d of our trial. Conversely, voluntary forage DMI by cows assigned to C decreased (treatment x time, $P < 0.01$) sharply and averaged 61 ± 8.9 g/kg $BW^{0.75}$ during that period (Figure 4.1). Abrupt introduction of prairie hay contaminated by sericea lespedeza seemed to exert an immediate but relatively minor effect on forage DMI from d 21 to d 24 (average difference between UC and C = 21 g DM/kg $BW^{0.75}$). Between d 25 and 30, average voluntary DMI of cows assigned to C declined dramatically (average difference between UC and C = 73 g DM/kg $BW^{0.75}$). This occurred even though both C and UC had similar CP and ADF concentrations. We speculated that the immediate decline in voluntary DMI that occurred between d 21 and 24 may be indicative of a relatively minor flavor aversion. Conversely, the precipitous drop in intake of C that followed (d 25 to 30) appeared to be driven by significant post-ingestive consequences of sericea lespedeza ingestion. This effect may have been associated with a ruminal build-up of tannin-protein complex that caused a general decrease in the activity of ruminal microorganisms.

Total-tract DM, CP, and NDF digestibilities were not different ($P \geq 0.29$) between C and UC (Table 4.2). In contrast, total digestible DMI by cows fed UC was more than 2-fold greater ($P < 0.01$) than that by cows fed C (64 vs. 29 g/kg $BW^{0.75}$ for UC and C, respectively). Clearly, beef cows that were offered C experienced a significant nutrient deficit compared to beef cows fed UC.

Implications

Our results were interpreted to indicate that tallgrass prairie hay contaminated with sericea lespedeza may be a useful model for the study of the appetite-suppressing effects of that plant. Furthermore, differences in voluntary DMI between contaminated and uncontaminated hays of similar chemical composition were manifested rapidly after introduction of C into beef cow diets. Palatability of tannins may be responsible for an initial but relatively minor flavor aversion; however, continued intake of contaminated forage may have resulted in a build-up of tannin-protein complexes in the rumen that suppressed forage DMI substantially. Using this model to investigate methods to mitigate the negative effects of consuming high-tannin forages appears promising.

Tables and Figures

Table 4.1: Chemical composition of Tallgrass prairie hay contaminated or uncontaminated by sericea lespedeza (DM basis)

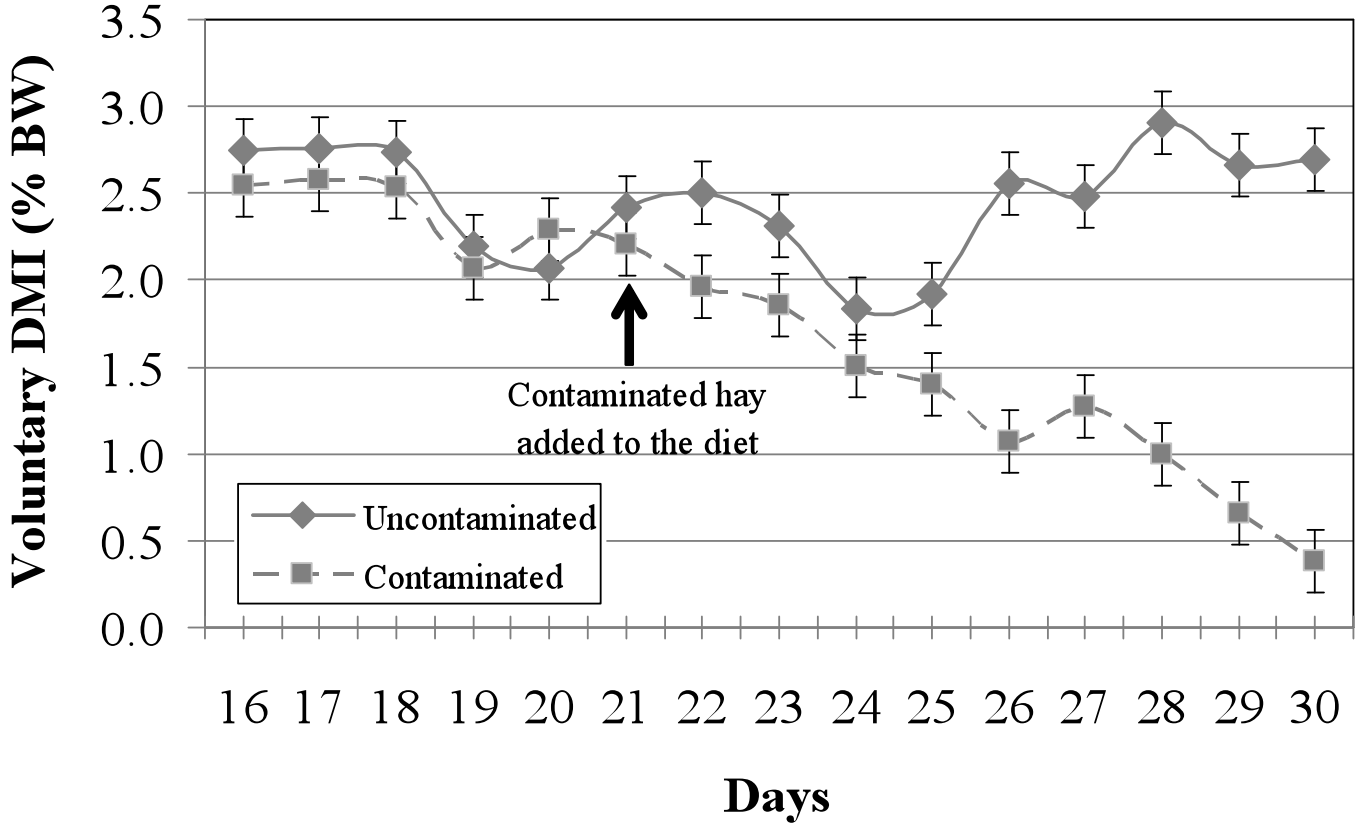
Item	DM (%)	OM (%)	CP (%)	ADF (%)	NDF (%)	Ca (%)	P (%)
Uncontaminated forage	93.95	85.40	4.49	39.76	65.67	0.85	0.11
Contaminated forage	93.08	85.75	4.75	41.15	66.28	0.63	0.09

Table 4.2: Total-tract nutrient digestion and total digestible DMI by beef cows fed tallgrass prairie hay contaminated by sericea lespedeza

Item	Uncontaminated Forage	Contaminated Forage	SEM	*P*-Value
Total-tract DM digestibility, %	51.9	50.4	1.82	0.59
Total-tract CP digestibility, %	24.8	26.6	1.98	0.53
Total-tract NDF digestibility, %	59.7	56.9	1.84	0.29
Total digestible DMI, g/kg $BW^{0.75}$	64.0[a]	29.0[b]	6.17	< 0.01

[a, b] Means within a row lacking common superscripts are different.

Figure 4.1: Effects of sericea lespedeza contamination on voluntary DMI of tallgrass prairie hay



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CHAPTER 5 - HIGH-TANNIN FORAGE UTILIZATION BY BEEF COWS II. EFFECTS OF CORN STEEP LIQUOR SUPPLEMENTATION ON INTAKE AND DIGESTION OF TALLGRASS PRAIRIE HAY CONTAMINATED WITH SERICEA LESPEDEZA (*LESPEDEZA CUNEATA*)

Abstract

Mature, non-pregnant, non-lactating beef cows (n = 24; initial BW = 546 ± 131 kg) were used to evaluate the effects of corn steep liquor (**CSL**; 32.8% CP, 45% DM) supplementation on voluntary DMI of tallgrass prairie hay contaminated with sericea lespedeza during a 29-d trial. Sericea lespedeza was 19.3% of forage DM by weight; condensed-tannin concentration in sericea lespedeza plants selected from contaminated hay ranged from 200 to 250 g/kg forage DM. Cows were assigned randomly to 1 of 4 feeding levels of CSL: 0, 0.6, 1.2, or 1.8 kg DM/d. Cows were individually fed contaminated hay for *ad libitum* intake using a Calan-gate feeding system. Cows were offered only contaminated hay during the first 14 d of the trial. Voluntary forage DMI was not different ($P = 0.52$) between treatments during that time and averaged 83 ± 2.2 g/kg $BW^{0.75}$. Beginning on d 15, supplemental CSL was abruptly introduced into cow diets at assigned feeding levels; it was offered once daily and was consumed by cows within 30 min. Cows supplemented with CSL ate more ($P \leq 0.01$) forage DM from d 15 to d 29 than unsupplemented cows; however, there was no difference ($P \geq 0.38$) in forage DMI between CSL feeding levels. Diet digestion was monitored using ADIA as an internal marker from d 23 to d 29. Total-tract DM digestibility was greater ($P < 0.01$) for cows fed 1.2 or 1.8 kg CSL than for cows fed 0 or 0.6 kg CSL. Total-tract CP digestion was least ($P < 0.01$) in cows fed no CSL (-1.5%), was slightly greater ($P < 0.01$) in cows fed 0.6 kg CSL (18.6%), and was greatest ($P < 0.01$) in cows fed either 1.2 or 1.8 kg CSL (51.7 and 52.3%, respectively). Total digestible DMI by cows fed 1.2 or 1.8 kg CSL was greater ($P \leq 0.03$; 75 and 88 g/kg $BW^{0.75}$, respectively) than that by cows fed 0 or 0.6 kg CSL (41 and 55 g/kg $BW^{0.75}$, respectively). Under the conditions of our study, CSL ameliorated the effects of condensed tannins on forage DMI and digestion in cows fed Tallgrass prairie hay contaminated with sericea lespedeza.

Introduction

Sericea lespedeza (*Lespedeza cuneata*) is a noxious weed that infests approximately 600,000 acres of native Tallgrass range in the Kansas Flint Hills (Eddy et al., 2003). Intake of sericea lespedeza by grazing livestock is poor, presumably due to the presence of tannins in the plant (Terrill et al., 1989; Mantz et al., 2009). Condensed tannins significantly reduce dietary protein and DM digestion by ruminants (Jones and Mangan, 1977; Terrill et al., 1989). These circumstances create negative post-ingestive consequences which deter consumption (Mantz et al., 2009). Poor intake of sericea lespedeza translates to negligible grazing pressure, which ensures that sericea lespedeza will be able to produce seed and continue to proliferate.

Increasing grazing pressure on sericea lespedeza may slow its advance and allow a measure of biological control using the most economically-important grazer (i.e., beef cattle) in the Flint Hills. Feedstuffs or feed additives with tannin-binding properties may allow grazing beef cattle to comfortably and voluntarily select this plant.

Jones and Mangan (1977) reported that feed-grade polyethylene glycol (**PEG**) may inhibit the formation of tannin protein complexes in the rumen. Mantz et al. (2009) reported that confined beef cattle fed 454 g of PEG daily ate more sericea lespedeza than cattle that were not fed PEG. Use of PEG by commercial beef producers has not been widely adopted for two reasons: 1) feeding PEG at the rates necessary to increase intake of sericea lespedeza is cost prohibitive and 2) feeding PEG at the rates necessary to increase intake of sericea lespedeza is disallowed from a regulatory standpoint (AAFCO, 2008). Therefore, it is advantageous to identify substances that are generally regarded as safe (GRAS; FDA, 2011), cost effective, and that mitigate the consequences of consuming a diet high in tannins.

Preliminary research in our laboratory indicated that corn steep liquor (**CSL**) has binding affinity for condensed tannins that equals or exceeds that of PEG. The objective of our study was to determine the effects of CSL supplementation on intake and digestion of Tallgrass prairie hay contaminated with sericea lespedeza.

Materials and Methods

All procedures used in the care, handling, and sampling of animals in our study were reviewed and approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol # 2650.5).

Preliminary data

We developed an *in vitro* method for estimating the binding affinity of various compounds for condensed tannins and the degree of protection from tannin binding that these compounds bestowed on true proteins. This method involved mixing purified tannin with a purified source of true protein (bovine serum albumin; **BSA**) in the presence or absence of potential tannin-mitigating compounds. The ratio of tannin to true protein in solution was held at 4:1 purified tannin to true protein, approximating the tannin:true protein ratio in the diets of beef cattle consuming sericea lespedeza only (Mantz et al., 2009). After the reaction was allowed to take place in the presence or absence of a mitigating agent, BSA in the solution that remained unbound by tannin was measured using a spectrophotometric technique (Sprint Rapid Protein Analyzer, CEM USA, Matthews, NC).

Among the mitigating agents that we evaluated were polyethylene glycol (**PEG**) and corn steep liquor (**CSL**) at doses of 16 mg / mg of true protein in the original sample. This dose of the mitigating agents created a ratio of mitigator-to-true protein that was approximately equal to the feeding rate of PEG recommended by Mantz et al. (2009) to increase consumption of sericea lespedeza by beef cattle.

In untreated samples, an average of 57.3% of the true protein was bound by tannins and would have been resistant to microbial protein digestion in the rumen (Table 5.1). The average amount of tannin-bound protein in the PEG-treated samples declined by approximately 16% relative to the untreated samples, indicating that PEG provided a modest degree of protection from tannin. Conversely, an equivalent dose of CSL appeared to fully protect BSA in the reaction vessel from interacting negatively with tannins. True protein availability of the CSL-treated samples was greater than the original amount of BSA placed in the reaction vessels. This was due to the fact that CSL contained a modest amount of true protein (Table 5.1).

These data were interpreted to suggest that CSL bound condensed tannins and protected true proteins to a greater degree than PEG. We hypothesized that beef cattle supplemented with a proper dose of CSL may be able to safely consume greater quantities of high-tannin forages than unsupplemented beef cattle.

Forage

Tallgrass prairie hay contaminated by sericea lespedeza was harvested from a single pasture in Greenwood County, Kansas, cut, sun-cured, packaged into 0.75 × 0.5 × 0.5 m bales (approximately 35 kg), and stored at the Kansas State University Commercial Cow-Calf Unit (Table 5.2). Forage was harvested in late July, which corresponded to the budding stage of sericea lespedeza and maximal concentration of extractable condensed tannins (Eckerle et al., 2010). Bales of contaminated hay used in the study were ground to a 10-cm particle size.

Plant-species composition within the study area was estimated using a modified step-point technique (Owensby, 1973); sericea lespedeza comprised 19.3% of all plants encountered during the procedure. Above-ground biomass of sericea lespedeza averaged 1,001 kg/acre. Concentrations of extractable condensed tannins in individual sericea lespedeza plants ranged from 200 to 250 g/kg forage DM.

Supplement

Corn steep liquor was purchased from Archer Daniels Midland in Columbus, NE, transported to the Kansas State University Commercial Cow-Calf Unit, and stored in a polyvinyl chloride container (Table 5.2).

Intake and digestibility measurements

Mature, non-pregnant, non-lactating beef cows (n = 24; initial BW = 546 ± 131 kg) were housed in a single pen (40 × 80 m) equipped with a Calan-gate feeding system. Gated feed bunks were covered but the remaining area of the pen was open to ambient air and wind. Cows were fitted with a single transponder capable of opening 1 gated feed bunk only and trained to use the Calan-gate feeding system over a period of 30 d. Cows were then stratified by body weight and BCS and assigned randomly to be fed 1 of 4 supplemental levels of CSL: 0, 0.6, 1.2,

and 1.8 kg DM/d. All cows were individually fed tallgrass prairie hay contaminated with sericea lespedeza for *ad libitum* intake for 14 d (130% of rolling 5-d average DMI). Cows were offered forage twice daily at 0600 and 1800. Daily forage refusals were collected and weighed at 0530. Daily voluntary DM intakes were determined by subtracting daily refusals from the total amount of hay offered. Intakes were expressed in g/kg $BW^{0.75}$.

Beginning on d 15, supplemental CSL was abruptly introduced into cow diets at assigned feeding levels; it was offered once daily and was consumed by cows within 30 min. Forage and supplement intake were monitored during the following 14 d. The purpose of the abrupt introduction of CSL into cow diets was to minimize the opportunity for ruminal microbes to adapt to nutrients in CSL.

Total-tract diet digestion was assessed from d 23 to 29 according to Olson et al. (2008). Forage samples were collected from d 23 to 28. Fecal grab samples were collected every 4 h on d 24 to 29. The collection interval was staggered 2 h each day to account for diurnal variation in fecal output and composition.

Daily forage, ort, and fecal samples were dried in a forced air-over (96 h; 50°C), weighed, and ground (#4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) to pass a 1-mm screen. Forage and CSL samples were composited across days; ort and fecal samples were composited across days within animal.

Forage, CSL, orts, and feces were analyzed for DM (16 h; 105 °C), OM (8 h; 450°C), and N (FP-528, LECO, St. Josephs, Michigan). These samples were also analyzed for NDF, ADF, and acid detergent insoluble ash (ADIA) using procedures described by Van Soest et al. (1991). Total tract nutrient digestion coefficients were calculated using ADIA as an internal marker (Cochran and Galyean, 1994; Olson et al., 2008).

Statistical analyses

Intake data were analyzed as a completely randomized design with repeated measures (PROC MIXED; SAS Inst. Inc., Cary, NC). The model included terms for treatment, day, and treatment × day. Fixed effects were tested using type-3 error rates. Treatment × day effects

were not detected ($P \geq 0.96$); therefore, main effects of treatment were reported as Least Squares means. Means were considered to be different when $P \leq 0.05$.

Digestibility data were analyzed as a completely randomized design (PROC GLM; SAS Inst. Inc., Cary, NC). Class variables included animal and treatment. The model included a term for treatment only. Type-3 error rates were used to test for differences in DM and CP digestibilities, as well as total-digestible DM intake. Least Squares means were separated by the method of Least Significant Difference and reported with pooled standard errors. Means were considered to be different when $P \leq 0.05$.

Results and Discussion

Prior to introduction of CSL (d 1 to 14), voluntary forage DMI did not differ ($P = 0.52$) between treatments and averaged 83 ± 2.2 g/kg $BW^{0.75}$. After introduction of CSL (d 15 to 29), supplemented cows ate more ($P \leq 0.01$) forage DM than unsupplemented cows; however, there was no difference ($P \geq 0.38$) in forage DMI between CSL feeding levels (Table 5.3). The smallest dose of CSL used in our trial (i.e., 0.6 kg DM/d) stimulated maximum intake of tallgrass prairie hay contaminated with sericea lespedeza in a short-term experiment.

It is possible that corn steep liquor could have stimulated intake of the protein-poor prairie hay by simply supplying more CP to the rumen; however, the purpose of the abrupt introduction of CSL into cow diets was to minimize the opportunity for ruminal microbes to adapt to the presence of nutrients in CSL. The amount of supplemental CP provided by CSL was modest (197, 394, and 525 g DM for 0.6, 1.2, and 1.6 kg CSL DM/d, respectively). Köster et al. (1996) estimated that approximately 540 g of supplemental CP was needed to maximize intake of low-quality tallgrass prairie hay in fully-adapted beef steers weighing 588 kg. Intake was maximized in our study at less than half of that level of supplementation. The vigorous, immediate increase in voluntary consumption of tallgrass prairie hay contaminated by sericea lespedeza that we observed may have been the result of rapid complexing between CSL and tannins within the rumen.

Total-tract DM digestibility was greater ($P < 0.01$) for cows fed 1.2 or 1.8 kg CSL than for cows fed 0 or 0.6 kg CSL (Table 5.3). Total-tract CP digestion was least ($P < 0.01$) in cows

fed no CSL, was slightly greater ($P < 0.01$) in cows fed 0.6 kg CSL, and was greatest ($P < 0.01$) in cows fed either 1.2 or 1.8 kg CSL. Total digestible DM intake by cows fed 1.2 or 1.8 kg CSL was greater than that by cows fed 0 or 0.6 kg CSL. In contrast to our intake data, it appeared that the CSL dose needed to optimize digestion characteristics of the diet was equal to or greater than 1.2 kg DM/d. Further research is warranted to evaluate the optimal CSL dose needed to mitigate the consequences of consuming a diet high in tannins.

Implications

Supplementation of corn steep liquor may increase tolerance of beef cows for high tannin-forages. In our study, supplemental corn steep liquor ameliorated the negative consequences of tannin consumption in a dose-dependent manner when fed to beef cows in confinement. The beef cows in our study had only limited opportunity to selectively avoid sericea lespedeza because it was offered in chopped form and in a mixture with other forage species. It is unknown if supplemental CSL can influence forage selection preference when cattle have the opportunity to eat either uncontaminated forage or forage contaminated by sericea lespedeza.

Tables and Figures

Table 5.1: Binding affinity of condensed tannins for bovine serum albumin (BSA) in the presence of potential tannin-binding agents

Sample	Mitigator	Mitigator Dose[a]	True Protein Availability (%)	Tannin-Bound Protein (%)[c]
Tannin + BSA	None	0	42.7	57.3
Tannin + BSA	PEG	16	59.0	41.0
Tannin + BSA	CSL	16	155.7	-
Tannin + BSA	Glycerol	16	44.6	55.4
Tannin + BSA	Corn distillers solubles	16	43.4	56.6

[a] Mitigator dose is expressed as mg/mg BSA in the original sample.
[b] True protein availability was expressed as % BSA in the original sample.
[c] Tannin-bound protein was expressed as the inverse of true protein availability.

Table 5.2: Chemical composition of tallgrass prairie hay (contaminated by sericea lespedeza) and corn steep liquor (DM basis).

Item	DM (%)	OM (%)	CP (%)	ADF (%)	NDF (%)	Ca (%)	P (%)
Contaminated forage	93.7	87.2	4.6	40.7	65.2	0.35	0.07
Corn steep liquor	44.7	42.5	32.8	--	0.4	0.03	0.62

Table 5.3: Effects of increasing dose of corn steep liquor on intake and digestion of tallgrass prairie hay contaminated by sericea lespedeza.

	Corn steep liquor intake, (kg DM /d)				
Item	0	0.6	1.2	1.8	SEM
Forage DMI, g/kg $BW^{0.75}$	69.9^{a}	80.7^{b}	80.9^{b}	84.6^{b}	3.09
Total-tract DM digestibility, %	52.6^{a}	55.6^{a}	65.6^{b}	66.3^{b}	2.08
Total-tract CP digestibility, %	-1.5^{a}	18.6^{b}	51.7^{c}	52.3^{c}	4.53
Total digestible DMI, g/kg $BW^{0.75}$	40.9^{a}	55.0^{a}	75.2^{bc}	87.6^{c}	6.20

[a, b, c] Means within a row lacking common superscripts are different.

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CHAPTER 6 - HIGH-TANNIN FORAGE UTILIZATION BY BEEF COWS III. EFFECTS OF CORN STEEP LIQUOR SUPPLEMENTATION ON VOLUNTARY SELECTION OF TALLGRASS PRAIRIE HAY CONTAMINTED WITH SERICEA LESPEDEZA (*LESPEDEZA CUNEATA*) AND UNCONTAMINATED TALLGRASS PRAIRIE HAY

Abstract

Mature, non-pregnant, non-lactating beef cows (n = 16; initial BW = 525 ± 76 kg) were used to evaluate the effects of corn steep liquor (**CSL**; 44.7% DM, 31.6% CP) supplementation on voluntary selection of uncontaminated tallgrass prairie hay (**UC**; 5.4% CP, 40% ADF) and tallgrass prairie hay contaminated with sericea lespedeza (**C**; 5.5% CP, 41% ADF) during a 24-d trial. These forages were similar in botanical composition with the exception of sericea lespedeza. Sericea lespedeza was 19.3% of forage DM by weight; condensed-tannin concentration in sericea lespedeza plants selected from C ranged from 200 to 250 g/kg forage DM. Cows were assigned randomly to be fed either 0 or 0.6 kg CSL/d (DM basis). Cows were individually penned and fed UC and C hay in separate feed bunks for *ad libitum* intake. Access to both forages was simultaneous. Supplement was offered once daily and was consumed by cows within 30 min. Cows were allowed to adapt to supplementation treatments for 10 d before forage intake measurements began. Uncontaminated hay DMI was not different ($P = 0.65$) between supplemented and unsupplemented cows from d 11 to 24. Conversely, cows supplemented with CSL ate 25% more ($P < 0.01$; 63 g/kg $BW^{0.75}$) C from d 11 to d 24 than unsupplemented cows (50 g/kg $BW^{0.75}$). In addition, cows supplemented with CSL ate more ($P = 0.05$; 105 g/kg $BW^{0.75}$) total forage DM from d 11 to d 24 than unsupplemented cows (94 g/kg $BW^{0.75}$). Diet digestion was monitored using ADIA as an internal marker from d 19 to 24. Total-tract DM and CP digestibilities were not different ($P \geq 0.17$) between treatments. Total digestible DM intake by cows fed CSL was 23% greater ($P < 0.01$; 64 g/kg $BW^{0.75}$) than that by unsupplemented cows (49 g/kg $BW^{0.75}$). Under the conditions of our study, a low level of supplemental CSL was associated with increased selection of tallgrass prairie hay contaminated

with sericea lespedeza by beef cows. We interpreted these data to suggest that supplemental CSL may increase beef cow tolerance for high-tannin forages.

Introduction

Sericea lespedeza (*Lespedeza cuneata*) is classified as a noxious weed throughout the Great Plains (USDA, 2010). It produces copious amounts of seed annually (Eddy et al., 2003). In addition, sericea lespedeza contains high levels of condensed tannins during much of the growing season, which deters grazing by large domestic herbivores (Eckerle et al., 2010). In the Kansas Flint Hills alone, this plant infests approximately 600,000 acres of native tallgrass range, reducing native grass production by up to 92% (Eddy et al., 2003). Increased grazing pressure on sericea lespedeza by beef cattle may slow its spread and facilitate some measure of biological control. Feedstuffs or feed additives with tannin-binding properties may promote voluntary consumption of this plant by grazing beef cattle.

Mantz et al. (2009) reported that confined beef cattle fed polyethylene glycol (**PEG**) daily ate more sericea lespedeza than cattle that were not fed PEG; however, use of PEG by commercial beef producers has been problematic. Feeding PEG at the rates necessary to increase intake of sericea lespedeza is cost prohibitive and disallowed from a regulatory standpoint (AAFCO, 2008). Eckerle et al. (2011) reported that low to moderate amounts of supplemental corn steep liquor (i.e., 0.6 to 1.8 kg/d) increased intake of tallgrass prairie hay contaminated with sericea lespedeza by beef cows fed in confinement. Corn steep liquor is an inexpensive, palatable, and abundant byproduct of wet-corn milling and is *Generally Regarded as Safe* (GRAS) by the U.S. Food and Drug Administration (FDA, 2011). It is unknown if beef cattle supplemented with corn steep liquor will readily consume forage contaminated by sericea lespedeza when uncontaminated forage is available simultaneously. Therefore, the objective of our study was to determine the effects of a low level of corn steep liquor fed to beef cows on voluntary selection of tallgrass prairie hay contaminated by sericea lespedeza when uncontaminated tallgrass prairie hay was also available.

Materials and Methods

All procedures used in the care, handling, and sampling of animals in our study were reviewed and approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol # 2650.5).

Tallgrass prairie hay contaminated with sericea lespedeza was harvested from a single pasture in Greenwood County, Kansas, cut, sun-cured, packaged into 0.75 × 0.5 × 0.5 m bales (approximately 35 kg), and stored at the Kansas State University Commercial Cow-Calf Unit. Forage was harvested in late July, which corresponded to the budding stage of sericea lespedeza and maximal concentration of extractable condensed tannins (Eckerle et al., 2010).

Plant-species composition within the study area was estimated using a modified step-point technique (Owensby, 1973); sericea lespedeza comprised 19.3% of all plants encountered during the procedure. Above-ground biomass of sericea lespedeza averaged 1,001 kg/acre. Concentrations of extractable condensed tannins in individual sericea lespedeza plants ranged from 200 to 250 g/kg forage DM.

Uncontaminated tallgrass prairie hay was harvested in Pottawatomie County, KS also late in July. Uncontaminated hay was verified to be free of sericea lespedeza, cut, sun-cured, packaged into 1.5 × 1.5 m cylindrical bales (approximately 450 kg), and stored at the Kansas State University Commercial Cow-Calf Unit. Species compositions of contaminated and uncontaminated forage were similar in all respects, except for the presence of sericea lespedeza.

Bales of each forage type were sampled to determine CP and ADF concentrations and were paired based on similarity in those values. Average CP and ADF concentrations of contaminated and uncontaminated hays are shown in Table 6.1. Purposeful selection for similarity in protein and fiber concentrations between forage types was intended to prevent confounding forage quality with effects on intake. Bales of contaminated and uncontaminated hay selected for the study were ground separately to a 10-cm particle size.

Mature, non-pregnant, non-lactating beef cows (n = 16; initial BW = 526 ± 76 kg; initial BCS = 4.8 ± 0.45) were assigned randomly to 1 of 2 dietary treatments: 0.0 or 0.6 kg/d corn steep liquor (**CSL**; DM basis; Table 6.1). Cows were individually confined in 5 × 20 m pens and

offered uncontaminated hay (**UC**) and contaminated hay (**C**) in separate feed bunks for *ad libitum* intake. Pens were arranged in 2 blocks of 8; feed bunks were covered but the remaining area of pens was open to ambient air and wind. Access to both C and UC was simultaneous and allowed cows the opportunity to display preference for one forage type over the other.

Supplemental CSL was offered to treated cows on d 1 to 24 at 0530; it was consumed completely within 30 min. During the entire study, cows had access to both forages offered at 130% of the 5-d rolling average DMI for each forage. Forages were fed twice daily at 0600 and 1800. Daily forage refusals were collected and weighed at 0530. Daily voluntary DM intakes of C and UC were determined by subtracting daily refusals from the total amount of hay offered. Intakes were expressed in g/kg $BW^{0.75}$. Voluntary intakes of C and UC were measured from d 1 to 24 and analyzed separately from d 1 to 14 (unadapted to CSL) and from d 15 to 24 (adapted to CSL).

Total-tract diet digestion was assessed from d 18 to 24 according to Olson et al. (2008). Forage samples were collected from d 18 to 23. Fecal grab samples were collected every 4 h from d 19 to 24. The collection interval was staggered 2 h each day to account for diurnal variation in fecal output and composition. Samples of refused C and UC were also collected on those days.

Daily forage, ort (for both hay types), and fecal samples were dried in a forced air-over (96 h; 50 °C), weighed, and ground (#4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) to pass a 1-mm screen. Samples of C, UC, and CSL were composited across days on an equal weight basis; ort and fecal samples were composited across days within animal on an equal weight basis.

Forage, CSL, ort, and fecal composite samples were analyzed for DM (16 h; 105 °C), OM (8 h; 450 °C), and N (FP-528, LECO, St. Josephs, Michigan). These samples were also analyzed for NDF, ADF, and acid detergent insoluble ash (ADIA) using procedures described by Van Soest et al. (1991). Total tract nutrient digestion coefficients were calculated using ADIA as an internal marker (Cochran and Galyean, 1994; Olson et al., 2008).

Intakes of C and UC from d 1 to 14 (unadapted to CSL) and from d 15 to 24 (adapted to CSL) were analyzed as a randomized complete block with repeated measures (PROC MIXED; SAS Inst. Inc., Cary, NC). The model included terms for treatment, day, block, all 2-way interactions, and treatment × day × block. Day was the repeated measures term and animal within treatment and day was used as the error term (CS option; SAS Inst. Inc., Cary, NC). Fixed effects were tested using type-3 error rates. Interactions were not detected ($P \geq 0.96$) for any of the variables we examined; therefore, main effects of treatment were reported as Least Squares means. Means were considered to be different when $P \leq 0.05$. Tendencies were discussed when $P > 0.05$ and < 0.10.

Digestibilities of DM and CP, as well as total digestible DM intake, were analyzed as a randomized complete block (PROC GLM; SAS Inst. Inc., Cary, NC). Class variables included animal, treatment, and block. The model included a term for treatment only. Fixed effects were tested using type-3 error rates. Least Squares means were separated using the method of Least Significant Difference and reported with pooled standard errors. Means were considered to be different when $P \leq 0.05$.

Results and Discussion

Intakes of C and UC were monitored from d 1 to 14 to ascertain how supplemented and unsupplemented cows were using these forages before ruminal microorganisms became fully adapted to CSL. Unsupplemented cows ate more ($P = 0.04$) UC than cows fed CSL from d 1 to 14 (Table 6.2). Conversely, unsupplemented cows tended to eat less ($P = 0.09$) C than cows fed CSL. Eckerle et al. (2011) reported also that beef cows increased intake of tallgrass prairie hay contaminated by sericea lespedeza immediately after CSL was introduced into the diet. These data were interpreted to suggest that beef cows may respond to supplemental CSL by increasing intake of hay contaminated with sericea lespedeza even when ruminal microorganisms are unadapted or minimally adapted to CSL.

Uncontaminated hay DMI was not different ($P = 0.65$) between supplemented and unsupplemented cows from d 15 to 24 (Table 6.2). Conversely, cows supplemented with CSL ate 25% more ($P < 0.01$) C from d 15 to d 24 than unsupplemented cows. Cows supplemented with CSL also ate more ($P = 0.05$) total forage DM from d 15 to d 24 than unsupplemented cows.

Beef cows supplemented with CSL voluntarily consumed more tallgrass prairie hay contaminated with sericea lespedeza than unsupplemented beef cows, even when uncontaminated hay was available concurrently. These data were interpreted to indicate that low levels of supplemental CSL may increase beef cow acceptance of and tolerance for high-tannin forages.

Total-tract DM and CP digestibilities were not different ($P \geq 0.17$) between treatments. Eckerle et al. (2011) reported that total-tract DM and CP digestibilities by beef cows fed tallgrass prairie hay contaminated with sericea lespedeza were maximized at CSL supplementation levels of 1.2 kg DM/d or greater. Further research is warranted to evaluate feeding rates of CSL that are needed for optimal digestion of low-quality tallgrass prairie hay contaminated with sericea lespedeza.

Total digestible DMI by cows fed CSL was 23% greater ($P < 0.01$) than that by unsupplemented cows. Eckerle et al. (2011) reported that unadapted cattle fed 1.2 to 1.8 kg of CSL/d (DM) had comparable total digestible DMI. Cows supplemented with CSL in our study appeared to have greater dietary energy availability than unsupplemented cows, even though the estimated increase in NE_m supply associated with supplementing CSL at 0.6 kg DM/d was only 1.1 Mcal/d (Kalscheur et al., 2008). Over a longer feeding period, this may have translated to improved performance.

Implications

Low-level supplementation of corn steep liquor may increase both acceptance of and tolerance for high tannin-forages by beef cows. Corn steep liquor fed at 0.6 kg DM/d ameliorated some of the negative consequences of tannin consumption on digestible DM intake. In addition, voluntary consumption of a high-tannin forage increased by 25% in supplemented vs. unsupplemented beef cows. It is unknown if supplemental CSL can promote voluntary selection of actively-growing sericea lespedeza by beef cattle grazing native rangeland in the Kansas Flint Hills.

Tables and Figures

Table 6.1: Chemical composition of tallgrass prairie hay (contaminated or uncontaminated by sericea lespedeza) and corn steep liquor fed to beef cows (DM basis).

Item	DM (%)	OM (%)	CP (%)	ADF (%)	NDF (%)	Ca (%)	P (%)
Uncontaminated forage	93.1	87.0	4.1	40.8	65.2	0.27	0.08
Contaminated forage	92.6	86.0	4.1	40.2	65.3	0.31	0.08
Corn steep liquor	45.1	43.1	31.6	--	0.5	0.04	0.63

Table 6.2: Effects of low-level corn steep liquor supplementation on forage intake and digestion by beef cows simultaneously offered tallgrass prairie hay that was contaminated with sericea lespedeza and that was uncontaminated by sericea lespedeza.

Item	Corn steep liquor intake, (kg DM /d)		SEM	*P*-Value
	0	0.6		
Before adaptation to CSL, d 1 to 14[a]				
Uncontaminated forage DMI, g/kg $BW^{0.75}$	68.2	62.2	2.02	0.04
Contaminated forage DMI, g/kg $BW^{0.75}$	28.9	33.6	1.94	0.09
Total forage DMI, g/kg $BW^{0.75}$	97.1	95.8	2.14	0.67
After adaptation to CSL, d 15 to 24[a]				
Uncontaminated forage DMI, g/kg $BW^{0.75}$	43.6	41.6	3.10	0.65
Contaminated forage DMI, g/kg $BW^{0.75}$	50.3	63.0	2.48	< 0.01
Total forage DMI, g/kg $BW^{0.75}$	93.9	104.7	3.90	0.05
Total-tract DM digestibility, %	50.5	53.9	1.66	0.17
Total-tract CP digestibility, %	17.1	18.5	2.15	0.64
Total digestible DMI, g/kg $BW^{0.75}$	48.7	63.7	3.49	< 0.01

[a] Supplemental CSL was fed from d 1 to 24. The first 14 d of the experiment were considered to represent an adaptation period to supplemental CSL. Animals were considered fully adapted to CSL after d 15.

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Appendix A - Tannin Extraction from Plant Fragments

Adapted from:

Makkar, H. 2003. Quantification of tannins in tree and shrub foliage: A laboratory manual. Boston, MA: Kluwer Academic Publishers.

Sample Preparation

1. Dry samples at 55°C until the DM content is above 90%
    a. Store samples in whirl-packs
2. Take a representative sample for analysis
    a. Grind sample (500g) to pass through a 2 mm screen
        i. Remove all components from mill and take 100 g of ground sample
            1. Grind this sample to pass through a 0.5 mm screen
    b. Be sure while grinding the temperature does not exceed 40°C

Extraction of Tannins

1. Place 200 mg of ground sample into a 50 ml beaker
2. Add 10 ml of 50 % methanol to each beaker
3. Place in an ultrasonic cleaner and run 2 times for 10 min each time
    a. Let samples stand for 5 min between each run
4. Transfer all contents to a centrifuge tube
    a. Centrifuge at 3,000 *g* at 4° C for 15 min

5. Collect supernatant
    a. Place on ice
6. Wash pellet from the tube
    a. Use two washes of 5 ml of 50 % methanol
    b. Place pellet back in the beaker and ultrasonic clean it for 20 min
    c. Repeat steps 3 and 4
7. Keep all supernatant chilled until use
8. Store at -20°C

Appendix B - Measurement of Condensed Tannins

Adapted from:

Makkar, H. 2003. Quantification of tannins in tree and shrub foliage: A laboratory manual. Boston, MA: Kluwer Academic Publishers.

Reagents

1. Butanol-HCl (butanol-HCl 95:5 v/v):
    a. 950 ml n-butanol
    b. 50 ml concentrated HCl (37%)
    c. Combine in a flask and store at room temperature.

2. Ferric reagent (2% ferric ammonium sulfate in 2N HCl):
    a. 2N HCl
        i. 16.6 ml of concentrated HCl
        ii. 84.4 ml distilled H_2O
    b. 2g Ferric ammonium sulfate

    In a flask, dissolve the ferric ammonium sulfate into the 2N HCl (100 ml). Store in a dark bottle away from light.

Procedure

1. Pipette 0.50 ml of tannin extract (diluted with 70% acetone) into a 100 mm x 12 mm test tube.
2. Add 3.0 ml of butanol-HCl
3. Add 100 µl of the ferric reagent
4. Vortex the tubes (45 seconds)
5. Cover each tube with a glass marble and put into a 100°C water bath for 60 min
6. Let tubes cool then read absorbance at 550 nm

Calculations

Condensed Tannins (% DM) as leucocyanidin**:

$$\frac{(\text{A 550 nm} \times 78.26 \times \text{Dilution Factor*})}{\text{\% Dry Matter}}$$

*Dilution factor is equal to one if no 70% acetone was added and the extract was made from a 200 mg sample in 10 ml of solvent. If 70% acetone was added the dilution factor is:

$$\frac{0.5\text{ml}}{\text{(Volume of extract taken)}}.$$

**Assumes that the effective $E^{1\%,\ 1cm,\ 550nm}$ of leucocyanidin is 460

Appendix C - Measurement of Protein-Precipitable Phenolics

Adapted from:

Makkar, H. 2003. Quantification of tannins in tree and shrub foliage: A laboratory manual. Boston, MA: Kluwer Academic Publishers.

Reagents

1. Acetate buffer (pH 4.8 - 4.9, 0.2 M):
    a. Pipette 11.4 ml of Glacial Acetic Acid into 800 ml of dH_2O
    b. Adjust pH with 4 N Sodium Hydroxide
    c. Bring final volume to 1 L
    d. Add 9.86 g NaCl to make the final concentration 0.17 M
2. Sodium dodecyl sulfate (SDS; 1% w/v):
    a. Dissolve 1 g SDS into 100 ml of dH_2O
3. SDS-triethalolamine (TEA; 1% SDS (w/v) and 7% TEA (v/v)):
    a. Add 7 ml TEA to 93 ml of dH_2O
    b. Dissolve 1 g SDS in solution
4. Ferric chloride reagent:
    a. Dissolve 0.81 g Ferric Chloride in 500 ml of 0.1 M HCl
    b. Filter and store in a dark bottle
5. 0.1 M HCl:
    a. Dilute 4.2 ml of concentrated HCl (37%) with 500 ml dH_2O
6. BSA solution:
    a. Dissolve 100 mg BSA into 100 ml of Acetate buffer

Procedure

Day One

1. Take 2 ml of BSA solution and add 50% methanol and increasing levels of tannin extract to make a final volume of 3 ml
2. Vortex contents and place in 4°C overnight

Day Two

1. Centrifuge at 3,000*g* for 10 min
2. Remove supernatant without disturbing the pellet
3. Add 1.5 ml of SDS to pellet and vortex until pellet dissolves

Measurement of Tannins in Tannin-Protein Complex

1. Take 1 ml of dissolved pellet and add 3 ml SDS-TEA solution
2. Add 1 ml of ferric chloride reagent
3. Record absorbance at 510 nm after 15-30 min
    a. Convert measurements to tannin concentrations using standard curve
    b. Multiply values by 1.5 to obtain tannin in the complex

Protein-Precipitable Phenolics as a Percentage of Total Phenolics

1. Take varying aliquots of tannin extract and add 1 ml of SDS
    a. May vary with initial amount of tannin in sample
2. Add 3 ml SDS-TEA
3. Add 1 ml ferric chloride reagent

4. Record absorbance at 510 nm

5. Convert to tannic acid equivalent using standard curve

Preparation of Standard Curve

Tube	Tannic Acid* Solution (ml)	SDS 1% (ml)	SDS-TEA (ml)	Ferric Chloride (ml)
Blank	0.0	1.0	3.0	1.0
Standard 1	0.1	0.9	3.0	1.0
Standard 2	0.2	0.8	3.0	1.0
Standard 3	0.3	0.7	3.0	1.0
Standard 4	0.4	0.6	3.0	1.0
Standard 5	0.5	0.5	3.0	1.0
Standard 6	0.6	0.4	3.0	1.0
Standard 7	0.7	0.3	3.0	1.0
Standard 8	0.8	0.2	3.0	1.0
Standard 9	0.9	0.1	3.0	1.0
Standard 10	1.0	0.0	3.0	1.0

*Tannic Acid Solution: 0.5mg/ml in 1% SDS

Appendix D - Inhibition and Reversal of Fraction 1 Protein-Tannin Complexes with Polyethylene Glycol (PEG)

Adapted from:

Jones, W. T., and J. L. Mangan. 1977. Complexes of the condensed tannins of saifoin (Onobrychis viciifolia Scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. J. Sci. Fd. Agric. 28:126-136.

Reagents

1) Tannin solution
    a) Dissolve 10 mg tannic acid into 200 µl of DH_2O
    b) Will need one per sample
    c) Can make a large batch to have enough for every sample

2) Protein solution
    a) Dissolve 2.5 mg BSA into 1000 µl DH_2O
    b) Will need one per sample
    c) Can make a large batch to have enough for every sample

Inhibition of Tannin-Protein Complex Formation

1) To each protein solution, add either 4 mg or 40 mg PEG
2) Vortex each sample until PEG is completely dissolved
3) Add tannin solution and vortex for 30 sec
4) Centrifuge samples at 16,000g for 30 min at 4°C

5) Remove supernatant for analysis using gel electrophoresis

6) Supernatant may also be used to determine true protein via a double Kjeldahl

Reversal of Tannin-Protein Complex Formation

1) Add tannin solution and vortex for 30 sec

2) Allow samples to incubate at room temperature for 10 min

3) After incubation add either 4 mg or 40 mg PEG

4) Vortex each sample until PEG is completely dissolved

5) Centrifuge samples at 16,000g for 30 min at 4°C

6) Remove supernatant for analysis using gel electrophoresis

7) Supernatant may also be used to determine true protein via a double Kjeldahl