# Reduced Lignin Alfalfa: Yield, Forage Nutritive Value, Stem and Leaf Characteristics, and Forage Digestibility by Horses

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## Table of Contents

Acknowledgements ..... i

Dedication ..... ii

Table of Contents ..... iii

List of Tables ..... v

List of Figures ..... vii

**Chapter 1: Review of Literature**

Forage Requirements for Horses ..... 1
Forage Utilization by the Horse ..... 3
Alfalfa as a Forage Source for Horses ..... 6
Effects of Lignification on Forage Digestibility ..... 7
Reduced Lignin Alfalfa ..... 8
Alfalfa Yield and Forage Nutritive Value ..... 10
Digestibility of Reduced Lignin Alfalfa ..... 12
Methods for Assessing *In Vivo* Forage Digestibility ..... 13
Factors Affecting Digestibility ..... 14
Assessing Digesta Passage Rates ..... 16
Marker Labeling, Sampling, and Administration ..... 22
Factors Affecting Digesta Passage Rates ..... 24
Summary and Conclusions ..... 26
Literature Cited ..... 28

**Chapter 2: Forage Accumulation and Nutritive Value of Reduced Lignin and Reference Alfalfa Cultivars**

Chapter Summary ..... 42
Introduction ..... 43
Materials and Methods ..... 46
Results and Discussion ..... 52
Summary and Conclusions ..... 68
Acknowledgements ..... 69
Literature Cited ..... 80

**Chapter 3: Morphology and Stem and Leaf Forage Nutritive Value of Reduced Lignin Alfalfa**

Chapter Summary ..... 86
Introduction ..... 87

Materials and Methods ..... 90
Results and Discussion ..... 93
Summary and Conclusions ..... 105
Acknowledgements ..... 106
Literature Cited ..... 114

**Chapter 4: Apparent Digestibility, Fecal Particle Size, and Mean Retention Time of Reduced Lignin Alfalfa Hay Fed to Horses**
Chapter Summary ..... 120
Introduction ..... 121
Materials and Methods ..... 123
Results and Discussion ..... 133
Summary and Conclusions ..... 140
Literature Cited ..... 149

**Bibliography** ..... 156

## List of Tables

Table 2.1. Alfalfa harvest schedule for 2015 and 2016 growing seasons in Becker, Rochester, Rosemount, and St. Paul, MN. ..... 70

Table 2.2. Average stage of maturity across multiple cuts for alfalfa grown in Becker (BE), Rochester (ROC), Rosemount (ROS), and St. Paul (SP), MN in 2015 and 2016 as determined by cultivar and cutting treatment. ..... 71

Table 2.3. Seasonal cumulative forage accumulation for alfalfa grown in Becker (BE), Rochester (ROC), Rosemount (ROS), and St. Paul (SP), MN in 2015 and 2016 as determined by cultivar and cutting treatment. ..... 72

Table 2.4. Crude protein, neutral detergent fiber, acid detergent lignin, and neutral detergent fiber digestibility for alfalfa cultivars grown in Becker (BE), Rochester (ROC), Rosemount (ROS), and St. Paul (SP), MN in 2015 and 2016. ..... 73

Table 2.5. Crude protein, acid detergent fiber, neutral detergent fiber, acid detergent lignin, and neutral detergent fiber digestibility for alfalfa grown in Becker (BE), Rochester (ROC), Rosemount (ROS), and St. Paul (SP), MN under various cutting treatments in 2015 and 2016. ..... 74

Table 3.1. Test of fixed effects for forage morphological characteristics and stem and leaf forage nutritive values, including stem yield, leaf yield, leaf to stem ratio (L:S), stem and leaf crude protein (CP), stem and leaf neutral detergent fiber (NDF), stem and leaf acid detergent lignin (ADL), and stem and leaf neutral detergent fiber digestibility (NDFD) for alfalfa cultivars grown in Becker and St. Paul, MN during the seeding (2015) and first production (2016) year. ..... 107

Table 3.2. Average stem yield, leaf yield, leaf to stem ratio (L:S), stem and leaf crude protein (CP), stem and leaf neutral detergent fiber (NDF), stem and leaf acid detergent lignin (ADL), and stem and leaf neutral detergent fiber digestibility (NDFD) for alfalfa cultivars grown in Becker and St. Paul, MN during the seeding (2015) and first production (2016) year. ..... 108

Table 3.3. Regression coefficients, associated $r^2$ values, and parameter estimates at a mid-vegetative (MSW 1) and late flower (MSW 6) maturity for models describing the responses of alfalfa stem yield, leaf yield, leaf to stem ratio (L:S), stem and leaf crude protein (CP), stem and leaf neutral detergent fiber (NDF), stem and leaf acid detergent lignin (ADL), and stem and leaf neutral detergent fiber digestibility

(NDFD) to forage mean stage by weight (MSW) for alfalfa cultivars grown in Becker and St. Paul, MN during the seeding and first production year. ..... 109

Table 4.1. Nutrient composition (DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; ADL, acid detergent lignin; DE, digestible energy) and forage characteristics of reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay fed to adult horses at maintenance. ..... 142

Table 4.2. Forage dry matter intake (DMI), water intake, total time to consumption (TTC), and DMI rate (DMIR) for adult horses fed reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay. ..... 143

Table 4.3. Apparent nutrient digestibility values (DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; ADL, acid detergent lignin) for reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay fed to adult horses at maintenance. ..... 144

Table 4.4. Fecal particle size distribution (% of DM) and mean fecal particle size (MFPS) for adult horses fed reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay. ..... 145

Table 4.5. Marker concentration values (average, avg; area under the curve, AUC; peak; and time to peak, TTP) and mean retention time (MRT) for liquid (Co) and particulate (Yb) phase matter in the digestive tract of adult horses fed reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay. ..... 146

## List of Figures

Figure 2.1. Monthly air temperature (°C), precipitation (cm), and 30-year historical average for Becker, Rochester, Rosemount, and St. Paul, MN during the 2015 (A) and 2016 (B) growing season. Weather data was obtained from http://mrcc.isws.illinois.edu/. ..... 76

Figure 2.2. Predicted alfalfa forage dry matter mass and 95% confidence intervals (shaded area) for reduced lignin 54HVX41 ($y_1$) and reference alfalfa cultivars ($y_2$) in response to average cumulative growing degree days. Vertical dashed lines correspond to treatment prescribed cutting intervals and their direct relationship to cumulative growing degree days during the 2016 growing season. ..... 78

Figure 2.3. Predicted alfalfa relative forage quality and 95% confidence intervals (shaded area) for reduced lignin 54HVX41 ($y_1$) and reference alfalfa cultivars ($y_2$) in response to average cumulative growing degree days. Vertical dashed lines correspond to treatment prescribed cutting intervals and their direct relationship to cumulative growing degree days during the 2016 growing season. Horizontal dashed lines correspond to the RFQ for reference alfalfa cultivars harvested under a 30-d cutting interval. ..... 79

Figure 3.1. Monthly air temperature (°C), precipitation (cm), and 30-year historical average for Becker and St. Paul, MN during the 2015 and 2016 growing season. Weather data was obtained from http://mrcc.isws.illinois.edu/. ..... 111

Figure 3.2. Predicted alfalfa stem acid detergent lignin and 95% confidence intervals (shaded area) for reduced lignin cultivar 54HVX41 ($y_1$) and reference alfalfa cultivar DKA43-22RR ($y_2$) in response to forage mean stage by weight during the seeding (A) and first production (B) year. ..... 112

Figure 3.3. Predicted alfalfa stem neutral detergent fiber digestibility and 95% confidence intervals (shaded area) for reduced lignin cultivar 54HVX41 ($y_1$) and reference alfalfa cultivar DKA43-22RR ($y_2$) in response to forage mean stage by weight during the seeding (A) and first production (B) year. ..... 113

Figure 4.1. Mean cobalt (Co) excretion for adult horses consuming reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay. ..... 147

Figure 4.2. Mean ytterbium (Yb) excretion for adult horses consuming reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay. ..... 148

# CHAPTER 1

## REVIEW OF LITERATURE

### Forage Requirements for Horses

As herbivores, horses obtain the nutrients necessary for everyday physiological processes through the consumption of plant material. Horses are uniquely designed in that they have foregut enzymatic digestion similar to monogastric species, but they also have an enlarged hindgut which allows for microbial post-gastric fermentation. This specialized digestive system enables the equine to successfully subsist on a forage-based diet.

Whether in the form of hay or pasture, forages represent a significant portion of the diet for all classes of post-weaned horses, and many adult horses can receive their entire digestible energy (DE) requirement from forages alone (NRC, 2007). Forages are typically characterized by a high dietary fiber content, which is comprised primarily of carbohydrates. These carbohydrates are produced during the process of photosynthesis, in which plants convert carbon dioxide to simple sugars using energy from the sun. These simple sugars, or monosaccharides, are then linked together to form di-, poly-, or oligosaccharides. Carbohydrates can be classified as either structural or nonstructural carbohydrates. Structural carbohydrates constitute the fibrous portion of plant material, making up much of the plant cell wall and providing the plant with the stability it needs to stand upright. Cellulose, hemicellulose, lignin, and pectin are all classified as structural carbohydrates. Nonstructural carbohydrates form the soluble portion of plant material and originate from the cell contents. The simple sugars glucose, fructose, and

sucrose, as well as the storage carbohydrates starch and fructan, are all nonstructural carbohydrates. Together, these structural and nonstructural carbohydrates constitute the main energy-yielding fractions of forage.

In the mature horse, following ingestion, feed travels down the length of the esophagus until it reaches the stomach. Within the stomach, feed mixes with digestive enzymes, which begin the digestion process (Lewis, 1995). From there, feed is propelled into the small intestine, where most of the soluble nutrients are broken down by digestive enzymes, absorbed across the gut wall, and metabolized for energy (Hintz et al., 1971). Digestive enzymes produced up until this point can be used for the digestion of proteins, fats, and nonstructural carbohydrates; however, structural carbohydrates cannot be broken down by endogenously-produced digestive enzymes (Janis, 1976; Åman and Graham, 1990). Instead, structural carbohydrates will pass through the small intestine to the large intestine, where they will undergo microbial fermentation (Hintz et al., 1971; Lewis, 1995). Through this fermentation process, the microbes in the hindgut can metabolize and break down structural carbohydrates and produce volatile fatty acids (VFAs), which can be absorbed by the horse and utilized as an energy source (Applegate and Hershberger, 1969; Hintz et al., 1971; Janis, 1976). This process of hindgut fermentation is essential, as VFA production can make a significant contribution to the horse's daily energy needs. Research has shown that fermentation in the cecum alone can supply 26 to 42% of a horse's DE requirement (Glinsky et al., 1976).

To successfully subsist on a forage-based diet, horses rely heavily on the microbial populations present in their gastrointestinal tract to break down the fibrous portion of the forage. As dietary forage concentrations increase, hindgut fermentation

and VFA production becomes increasingly important (Hintz et al., 1971). Microbial populations in the hindgut therefore play a critical role in increasing nutrient availability to the horse. Forages should never comprise less than 50% of the total diet for adult horses, as the fiber content in forages plays an essential role in maintaining normal microbial function (NRC, 2007). Reducing the amount of dietary forage and increasing the amount of concentrates given to a horse will alter the microbial profile of the gastrointestinal tract (Goodson et al., 1988; Julliand et al., 2001). Although concentrates are high in soluble carbohydrates and would typically be broken down and absorbed in the small intestine, high levels of concentrates can exceed the digestion capacity of the small intestine and result in an influx of soluble carbohydrates to the hindgut (Potter et al., 1992). In the hindgut, non-degraded starch is readily available for fermentation by starch-utilizing bacteria, resulting in the production and accumulation of lactic acid and a reduction in hindgut pH (Goodson et al., 1988; Julliand et al., 2001; Medina et al., 2002). This drop in pH disrupts the balance of microbial populations in the hindgut and changes the microbial profile by reducing the concentration and activity of the fiber-digesting microbial populations, altering VFA production, and decreasing fiber digestion in the hindgut (Julliand et al., 2001; Medina et al., 2002). The accumulation of lactic acid can also result in lactate acidosis, which has been associated with laminitis in the horse (Garner et al., 1977). Therefore, the importance of fiber in the equine diet should not be underestimated. When structural carbohydrates are fed in a greater proportion than nonstructural carbohydrates, gastrointestinal pH, motility, and function remain normal, decreasing the risk of additional health concerns (Medina et al., 2002).

## Forage Utilization by the Horse

While the anatomy of the equine gastrointestinal tract provides horses with a large capacity for fiber fermentation, considerable variation exists in forage quality and not all forage sources will provide equal concentrations of nutrients to the horse. Furthermore, in order to be fully utilized and provide benefit to the animal, nutrients must not only be present in the forage but must also be made available through the process of digestion and absorption. This concept of nutrient availability is often broadly referred to as digestibility, which has been defined as the fraction of a feedstuff that is lost on passage through the digestive tract (Schneider and Flatt, 1975; Cochran and Galyean, 1994). There are a number of different methods that can be used to evaluate forage quality and the availability of nutrients. Common methods include chemical analysis, *in vitro* digestibility, and *in vivo* digestibility.

Chemical analysis of feeds is routinely measured via the detergent analysis system, which was first developed by Van Soest in the 1960s (Van Soest et al., 1966). The Van Soest system chemically partitions the plant cell components based on expected nutritional availability (NRC, 2007). The cell contents of the plant include proteins, lipids, and nonstructural carbohydrates and are considered highly available. The plant cell wall contains the partially digestible or indigestible portions of the cell and includes primarily structural carbohydrates. Under this system (Van Soest et al., 1991), feed samples are first washed in a neutral detergent that removes nonstructural carbohydrates, protein, and pectin. The remaining fraction includes hemicellulose, cellulose, and lignin, and is referred to as neutral detergent fiber (NDF). Samples are then washed with an acid detergent solution that removes hemicellulose, leaving cellulose and lignin; this fraction is referred to as acid detergent fiber (ADF). Lignin concentrations can be determined by

treating the sample with sulfuric acid or permanganate and subtracting the ash content; this fraction is referred to as acid detergent lignin (ADL).

*In vitro* digestibility measurements offer a way to measure nutrient availability and are traditionally used to simulate digestion by ruminants (Tilley and Terry, 1963; Van Soest et al., 1966; Applegate and Hershberger, 1969). In this procedure, samples undergo a two-stage process where feed is first digested in rumen fluid and buffered for 48 hours to stimulate microbial fermentation and then digested in either pepsin (Tilley and Terry, 1963) or neutral detergent solution (Van Soest et al., 1966). To apply these methods to equine digestion, inoculum should come from cecal fluid rather than rumen fluid. Work by Applegate and Hershberger (1969) and Koller et al. (1978) validated the use of cecal fluid as an inoculum source in equine *in vitro* digestibility experiments. However, due to the challenge associated with obtaining cecal fluid, it has been of interest to find an alternative inoculum source. Lowman et al. (1999) established equine feces as an acceptable source of inoculum for gas production *in vitro* digestibility work, and several researchers have successfully used horse feces for *in vitro* digestion experiments (Abdouli and Attia, 2007; Lattimer et al., 2007; Earing et al., 2010). Abdouli and Attia (2007) developed a two-stage method for *in vitro* digestibility determination for horses using an initial step of pepsin and amylase digestion followed by a microbial fermentation step via the Tilley and Terry (1963) method. Using these methods to simulate hindgut fermentation using the DaisyII incubator has shown similarity to *in vivo* measurements (Lattimer et al., 2007; Earing et al., 2010). However, while Earing et al. (2010) found comparable dry matter digestibility (DMD) for diets consisting of alfalfa hay with oats, timothy hay with oats, and timothy hay diets between

*in vivo* and *in vitro* methods, different digestibility values were observed between the two methods with an alfalfa hay diet, and neutral detergent fiber digestibility (NDFD) estimates differed between methods. Further research using a wider range of forages and methods is needed to determine if *in vivo* and *in vitro* digestibility methods produce similar results for horses, and to establish *in vitro* digestibility as a viable technique for estimating digestibility in the horse.

*In vivo* digestibility is considered the gold standard for evaluating forage quality and nutrient availability. Records of digestibility experiments began as early as the 1860's (Schneider and Flatt, 1975) and still continue today. A typical digestibility trial involves measuring complete nutrient intakes and outputs. The difference between nutrient intake and output can be used to estimate the percentage of nutrient degradation and absorption within the digestive tract. To date, numerous studies have been published containing digestibility estimates for a wide variety of feedstuffs fed to horses. Estimates for forages can vary widely and are dependent on a number of factors, including forage type, plant species, maturity, and harvest conditions (NRC, 2007).

## Alfalfa as a Forage Source for Horses

While the nutritional needs of many adult horses can be met with grass forages (Bott et al., 2013), there are certain classes of horses within the equine population that have advanced dietary requirements which can only be met with a higher quality, more nutrient-dense diet (NRC, 2007). These classes include breeding stock such as broodmares and stallions, "hard keepers" such as thin or older horses, and performance horses in heavy work. For these groups of horses, a higher quality or more nutrient-dense forage is often beneficial, as it can fulfill these increased requirements while still

providing enough dietary fiber to maintain a normal gastrointestinal environment. Compared to grasses, legumes such as alfalfa are typically lower in NDF and contain greater concentrations of protein, energy, and essential vitamins and minerals like calcium (Fonnesbeck et al., 1967; Gibbs et al., 1988; Crozier et al., 1997; Wilman and Moghaddam, 1998; Sturgeon et al., 2000; Woodward et al., 2011). Legumes are also generally more digestible than grasses, making them a preferred forage source for these classes of horses. Researchers comparing digestibility between legumes and grasses have reported average DMD ranging from 44 to 59% for grass hays and 58 to 73% for alfalfa hays (Cuddeford et al., 1995; Crozier et al., 1997; LaCasha et al., 1999; Sturgeon et al., 2000; Pearson et al., 2006; Edouard et al., 2008; Potts et al., 2010).

## Effects of Lignification on Forage Digestibility

Although alfalfa is widely used as a forage for horses and other herbivores with advanced dietary requirements, the digestibility and utilization of alfalfa by these animals is hampered by its lignin content (Sewalt et al., 1997; Casler et al., 2002). Classified as one of the structural carbohydrates, lignin is a complex structural polymer that is the second most abundant component of secondary plant cells walls (Li et al., 2015b). Along with the other structural carbohydrates, lignin is essential for providing the strength and rigidity necessary for the plant to stand upright (Inoue et al., 1998; Guo et al., 2001a). As a plant matures, lignin concentrations increase, filling the space between cellulose, hemicellulose, and pectin molecules and forming cross-linkages with hemicellulose (Albrecht et al., 1987; Jung et al., 1997b; Inoue et al., 1998; Casler and Vogel, 1999).

While it is essential for normal plant growth, the deposition of lignin into plant cell walls can reduce the feeding value of alfalfa by negatively affecting microbial

degradation and the digestion of feed by intestinal enzymes (Buxton and Hornstein, 1986; Liu and Yu, 2011). Lignification has been reported to be the major factor limiting both the *in vitro* digestibility of plant cell-wall polysaccharides (Morrison, 1979; Albrecht et al., 1987; Jung et al., 2012) and the *in vitro* DMD of whole plant forage (Casler, 1986, 1987; Reddy et al., 2005). These negative effects have primarily been associated with lignin concentration, as numerous studies have found strong negative correlations between lignin concentrations and forage digestibility (Albrecht et al., 1987; Casler, 1987; Jung et al., 1997b; a; Reddy et al., 2005).

Major improvements in the digestibility and quality of alfalfa as a forage source may be possible if plants can be selected that exhibit a slower decline in loss of digestibility (Buxton et al., 1985; Sanderson et al., 1989; Jung and Lamb, 2006; Lamb et al., 2014). With such a strong influence on forage digestibility, small decreases in the lignin concentration of forages can be expected to improve the fiber digestibility at any plant maturity stage (Casler, 1987; Undersander et al., 2009). Predictions by Casler (1987) estimated that a single unit decrease (g $kg^{-1}$) in the concentration of acid detergent lignin (ADL) of smooth bromegrass (*Bromus inermis* L.) would result in a 7.0 unit increase in *in vitro* DMD. Feeding and grazing studies have shown that small changes in forage digestibility can significantly impact animal performance. For a number of grass cultivars, Casler and Vogel (1999) reported a positive relationship between *in vitro* DMD improvement and animal daily gains, with a 1% increase in *in vitro* DMD resulting in a 3.2% increase in daily weight gains for beef cattle.

## Reduced Lignin Alfalfa

Several experimental lines of alfalfa have been developed with down-regulation of the caffeic acid 3-O-methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCOMT) lignin biosynthetic genes (Inoue et al., 1998; Guo et al., 2001a; Marita et al., 2003; Getachew et al., 2011). Initial comparisons in the forage quality of these reduced-lignin alfalfas have shown potential for increased digestibility. Experimental populations of COMT and/or CCOMT down-regulated alfalfa have shown a 4 to 29% decrease in stem lignin concentration and a 1 to 24% decrease in whole plant lignin concentration compared to reference alfalfa varieties (Guo et al., 2001a; b; Marita et al., 2003; Reddy et al., 2005; Undersander et al., 2009; Getachew et al., 2011). The wide variation in lignin reduction reported could be due to the specific down-regulated gene (Guo et al., 2001a; b; Marita et al., 2003; Undersander et al., 2009; Getachew et al., 2011), the methods used for lignin analysis (Guo et al., 2001a; b; Jung et al., 2012), or the plant growing conditions (Baucher et al., 1999).

Populations of reduced lignin alfalfa have shown an increase in *in vitro* DMD, *in situ* rumen digestibility, and *in vitro* NDFD (Guo et al., 2001b; Reddy et al., 2005; Mertens and McCaslin, 2008; Weakley et al., 2008; Undersander et al., 2009; Getachew et al., 2011). Reddy et al. (2005) reported a strong negative linear relationship between *in situ* digestibility and ADL levels across all reduced lignin lines. In addition to increased digestibility, reduced lignin alfalfa populations have also shown reduced NDF concentrations and greater non-fiber carbohydrate concentrations compared to control lines (Guo et al., 2001b; Reddy et al., 2005; Getachew et al., 2011; Li et al., 2015a), while crude protein (CP) concentrations remained similar for reduced lignin and reference alfalfa lines (Getachew et al., 2011; Li et al., 2015a).

## Alfalfa Yield and Forage Nutritive Value

Research with experimental populations of reduced lignin alfalfa has shown their potential to improve forage quality and digestibility. However, field evaluations under diverse conditions are needed to determine the performance of new commercial alfalfa cultivars containing the reduced lignin trait, especially with regard to forage accumulation and nutritive value under different harvest frequencies. As the interval between alfalfa harvests increases, annual DM forage accumulation also increases (Brink and Marten, 1989; Kallenbach et al., 2002; Putnam et al., 2005; Probst and Smith, 2011; Min, 2016). At the same time, increasing the interval between harvests also results in a greater forage maturity and decreased forage nutritive value, including decreased CP, increased NDF and ADL, and decreased DMD and NDFD (Weir et al., 1960; Nordkvist and Åman, 1986; Hall et al., 2000; Kallenbach et al., 2002; Yu et al., 2003; Brink et al., 2010; Palmonari et al., 2014; Min, 2016).

This improvement in forage accumulation at the expense of forage nutritive value is often referred to as the yield-quality tradeoff, and can largely be attributed to the maturity stage at which alfalfa is harvested. It has been well established that increasing forage maturity results in increasing fiber, declining CP, and decreasing digestibility within alfalfa herbage (Albrecht et al., 1987; Sanderson and Wedin, 1988; Sanderson et al., 1989; Griffin et al., 1994; Hall et al., 2000; Kallenbach et al., 2002; Lamb et al., 2007, 2012; Brink et al., 2010; Palmonari et al., 2014). As such, highest herbage nutritive value and intake potential usually occur with pre-flowering alfalfa, while alfalfa harvested at later maturity stages has been shown to have a lower nutritive value (Sheaffer et al., 1988, 2000; Kallenbach et al., 2002; Lamb et al., 2007, 2012; Brink et al., 2010).

Therefore, when alfalfa is used for livestock feeding, harvest at bud to early flower is recommended to provide forage with a high to medium nutrient concentration (Lamb et al., 2003).

The decline in the quality of alfalfa herbage with advancing maturity can be attributed to a decrease in leaf and increase in stem proportion as the plant matures (Buxton et al., 1985; Albrecht et al., 1987; Sanderson and Wedin, 1988; Julier and Huyghe, 1997; Sheaffer et al., 2000; Lamb et al., 2003, 2012; Milić et al., 2011; Yari et al., 2012, 2014). Increasing forage maturity and canopy height results in defoliation through leaf senescence and abscission from the lower portions of the plant due to shading and disease (Buxton et al., 1985; Albrecht et al., 1987; Sheaffer et al., 1988). This increase in leaf loss is coupled with increases in stem growth, resulting in an increased contribution of the stem to the total herbage amount and a decreased leaf to stem ratio.

Shifts in the proportions of leaf and stem material result in significant changes in herbage quality largely because of the differences in forage nutritive value within the stem and leaf fractions of the plant. Alfalfa leaves are protein-rich and low in cell wall concentration, and therefore have a high nutritive value and are highly digestible; in contrast, alfalfa stems exhibit low digestibility as a result of high concentrations of cell wall polysaccharides and lignin (Buxton et al., 1985; Buxton and Hornstein, 1986; Albrecht et al., 1987; Buxton and Russell, 1988; Julier and Huyghe, 1997; Jung et al., 1997b; Milić et al., 2011; Marković et al., 2012; Yari et al., 2012; Lamb et al., 2012). As maturity advances, herbage digestibility declines and cell wall concentrations increase at much slower rates in leaves compared to stems (Kilcher and Heinrichs, 1974; Buxton et

al., 1985; Buxton and Hornstein, 1986; Albrecht et al., 1987; Sheaffer et al., 2000; Marković et al., 2012). Therefore, because stems not only increase in proportion but also decrease in digestibility faster with advancing maturity compared to leaves, they exert a larger influence and have a greater detrimental impact on total herbage quality than leaves do (Buxton et al., 1985; Buxton and Hornstein, 1986; Sanderson et al., 1989; Sheaffer et al., 2000; Jung and Lamb, 2006; Lamb et al., 2012).

To evaluate the effects of varied harvest frequencies and changing forage maturity on yield and quality for reduced lignin alfalfa, two field studies was established in Minnesota. The objectives of the first study were to evaluate forage accumulation and forage nutritive value for reduced lignin and reference alfalfa cultivars when subject to diverse cutting treatments during the establishment and first production year. Information on the methods and results for this study are reported in Chapter 2. The objectives of the second study were to characterize changes in morphological development and forage nutritive value within leaf and stem fractions for reduced lignin and reference alfalfa cultivars over time. Information on the methods and results for this study are reported in Chapter 3.

**Digestibility of Reduced Lignin Alfalfa**

In addition to the need for an evaluation of the performance of reduced lignin alfalfa cultivars in the field, it remains to be seen if the improvements in *in vitro* DMD and NDFD for reduced lignin alfalfa will translate to greater *in vivo* digestibility when fed to the animal directly. Preliminary results evaluating reduced lignin alfalfa hay found that when reduced lignin and traditional alfalfa hays were fed to lambs, DMD and NDFD were greater for the reduced lignin hays (Mertens and McCaslin, 2008). Similarly, when

reduced lignin alfalfa hays were included as 50% of the ration for lactating dairy cows, NDFD was significantly increased for both of the reduced lignin hays, and the additional forage digestibility for the COMT down-regulated hay resulted in 1.3 kg more milk production per head per day compared to the control diet (Weakley et al., 2008). While this information is promising, information on forage digestibility for current, commercially available reduced lignin alfalfa cultivars is not yet available, and digestibility changes have not yet been evaluated in the equine model.

## Methods for Assessing *In Vivo* Forage Digestibility

Traditional *in vivo* digestibility experiments typically involve total fecal collections. In these experiments, animals are first adapted to the feedstuff of interest, then placed in metabolism crates or stalls or fitted with collection devices to allow for total fecal collections. During the total fecal collection period, daily consumption records are kept and all voided feces are collected. Collection periods ranging from 3 to 10 days have been reported in the literature (Smolders et al., 1990; Lindberg et al., 2006). Research investigating fecal collection period lengths has demonstrated that a period as short as 3 days may be sufficient to obtain reasonable estimates of *in vivo* digestibility in the horse (Hintz and Loy, 1966; Goachet et al., 2009). However, Schaafstra et al. (2015) reported decreasing variability as collection periods were increased to 5 days, after which variation remained fairly constant. Therefore, to minimize variability, a collection period of 5 days is often recommended, and collection periods ranging from 5 to 7 days are commonly used throughout the literature. Following the total fecal collection period, feed and fecal samples are analyzed for nutrient composition. Using feed consumption and fecal excretion data, nutrient intake and excretion can be calculated. For any given

nutrient, apparent digestibility can be determined by calculating the difference between the amount of nutrient consumed and the amount of nutrient excreted in the feces.

Total fecal collections are labor intensive, time consuming, and expensive, often resulting in small animal numbers which may limit the power of the study (Sales, 2012). Additionally, certain situations may limit their use, like when studying horses that are grazing or exercising. Because of this, alternative methods for estimating digestibility have been investigated to replace or modify the use of total fecal collections. Partial collection techniques involve periodic fecal sampling, but have only been used with limited success. Goachet et al. (2009) used ADL and acid insoluble ash (AIA) as markers to compare digestibility estimates obtained using partial and total fecal collection methods. In this experiment, ADL underestimated digestibility for all dietary constituents measured except dry matter, and AIA overestimated the digestibility of all dietary constituents. More research comparing the effectiveness of partial and total fecal collection techniques is likely needed to ensure accuracy before partial collection techniques are widely adopted for digestibility determinations.

## Factors Affecting Digestibility

It has been well established that the nutritive value of a feedstuff affects digestibility. For forages, diets containing higher fiber concentrations have been consistently associated with decreased digestibility (Cuddeford et al., 1995; Crozier et al., 1997; Pearson et al., 2001; Miyaji et al., 2011). Similarly, the type of diet (*i.e.* forage vs. concentrate) can also affect digestibility. The addition of concentrates to the equine diet has been shown to increase overall dietary DMD, regardless of the inclusion rate or concentrate source (Pagan et al., 1998; Palmgren Karlsson et al., 2000; Drogoul et al.,

2001; Hussein et al., 2004; Jouany et al., 2008). Fiber digestion was not found to be affected by an inclusion of low levels of concentrates in the diet (Palmgren Karlsson et al., 2000; Hussein et al., 2004). However, grain supplementation did decrease fecal pH (Hussein et al., 2004), and a high inclusion of dietary concentrates has been shown to have a negative impact on dietary fiber digestion (Pagan et al., 1998; Palmgren Karlsson et al., 2000; Drogoul et al., 2001; Jouany et al., 2008). While the quality of fiber present in forage versus concentrate sources could play a role in fiber digestion (Pagan et al., 1998), these results could also suggest that high levels of concentrates decrease the stability of the microbial community in the hindgut, reducing the rate of fiber fermentation (Medina et al., 2002; Jouany et al., 2008).

Other external factors, such as intake level, can also have an effect on digestibility. While Martin-Rosset and Dulphy (1987) and Martin-Rosset et al. (1990) found no difference in digestibility with varying intake levels, Cuddeford et al. (1995) compared digestibility among horses, ponies, and donkeys and found that animals with lower intakes appeared to digest feed more effectively. Similarly, Miyaji et al. (2014) reported an increase in the apparent digestibility of DM, NDF, and ADF for horses fed grass hay at lower intakes.

Changes in intake can often result in changes in digestibility through alterations in digesta passage rates. Broadly, passage rate is defined as the flow of material through the gastrointestinal tract per unit of time. Various terms have been used in the literature to describe digesta passage. Minimum retention time (or transit time) describes the time between feeding and the first appearance of a meal in the feces, while maximum retention time represents the time between feeding and the last excretion of a meal (Van

Weyenberg et al., 2006). The most common term used to describe digesta passage rates is mean retention time (MRT), which denotes the duration the average feed particle is retained within the gastrointestinal tract (Owens and Hanson, 1992; Van Weyenberg et al., 2006). Generally, longer digesta retention times have been associated with increased diet digestibility, likely as a result of increased enzymatic digestion in the small intestine and/or more extensive microbial fermentation in the hindgut (Mertens and Ely, 1982; Pearson and Merritt, 1991; Cuddeford et al., 1995; Van Weyenberg et al., 2006). Digestibility, intake, and passage rate kinetics are all closely linked. Often, the study of digesta flow through the gastrointestinal tract can provide a crucial link between digestion and nutrition, making it an important factor to consider when studying the digestive process.

**Assessing Digesta Passage Rates**

Several approaches have been used by researchers to quantitate digesta passage rates. Direct measures of digesta passage can be gathered using cannulas (Drogoul et al., 2000; Austbø and Volden, 2006; Jouany et al., 2008), or animals can be euthanized and samples collected directly from each segment of the gastrointestinal tract (Hintz et al., 1971; Gibbs et al., 1988; Miyaji et al., 2008a, 2014). However, the use of markers to estimate digesta passage has proven to be a more suitable method and has reduced the use of invasive or terminal procedures. This type of experimentation involves feeding or dosing a marked feed, collecting feces, and measuring the excretion of the marker over time. A marker is defined as a reference compound used to monitor the chemical and physical flow of digesta (Owens and Hanson, 1992). Criteria that have been used to define an ideal marker include: it must be strictly non-absorbable, inert substances; it

must be intimately associated with and uniformly distributed throughout the material it is to mark; it must not modify any physical characteristics or fermentation kinetics of the feed fraction it is to mark; it must not affect, or be affected by, the gastrointestinal tract or its microbial population; and its method of estimation in samples must be specific and sensitive without interfering with other analyses (Bernard and Doreau, 2000; Sales, 2012).

Because fluid and particulate matter move through the gastrointestinal tract differently, separate markers are needed to estimate the passage of each phase (Van Weyenberg et al., 2006). The liquid phase is made up of the soluble components of the digesta, whereas the particulate phase is comprised of any insoluble, undigested particles. Generally, fluid and fine particles will proceed more rapidly through the gastrointestinal tract, particularly in the hindgut, where larger particles are selectively retained to allow for further microbial fermentation (Sellers and Lowe, 1986; Drogoul et al., 2000, 2001). Fluid phase markers must be water soluble and dissolve upon ingestion, remaining in solution in the fluid phase of digesta. Common fluid phase markers include chromium-ethylenediaminetetraacetic acid (Cr-EDTA) and cobalt-ethylenediaminetetraacetic acid (Co-EDTA; Udén et al., 1980). Particulate phase markers can be further classified as internal or external. Markers are considered internal when the marker is a naturally occurring part of the plant, or external when the marker is artificially associated with the plant material (Bernard and Doreau, 2000). Internal markers are usually indigestible components of a feed, including acid insoluble ash (AIA), acid detergent lignin (ADL), indigestible acid detergent fiber (IADF), or n-alkanes (Penning and Johnson, 1983a; b; Dove and Mayes, 1991; Owens and Hanson, 1992; Miraglia et al., 1999). External

markers used in equine studies have varied widely, ranging from Styrofoam particles and colored beads to chromium mordanted-fiber and rare-earth labeled feeds (Hintz and Loy, 1966; Pearson and Merritt, 1991; Pearson et al., 2001; Drogoul et al., 2001; Moore-Colyer et al., 2003; Miyaji et al., 2008b, 2014). Commonly used external markers include chromic oxide ($Cr_2O_3$), chromium-mordanted fiber, and rare-earth elements.

***Cr-EDTA and Co-EDTA***. Both Cr-EDTA and Co-EDTA are water soluble and are commonly used as fluid phase markers in rate of passage studies. The presence of EDTA prevents the absorption of the chromium or cobalt molecule across the gut wall; instead, the marker will pass along the gastrointestinal tract with the fluid phase of the digesta. Evaluation of Cr-EDTA and Co-EDTA as markers has shown that both markers give similar results for fluid passage rate and appear to be suitable markers for the liquid phase, with recovery rates ≥ 90% (Udén et al., 1980; Nyberg et al., 1995; Cuddeford et al., 1995; Drogoul et al., 2000).

***AIA and ADL***. Compounds like AIA and ADL are naturally occurring in feedstuffs and are generally considered to be indigestible. Reports concerning their use as internal markers have been somewhat contradictory. When AIA was used as an internal marker, Orton et al. (1985) and Miraglia et al. (1999) found no differences in digestibility estimates between total collection and AIA methods. However, others have found that digestion coefficients determined based off of AIA content were overestimated compared to those determined by total fecal collection (Cuddeford and Hughes, 1990; Almeida et al., 2001; Goachet et al., 2009). Studies using ADL as an internal marker have shown that apparent digestibility coefficients appeared to be underestimated (Miraglia et al., 1999; Almeida et al., 2001; Goachet et al., 2009) and recovery in the

feces is often low (Miraglia et al., 1999). As such, the use of AIA or ADL as internal markers has not been widely accepted.

***N-alkanes***. N-alkanes are another internal marker that has been investigated as a potential marker for digestibility and passage rate studies (Dove and Mayes, 1991; Mayes and Dove, 2000; Stevens et al., 2002). Odd-chain alkanes, which are naturally present in the cuticular wax of forage plants, can be used as internal markers to predict digestibility, while even-chain alkanes, typically provided from external sources, can be fed and used to predict fecal output (Mayes and Dove, 2000). Although some disappearance of alkanes from the digestive tract was reported, Ordakowski et al. (2001) and Stevens et al. (2002) found similar DMD estimates using fecal collection and n-alkane methods when n-alkane estimates were corrected for fecal recovery; both concluded that n-alkanes could be used as internal markers for equine digestibility research provided fecal recoveries could be calculated. Chavez et al. (2014) also investigated the use of n-alkanes to measure intake and digestibility, and concluded that n-alkanes could be useful for determining intake and digestibility under grazing conditions.

***Chromic oxide***. Chromic oxide has been used as an external, particulate phase marker in passage rate studies. While Todd et al. (1995) found digestibility estimates based on $Cr_2O_3$ to be comparable to fecal collection methods, others have reported that the flow kinetics of $Cr_2O_3$ are not representative of the digesta fraction (Owens and Hanson, 1992), and incomplete fecal recovery and poor mixing with forages and grains has been noted (Sales, 2012). Additionally, if total fecal collections are not used and marker recovery cannot be calculated, the diurnal variation of fecal chromium limits the

usefulness of $Cr_2O_3$ as a marker (Parkins et al., 1982; Cuddeford and Hughes, 1990; Holland et al., 1998).

***Chromium-mordanted fiber***. Chromium forms strong ligands with plant cell wall constituents, and the mordanting process used to produce chromium-mordanted fiber irreversibly binds the chromium to the fibrous portion of the cell wall. Udén et al. (1980) and Beauchemin and Buchanan-Smith (1989) tested the stability of chromium-mordanted fiber when exposed to 0.01M HCl solution or 48-h *in vitro* digestion and reported marker recoveries ≥ 88%. Due to this high binding capacity, chromium-mordanted fiber has frequently been used as a particulate marker for *in vivo* digestion trials, which have reported fecal recovery of chromium ranging from 77 to 99% when fed to steers or dairy cattle (Udén et al., 1980; Mader et al., 1984) and 88 to 90% when fed to horses, ponies, and donkeys (Pearson and Merritt, 1991; Cuddeford et al., 1995). However, although chromium binds well to particulate material, the binding of high levels of chromium to the cell wall has been shown to adversely affect particulate passage rates by reducing DM digestibility (Udén et al., 1980; Mader et al., 1984; Beauchemin and Buchanan-Smith, 1989) and increasing feed density of the labeled particles (Ehle, 1984).

***Rare-earth elements***. The use of rare-earth metals as particulate markers has become increasingly more common because they are indigestible, will bind tightly to plant materials, and are present in very low concentration in forages (Bernard and Doreau, 2000). The elements commonly used as markers in passage rate research include dysprosium (Dy), europium (Eu), cerium (Ce), lanthanum (La) samarium (Sa), and ytterbium (Yb; Crooker et al., 1982; Teeter et al., 1984; Poore et al., 1990). The metallic properties of these elements allow them to be easily complexed with fibrous plant

materials; binding occurs between the functional groups (specifically carboxyl and amino groups) of the fibrous constituents and the metal ion. Total binding capacity is largely dependent on the NDF concentration, with higher levels of NDF resulting in a tighter bond (Bernard and Doreau, 2000). Early information investigating the use of rare-earth metals as markers suggested that the elements could become mobile during the digestive process and migrate away from the originally labeled material, especially under acidic conditions (Crooker et al., 1982; Mader et al., 1984; Teeter et al., 1984; Beauchemin and Buchanan-Smith, 1989). Migration away from labeled material and possible association with smaller particle size material or soluble compounds could bias passage estimates and result in underestimation of true passage rate (Beauchemin and Buchanan-Smith, 1989). However, more recent studies have demonstrated only minimal migration, with fecal marker recoveries ≥ 94% (Drogoul et al., 2000; Moore-Colyer et al., 2003), and have suggested that rare-earth metals could be used successfully as external markers (Hartnell and Satter, 1979; Ledoux et al., 1985; Austbø and Volden, 2006; Rosenfeld et al., 2006). The extent of migration from a feed can be reduced through extensive rinsing following marker attachment, which will remove unbound or loosely bound markers (Teeter et al., 1984; Owens and Hanson, 1992). Labeling feedstuffs with rare-earth metals has also been suggested to induce varying levels of digestive modifications, with several studies reporting reductions in *in situ* DMD for feeds labeled with Yb (Mader et al., 1984; Teeter et al., 1984; Beauchemin and Buchanan-Smith, 1989). Effects on digestibility increase as the level of bound rare-earth increases, likely as a result of negative interactions between marker and microbe attachment; as such, it has been recommended that no more label be bound to the feedstuff than necessary (Bernard and Doreau, 2000).

## Marker Labeling, Sampling, and Administration

Before an external marker can be used it must first be bound to the feedstuff of interest. Many of the techniques used to label forages with external markers require the forage of interest to be boiled, or washed, in a detergent solution to remove all soluble matter prior to marker attachment (Drogoul et al., 2000, 2001). Thus, the marker is applied only to the NDF fraction of the feedstuff. This washing step prevents markers from binding to the soluble portion of the forage and moving through the gastrointestinal tract with the fluid phase rather than the particulate phase.

Numerous labeling techniques have been utilized to bind markers to feedstuffs; the most common methods involve immersion, spraying, or mordanting (Mader et al., 1984; Teeter et al., 1984; Beauchemin and Buchanan-Smith, 1989). A study by Mader et al. (1984) compared labeling methods for Yb-labelled wheat and found that labeling method did not alter MRT estimates. However, the time to first excretion of the marker was less for the spraying technique compared to the immersion technique, suggesting that the Yb may have been more loosely bound to the forage when the marker was applied by spraying (Mader et al., 1984). Unlike spraying, the immersion technique allows for extensive rinsing following marker attachment, which can be used to remove unbound or loosely bound markers and reduce marker migration within the gastrointestinal tract (Teeter et al., 1984; Owens and Hanson, 1992; Bernard and Doreau, 2000).

Regardless of marker selection, all marker procedures use one of two types of dosing (continuous or single pulse-dose) and one of two types of sampling procedures (time sequence or total collection; Owens and Hanson, 1992). Under the pulse-dose technique, marked feeds are usually provided in a single meal or dose; this technique is

typically used to estimate digesta volume and retention time. Continuous dosing involves repeated consumption of marked feeds to maintain marker levels over time and is primarily used to measure instantaneous flow at a specific point within the digestive tract (Owens and Hanson, 1992). The most common dosing technique in equine research involves a pulse-dosed marker followed by time sequence sampling; this technique is commonly used to estimate MRT.

To administer markers, many studies simply mix the labeled feeds with a portion of the daily diet (Drogoul et al., 2001; Austbø and Volden, 2006; Rosenfeld et al., 2006; Jouany et al., 2008; Rosenfeld and Austbø, 2009). Others have mixed the marked feed with a small amount of a highly palatable treat or feed (Pearson and Merritt, 1991; Pearson et al., 2001, 2006; Moore-Colyer et al., 2003; Clauss et al., 2014). Fluid phase markers can also be top dressed, given as a drench by syringe at the back of the throat (Pearson and Merritt, 1991; Pearson et al., 2001, 2006), or administered via a stomach tube (Drogoul et al., 2000, 2001).

When sampling feces for marker concentration, total fecal collections (Pearson and Merritt, 1991; Drogoul et al., 2001; Moore-Colyer et al., 2003; Pearson et al., 2006; Miyaji et al., 2008a, 2011) or partial sampling (Austbø and Volden, 2006; Rosenfeld et al., 2006; Goachet et al., 2009, 2010) methods can be used. Total fecal collections offer the advantage that total marker excretion can be determined to allow for percent recovery calculations. However, the use of partial sampling collection techniques for estimating MRT has also been verified (Goachet et al., 2009). Partial collections are less labor intensive and are useful for situations when total collections cannot be conducted.

Retention times are calculated based on the amount or concentration of marker excreted at a specific time point. The two most common calculations to determine MRT are based on equations by Blaxter et al. (1956), which is based on marker amounts, or by Thielemans et al. (1978), which is based on marker concentrations. The equations of Blaxter et al. (1956) and Thielmans et al. (1978) often result in very similar estimates of MRT (Earing, 2011; Hansen, 2014). Consequently, both are used extensively throughout the literature and are considered accurate estimates for determination of digesta MRT.

## Factors Affecting Digesta Passage Rates

Particulate MRT reported in the literature for horses range from 14 hours to over 50 hours, depending on the marker used, collection methods, diets, and experimental variation (Van Weyenberg et al., 2006). Digesta passage rates are largely influenced by dietary type and composition. Pagan et al. (1998) observed a longer MRT when mature thoroughbred geldings were fed a mixed diet compared to an all-forage diet, and Drogoul et al. (2001) found that increasing the hay:grain ratio increased passage rates through the entire digestive tract. In addition to mixed diets, differences in digesta passage rates among forage-only diets have also been reported. Low quality forage diets often contain high concentrations of fiber and will pass more slowly through the digestive tract, resulting in a longer MRT (Pearson and Merritt, 1991; Pearson et al., 2001; Guay et al., 2002; Clauss et al., 2014). For example, at similar levels of intake, Pearson et al. (2001) found MRT to be 30.5 and 36 h when ponies were fed alfalfa and oat straw, respectively, and Clauss et al. (2014) reported MRTs of 23 and 31 h for horses fed high-quality and low-quality grass hay, respectively. However, other studies have shown that high-fiber forages decreased retention times compared to low-fiber forages (Moore-Colyer et al.,

2003; Pearson et al., 2006). Miyaji et al. (2011) found no difference in particulate or liquid MRT when horses were fed late vegetative or late bloom timothy hay at the same intake level. These variations in passage rate results could be attributed to a number of factors, including feeding level, fiber length, forage variety, plant maturity, or moisture content (Van Weyenberg et al., 2006).

Intake level has also been shown to influence passage rate in horses, with decreased intakes generally resulting in an increased MRT (Pearson and Merritt, 1991; Pearson et al., 2001; Clauss et al., 2014). Cuddeford et al. (1995) and Pearson and Merritt (1991) both reported a greater MRT for diets with lower intake levels. Similarly, Miyaji et al. (2014) reported a 9.7 h reduction in MRT with a 1.5-fold increase in DM intake. Other studies have reported that *ad libitum* access resulted in an increase in DMI and a subsequent decrease in MRT (Pearson et al., 2001, 2006; Guay et al., 2002; Miyaji et al., 2011). When forage intakes for horses are restricted, particulate MRT have been shown to increase by 9 to 43% and liquid MRT by 11 to 31% (Pearson et al., 2001; Miyaji et al., 2011, 2014). Clauss et al. (2014) fed grass hay to ponies at varying intake levels and found increasing particulate and liquid MRTs as intake levels decreased from *ad libitum* down to 30%.

The level of feed processing can also have an effect on digesta passage rates in horses. While grain processing method was not found to affect MRT (Rosenfeld et al., 2006; Rosenfeld and Austbø, 2009), grinding and pelleting forages increased particulate MRT in horses compared to chopped forages fed at the same level of intake (Drogoul et al., 2000; Miyaji et al., 2011). Drogoul et al. (2000) also reported increases in liquid MRT when feeding ground and pelleted hay compared to chopped hay, whereas Miyaji et

al. (2011) noted no statistical differences in liquid MRT due to hay processing. Moore-Colyer et al. (2003) found decreased particulate MRT for chopped grass silage compared to unchopped silage, but found no differences in MRT between chopped and unchopped grass hay.

The relationship between dietary characteristics and MRT is not fully understood, and is often further complicated by external factors such as exercise level, physiological status of the animal, or water consumption (Orton et al., 1985; Pagan et al., 1998). It is clear that numerous factors can affect digesta passage rates, which in turn can affect digestibility. For example, if horses consume a high fiber, restricted diet, increasing MRT may increase nutrient availability, compensating for the low nutrient intake. Because digestibility and passage rate are closely linked, it is important to consider both factors together when completing nutritional research. Doing so will provide a more comprehensive view, hopefully increasing the understanding behind what might be affecting the animal.

## Summary and Conclusions

Although alfalfa is a preferred forage source for horses and other herbivores with advanced dietary requirements, the digestibility and utilization of alfalfa by these animals is hampered by its lignin content. Populations of reduced lignin alfalfa are now available, and research with these experimental populations has shown their potential to improve forage quality and digestibility. However, field evaluations under diverse conditions are needed to determine the performance of new commercial alfalfa cultivars containing the reduced lignin trait, especially with regard to forage accumulation and nutritive value under different harvest frequencies. To evaluate the effects of varied harvest frequencies

and changing forage maturity on yield, quality, and stem and leaf characteristics for reduced lignin alfalfa, two field studies were completed. Information on these studies can be found in Chapter 2 and Chapter 3.

In addition to the need for an evaluation of the performance of reduced lignin alfalfa cultivars in the field, it remains to be seen if the improvements in *in vitro* DMD and NDFD for reduced lignin alfalfa will translate to greater *in vivo* digestibility when fed to the animal directly. A variety of methods have been successfully used to evaluate digestibility and related nutritional parameters, including fecal particle size and digesta passage rates. While preliminary information surrounding the forage nutritive value and digestibility of reduced lignin alfalfa is promising, information on forage digestibility for current, commercially available reduced lignin alfalfa cultivars is not yet available, and digestibility changes have not yet been evaluated in the equine model. Therefore, a digestibility study in which reduced lignin and reference alfalfa hays were fed to horses was completed. The objectives for the study were to evaluate apparent digestibility and other digestibility-related parameters, including fecal particle size and retention time, when feeding reduced lignin and reference alfalfa hays to adult horses. Information on the methods and results for this study are reported in Chapter 4.

## LITERATURE CITED


Abdouli, H., and S.B. Attia. 2007. Evaluation of a two-stage in vitro technique for estimating digestibility of equine feeds using horse faeces as the source of microbial inoculum. Animal Feed Science and Technology 132(1): 155–162. doi: 10.1016/j.anifeedsci.2006.03.005.

Albrecht, K.A., W.F. Wedin, and D.R. Buxton. 1987. Cell-wall composition and digestibility of alfalfa stems and leaves. Crop Science 27(4): 735–741. doi: 10.2135/cropsci1987.0011183X002700040027x.

Almeida, M.I., J. Lopez Donzeles, M.I. Leao, and P.R. Cecon. 2001. Internal and external markers to estimate the apparent digestibility of nutrients in horse diets. Proceedings of the 17th Equine Nutrition and Physiology Symposium. Equine Science Society, Lexington, KY. p. 477–481

Åman, P., and H. Graham. 1990. Chemical evaluation of polysaccharides in animal feeds. In: Wiseman, J. and Cole, D.J.A., editors, Feedstuff Evaluation. Butterworth-Heinemann. p. 161–177

Applegate, C.S., and T.V. Hershberger. 1969. Evaluation of and Cecal Fermentation Techniques for Estimating the Nutritive Value of Forages for Equine. Journal of Animal Science 28(1): 18–22. doi: 10.2527/jas1969.28118x.

Austbø, D., and H. Volden. 2006. Influence of passage model and caecal cannulation on estimated passage kinetics of roughage and concentrate in the gastrointestinal tract of horses. Livestock Science 100(1): 33–43. doi: 10.1016/j.livprodsci.2005.11.002.

Baucher, M., M.A. Bernard-vailhé, B. Chabbert, J.-M. Besle, C. Opsomer, et al. 1999. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (Medicago sativa L.) and the effect on lignin composition and digestibility. Plant Mol Biol 39(3): 437–447. doi: 10.1023/A:1006182925584.

Beauchemin, K.A., and J.G. Buchanan-Smith. 1989. Evaluation of markers, sampling sites and models for estimating rates of passage of silage or hay in dairy cows. Animal Feed Science and Technology 27(1): 59–75. doi: 10.1016/0377-8401(89)90129-6.

Bernard, L., and M. Doreau. 2000. Use of rare earth elements as external markers for mean retention time measurements in ruminants. Reprod. Nutr. Dev. 40(2): 89–101. doi: 10.1051/rnd:2000122.

Blaxter, K.L., N.M. Graam, and F.W. Wainman. 1956. Some observations on the digestibility of food by sheep and on related problems. Brit. J. Nutrition 10: 69–91.

Bott, R.C., E.A. Greene, K. Koch, K.L. Martinson, P.D. Siciliano, et al. 2013. Production and Environmental Implications of Equine Grazing. Journal of Equine Veterinary Science 33(12): 1031–1043. doi: 10.1016/j.jevs.2013.05.004.

Brink, G., M. Hall, G. Shewmaker, D. Undersander, N. Martin, et al. 2010. Changes in alfalfa yield and nutritive value within individual harvest periods. Agronomy Journal 102(4): 1274–1282. doi: 10.2134/agronj2010.0080.

Brink, G.E., and G.C. Marten. 1989. Harvest management of alfalfa—Nutrient yield vs. forage quality, and relationship to persistence. Journal of Production Agriculture 2(1): 32–36. doi: 10.2134/jpa1989.0032.

Buxton, D.R., and J.S. Hornstein. 1986. Cell-wall concentration and components in stratified canopies of alfalfa, birdsfoot trefoil, and red clover. Crop Science 26(1): 180–184. doi: 10.2135/cropsci1986.0011183X002600010043x.

Buxton, D.R., J.S. Hornstein, W.F. Wedin, and G.C. Marten. 1985. Forage Quality in Stratified Canopies of Alfalfa, Birdsfoot Trefoil, and Red Clover. Crop Science 25(2): 273–279. doi: 10.2135/cropsci1985.0011183X002500020016x.

Buxton, D.R., and J.R. Russell. 1988. Lignin Constituents and Cell-Wall Digestibility of Grass and Legume Stems. Crop Science 28(3): 553. doi: 10.2135/cropsci1988.0011183X002800030026x.

Casler, M.D. 1986. Causal effects among forage yield and quality measures of smooth bromegrass. Can. J. Plant Sci. 66(3): 591–600. doi: 10.4141/cjps86-079.

Casler, M.D. 1987. In vitro digestibility of dry matter and cell wall constituents of smooth bromegrass forage. Crop Science 27(5): 931–934. doi: 10.2135/cropsci1987.0011183X002700050021x.

Casler, M.D., D.R. Buxton, and K.P. Vogel. 2002. Genetic modification of lignin concentration affects fitness of perennial herbaceous plants. Theor Appl Genet 104(1): 127–131. doi: 10.1007/s001220200015.

Casler, M.D., and K.P. Vogel. 1999. Accomplishments and impact from breeding for increased forage nutritional value. Crop Science 39(1): 12–20. doi: 10.2135/cropsci1999.0011183X003900010003x.

Chavez, S.J., P.D. Siciliano, and G.B. Huntington. 2014. Intake estimation of horses grazing tall fescue or fed tall fescue hay. Journal of Animal Science 92(5): 2304–2308. doi: 10.2527/jas.2013-7119.

Clauss, M., K. Schiele, S. Ortmann, J. Fritz, D. Codron, et al. 2014. The effect of very low food intake on digestive physiology and forage digestibility in horses. J Anim Physiol Anim Nutr 98(1): 107–118. doi: 10.1111/jpn.12053.

Cochran, R.C., and M.L. Galyean. 1994. Measurement of in vivo forage digestion by ruminants. Forage Quality, Evaluation, and Utilization. ASA, CSSA, and SSSA, Madison, WI. p. 613–643

Crooker, B.A., J.H. Clark, and R.D. Shanks. 1982. Rare earth elements as markers for rate of passage measurements of individual feedstuffs through the digestive tract of ruminants. J. Nutr 112: 1353–1361.

Crozier, J.A., V.G. Allen, N.E. Jack, J.P. Fontenot, and M.A. Cochran. 1997. Digestibility, apparent mineral absorption, and voluntary intake by horses fed alfalfa, tall fescue, and caucasian bluestem. Journal of animal science 75(6): 1651–1658. doi: 10.2527/1997.7561651x.

Cuddeford, D., and D. Hughes. 1990. A comparison between chromium-mordanted hay and acid-insoluble ash to determine apparent digestibility of a chaffed, molassed hay/straw mixture. Equine Veterinary Journal 22(2): 122–125. doi: 10.1111/j.2042-3306.1990.tb04223.x.

Cuddeford, D., R.A. Pearson, R.F. Archibald, and R.H. Muirhead. 1995. Digestibility and gastro-intestinal transit time of diets containing different proportions of alfalfa and oat straw given to Thoroughbreds, Shetland ponies, Highland ponies and donkeys. Animal Science 61(2): 407–417. doi: 10.1017/S1357729800013953.

Dove, H., and R.W. Mayes. 1991. The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. Aust. J. Agric. Res. 42(6): 913–952. doi: 10.1071/ar9910913.

Drogoul, C., A. de Fombelle, and V. Julliand. 2001. Feeding and microbial disorders in horses: 2: Effect of three hay:grain ratios on digesta passage rate and digestibility in ponies. Journal of Equine Veterinary Science 21(10): 487–491. doi: 10.1016/S0737-0806(01)70211-0.

Drogoul, C., C. Poncet, and J.L. Tisserand. 2000. Feeding ground and pelleted hay rather than chopped hay to ponies: 1. Consequences for in vivo digestibility and rate of passage of digesta. Animal Feed Science and Technology 87(1): 117–130. doi: 10.1016/S0377-8401(00)00187-5.

Earing, J. 2011. Comparison of Digestive Function in Young and Mature Horses. http://uknowledge.uky.edu/gradschool_diss/142.

Earing, J.E., B.D. Cassill, S.H. Hayes, E.S. Vanzant, and L.M. Lawrence. 2010. Comparison of in vitro digestibility estimates using the DaisyII incubator with in vivo digestibility estimates in horses. Journal of Animal Science 88(12): 3954–3963. doi: 10.2527/jas.2010-2989.

Edouard, N., G. Fleurance, W. Martin-Rosset, P. Duncan, J.P. Dulphy, et al. 2008. Voluntary intake and digestibility in horses: effect of forage quality with

emphasis on individual variability. Animal 2(10): 1526–1533. doi: 10.1017/S1751731108002760.

Ehle, F.R. 1984. Influence of Feed Particle Density on Paniculate Passage from Rumen of Holstein Cow. Journal of Dairy Science 67(3): 693–697. doi: 10.3168/jds.S0022-0302(84)81354-5.

Fonnesbeck, P.V., R.K. Lydman, V. Noot, G. W, and L.D. Symons. 1967. Digestibility of the Proximate Nutrients of Forage by Horses. Journal of Animal Science 26(5): 1039–1045. doi: 10.2527/jas1967.2651039x.

Garner, H.E., D.P. Hutcheson, J.R. Coffman, A.W. Hahn, and C. Salem. 1977. Lactic Acidosis: a Factor Associated with Equine Laminitis. Journal of Animal Science 45(5): 1037–1041. doi: 10.2527/jas1977.4551037x.

Getachew, G., A.M. Ibáñez, W. Pittroff, A.M. Dandekar, M. McCaslin, et al. 2011. A comparative study between lignin down regulated alfalfa lines and their respective unmodified controls on the nutritional characteristics of hay. Animal Feed Science and Technology 170(3–4): 192–200. doi: 10.1016/j.anifeedsci.2011.09.009.

Gibbs, P.G., G.D. Potter, G.T. Schelling, J.L. Kreider, and C.L. Boyd. 1988. Digestion of Hay Protein in Different Segments of the Equine Digestive Tract. Journal of Animal Science 66(2): 400–406. doi: 10.2134/jas1988.662400x.

Glinsky, M.J., R.M. Smith, H.R. Spires, and C.L. Davis. 1976. Measurement of volatile fatty acid production rates in the cecum of the pony. Journal of animal science 42(6): 1465–1470.

Goachet, A.G., C. Philippeau, M. Varloud, and V. Julliand. 2009. Adaptations to standard approaches for measuring total tract apparent digestibility and gastro-intestinal retention time in horses in training. Animal feed science and technology 152(1): 141–151.

Goachet, A.-G., M. Varloud, C. Philippeau, and V. Julliand. 2010. Long-term effects of endurance training on total tract apparent digestibility, total mean retention time and faecal microbial ecosystem in competing Arabian horses. Equine Veterinary Journal 42: 387–392. doi: 10.1111/j.2042-3306.2010.00188.x.

Goodson, J., W.J. Tyznik, J.H. Cline, and B.A. Dehority. 1988. Effects of an abrupt diet change from hay to concentrate on microbial numbers and physical environment in the cecum of the pony. Appl. Environ. Microbiol. 54(8): 1946–1950.

Griffin, T.S., K.A. Cassida, O.B. Hesterman, and S.R. Rust. 1994. Alfalfa Maturity and Cultivar Effects on Chemical and In Situ Estimates of Protein Degradability. Crop Science 34(6): 1654–1661. doi: 10.2135/cropsci1994.0011183X003400060043x.

Guay, K.A., H.A. Brady, V.G. Allen, K.R. Pond, D.B. Wester, et al. 2002. Matua bromegrass hay for mares in gestation and lactation. Journal of Animal Science 80(11): 2960–2966. doi: 10.2527/2002.80112960x.

Guo, D., F. Chen, K. Inoue, J.W. Blount, and R.A. Dixon. 2001a. Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: Impacts on lignin structure and implications for the biosynthesis of G and S lignin. The Plant Cell 13(1): 73–88. doi: 10.2307/3871154.

Guo, D., F. Chen, J. Wheeler, J. Winder, S. Selman, et al. 2001b. Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. Transgenic Res 10(5): 457–464. doi: 10.1023/A:1012278106147.

Hall, M.H., W.S. Smiles, and R.A. Dickerson. 2000. Morphological development of alfalfa cultivars selected for higher quality. Agronomy Journal 92(6): 1077–1080. doi: 10.2134/agronj2000.9261077x.

Hansen, T. 2014. Modeling Digestibility and Rate of Passage in Horses. http://uknowledge.uky.edu/animalsci_etds/37.

Hartnell, G.F., and L.D. Satter. 1979. Extent of Particulate Marker (Samarium, Lanthanum and Cerium) Movement from One Digesta Particle to Another. Journal of Animal Science 48(2): 375–380. doi: 10.2527/jas1979.482375x.

Hintz, H.F., D.E. Hogue, E.F. Walker, J.E. Lowe, and H.F. Schryver. 1971. Apparent digestion in various segments of the digestive tract of ponies fed diets with varying roughage-grain ratios. Journal of Animal Science 32(2): 245–248.

Hintz, H.F., and R.G. Loy. 1966. Effects of Pelleting on the Nutritive Value of Horse Rations. Journal of Animal Science 25(4): 1059–1062. doi: 10.2527/jas1966.2541059x.

Holland, J.L., D.S. Kronfeld, D. Sklan, and P.A. Harris. 1998. Calculation of fecal kinetics in horses fed hay or hay and concentrate. Journal of animal science 76(7): 1937–1944. doi: 10.2527/1998.7671937x.

Hussein, H.S., L.A. Vogedes, G.C.J. Fernandez, and R.L. Frankeny. 2004. Effects of cereal grain supplementation on apparent digestibility of nutrients and concentrations of fermentation end-products in the feces and serum of horses consuming alfalfa cubes. Journal of Animal Science 82(7): 1986–1996. doi: 10.2527/2004.8271986x.

Inoue, K., V.J.H. Sewalt, G.M. Ballance, W. Ni, C. Stürzer, et al. 1998. Developmental expression and substrate specificities of alfalfa caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase in relation to lignification. Plant Physiol. 117(3): 761–770. doi: 10.1104/pp.117.3.761.

Janis, C. 1976. The Evolutionary Strategy of the Equidae and the Origins of Rumen and Cecal Digestion. Evolution 30(4): 757–774. doi: 10.2307/2407816.

Jouany, J.-P., J. Gobert, B. Medina, G. Bertin, and V. Julliand. 2008. Effect of live yeast culture supplementation on apparent digestibility and rate of passage in horses fed a high-fiber or high-starch diet. Journal of Animal Science 86(2): 339–347. doi: 10.2527/jas.2006-796.

Julier, B., and C. Huyghe. 1997. Effect of growth and cultivar on alfalfa digestibility in a multi-site trial. Agronomie 17(9–10): 481–489.

Julliand, V., A. de Fombelle, C. Drogoul, and E. Jacotot. 2001. Feeding and microbial disorders in horses: Part 3—Effects of three hay:grain ratios on microbial profile and activities. Journal of Equine Veterinary Science 21(11): 543–546. doi: 10.1016/S0737-0806(01)70159-1.

Jung, H.G., and J.F.S. Lamb. 2006. Stem Morphological and Cell Wall Traits Associated with Divergent In Vitro Neutral Detergent Fiber Digestibility in Alfalfa Clones. Crop Science 46(5): 2054–2061. doi: 10.2135/cropsci2005.12.0470.

Jung, H.G., D.R. Mertens, and A.J. Payne. 1997a. Correlation of acid detergent lignin and klason lignin with digestibility of forage dry matter and neutral detergent fiber. Journal of Dairy Science 80(8): 1622–1628. doi: 10.3168/jds.S0022-0302(97)76093-4.

Jung, H.-J.G., D.A. Samac, and G. Sarath. 2012. Modifying crops to increase cell wall digestibility. Plant Science 185–186: 65–77. doi: 10.1016/j.plantsci.2011.10.014.

Jung, H.G., C.C. Sheaffer, D.K. Barnes, and J.L. Halgerson. 1997b. Forage quality variation in the U.S. alfalfa core collection. Crop Science 37(4): 1361–1366. doi: 10.2135/cropsci1997.0011183X003700040054x.

Kallenbach, R.L., C.J. Nelson, and J.H. Coutts. 2002. Yield, quality, and persistence of grazing- and hay-type alfalfa under three harvest frequencies. Agronomy Journal 94(5): 1094–1103. doi: 10.2134/agronj2002.1094.

Kilcher, M.R., and D.H. Heinrichs. 1974. Contribution of Stems and Leaves to the Yield and Nutrient Level of Irrigated Alfalfa at Different Stages of Development. Can. J. Plant Sci. 54(4): 739–742. doi: 10.4141/cjps74-125.

Koller, B.L., H.F. Hintz, J.B. Robertson, and P.J. Van Soest. 1978. Comparative Cell Wall and Dry Matter Digestion in the Cecum of the Pony and the Rumen of the Cow Using and Nylon Bag Techniques. Journal of Animal Science 47(1): 209–215. doi: 10.2527/jas1978.471209x.

LaCasha, P.A., H.A. Brady, V.G. Allen, C.R. Richardson, and K.R. Pond. 1999. Voluntary intake, digestibility, and subsequent selection of Matua bromegrass,

coastal bermudagrass, and alfalfa hays by yearling horses. Journal of animal science 77(10): 2766–2773. doi: 10.2527/1999.77102766x.

Lamb, J.F.S., G.J. Hans-Joachim, and H. Riday. 2012. Harvest Impacts on Alfalfa Stem Neutral Detergent Fiber Concentration and Digestibility and Cell Wall Concentration and Composition. Crop Science 52(5): 2402–2412. doi: 10.2135/cropsci2012.01.0027.

Lamb, J.F.S., H.-J.G. Jung, and D.A. Samac. 2014. Environmental Variability and/or Stability of Stem Fiber Content and Digestibility in Alfalfa. Crop Science 54(6): 2854–2863. doi: 10.2135/cropsci2014.04.0323.

Lamb, J.F.S., H.-J.G. Jung, C.C. Sheaffer, and D.A. Samac. 2007. Alfalfa Leaf Protein and Stem Cell Wall Polysaccharide Yields under Hay and Biomass Management Systems. Crop Science 47(4): 1407–1415. doi: 10.2135/cropsci2006.10.0665.

Lamb, J.F.S., C.C. Sheaffer, and D.A. Samac. 2003. Population Density and Harvest Maturity Effects on Leaf and Stem Yield in Alfalfa. Agronomy Journal 95(3): 635–641. doi: 10.2134/agronj2003.6350.

Lattimer, J.M., S.R. Cooper, D.W. Freeman, and D.L. Lalman. 2007. Effect of yeast culture on in vitro fermentation of a high-concentrate or high-fiber diet using equine fecal inoculum in a Daisy II incubator. Journal of Animal Science 85(10): 2484–2491. doi: 10.2527/jas.2006-655.

Ledoux, D.R., J.E. Williams, T.E. Stroud, and M. Ellersieck. 1985. Ytterbium as a particulate phase marker for measurement of digesta passage rate in ruminants. Nutrition reports international 32(1): 111–115.

Lewis, L.D. 1995. Equine clinical nutrition: feeding and care. Williams & Wilkins, Baltimore, MD.

Li, Z., Z. Li, D. Combs, and D. Undersander. 2015a. Effect of reduced lignin alfalfa on forage quality at three harvest intervals. Journal of Animal Science/Journal of Dairy Science, Orlando, FL. p. 675

Li, X., Y. Zhang, A. Hannoufa, and P. Yu. 2015b. Transformation with TT8 and HB12 RNAi constructs in model forage (Medicago sativa, alfalfa) affects carbohydrate structure and metabolic characteristics in ruminant livestock systems. J. Agric. Food Chem. 63(43): 9590–9600. doi: 10.1021/acs.jafc.5b03717.

Lindberg, J.E., B. Essen-Gustavsson, K. Dahlborn, M. Gottlieb-Vedi, and A. Jansson. 2006. Exercise response, metabolism at rest and digestibility in athletic horses fed high-fat oats. Equine Veterinary Journal 38(S36): 626–630.

Liu, N., and P. Yu. 2011. Molecular clustering, interrelationships and carbohydrate conformation in hull and seeds among barley cultivars. Journal of Cereal Science 53(3): 379–383. doi: 10.1016/j.jcs.2011.02.011.

Lowman, R.S., M.K. Theodorou, J.J. Hyslop, M.S. Dhanoa, and D. Cuddeford. 1999. Evulation of an in vitro batch culture technique for estimating the in vivo digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum. Animal Feed Science and Technology 80(1): 11–27. doi: 10.1016/S0377-8401(99)00039-5.

Mader, T.L., R.G. Teeter, and G.W. Horn. 1984. Comparison of Forage Labeling Techniques for Conducting Passage Rate Studies. Journal of Animal Science 58(1): 208–212. doi: 10.2527/jas1984.581208x.

Marita, J.M., J. Ralph, R.D. Hatfield, D. Guo, F. Chen, et al. 2003. Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase. Phytochemistry 62(1): 53–65. doi: 10.1016/S0031-9422(02)00434-X.

Marković, J.P., R.T. Scaron, trbanović, D.V. Terzić, D.J. Djokić, et al. 2012. Changes in lignin structure with maturation of alfalfa leaf and stem in relation to ruminants nutrition. AJAR 7(2): 257–264. doi: 10.5897/AJAR11.1485.

Martin-Rosset, W., M. Doreau, S. Boulot, and N. Miraglia. 1990. Influence of level of feeding and physiological state on diet digestibility in light and heavy breed horses. Livestock Production Science 25(3): 257–264. doi: 10.1016/0301-6226(90)90021-W.

Martin-Rosset, W., and J.P. Dulphy. 1987. Digestibility interactions between forages and concentrates in horses: Influence of feeding level - Comparison with sheep. Livestock Production Science 17: 263–276. doi: 10.1016/0301-6226(87)90071-6.

Mayes, R.W., and H. Dove. 2000. Measurement of dietary nutrient intake in free-ranging mammalian herbivores. Nutrition Research Reviews 13(1): 107–138. doi: 10.1079/095442200108729025.

Medina, B., I.D. Girard, E. Jacotot, and V. Julliand. 2002. Effect of a preparation of on microbial profiles and fermentation patterns in the large intestine of horses fed a high fiber or a high starch diet. Journal of Animal Science 80(10): 2600–2609. doi: 10.2527/2002.80102600x.

Mertens, D.R., and L.O. Ely. 1982. Relationship of Rate and Extent of Digestion to Forage Utilization-A Dynamic Model Evaluation. Journal of Animal Science 54(4): 895–905. doi: 10.2527/jas1982.544895x.

Mertens, D.R., and M. McCaslin. 2008. Evaluation of alfalfa hays with down-regulated lignin biosynthesis. Journal of Dairy Science. p. 170

Milić, D., D. Karagić, S. Vasiljević, A. Mikić, B. Mijić, et al. 2011. Leaf and stem chemical composition of divergent alfalfa cultivars. Biotechnology in Animal Husbandry 27(4): 1505–1511.

Min, D. 2016. Effects of cutting interval between harvests on dry matter yield and nutritive value in alfalfa. American Journal of Plant Sciences 07(08): 1226–1231. doi: 10.4236/ajps.2016.78118.

Miraglia, N., D. Bergero, B. Bassano, M. Tarantola, and G. Ladetto. 1999. Studies of apparent digestibility in horses and the use of internal markers. Livestock Production Science 60(1): 21–25. doi: 10.1016/S0301-6226(99)00043-3.

Miyaji, M., K. Ueda, H. Hata, and S. Kondo. 2011. Effects of quality and physical form of hay on mean retention time of digesta and total tract digestibility in horses. Animal Feed Science and Technology 165(1): 61–67. doi: 10.1016/j.anifeedsci.2011.02.003.

Miyaji, M., K. Ueda, H. Hata, and S. Kondo. 2014. Effect of grass hay intake on fiber digestion and digesta retention time in the hindgut of horses. Journal of Animal Science 92(4): 1574–1581. doi: 10.2527/jas.2013-6676.

Miyaji, M., K. Ueda, Y. Kobayashi, H. Hata, and S. Kondo. 2008a. Fiber digestion in various segments of the hindgut of horses fed grass hay or silage. Animal Science Journal 79(3): 339–346. doi: 10.1111/j.1740-0929.2008.00535.x.

Miyaji, M., K. Ueda, H. Nakatsuji, T. Tomioka, Y. Kobayashi, et al. 2008b. Mean retention time of digesta in the different segments of the equine hindgut. Animal Science Journal 79(1): 89–96. doi: 10.1111/j.1740-0929.2007.00502.x.

Moore-Colyer, M.J.S., H.J. Morrow, and A.C. Longland. 2003. Mathematical modelling of digesta passage rate, mean retention time and apparent digestibility of two different lengths of hay and big-bale grass silage in ponies. British Journal of Nutrition 90(1): 109–118. doi: 10.1079/BJN2003869.

Morrison, I.M. 1979. Carbohydrate chemistry and rumen digestion. Proceedings of the Nutrition Society 38(03): 269–274. doi: 10.1079/PNS19790048.

Nordkvist, E., and P. Åman. 1986. Changes during growth in anatomical and chemical composition and in-vitro degradability of lucerne. J. Sci. Food Agric. 37(1): 1–7. doi: 10.1002/jsfa.2740370102.

NRC. 2007. Nutrient Requirements of Horses. 6th ed. The National Academies Press, Washington, D.C.

Nyberg, M.A., G.D. Potter, P.G. Gibbs, J. Schumacher, M. Murray-Gerzik, et al. 1995. Flow rate through the equine small intestine determined with soluble and insoluble indicators. 14th Equine Nutrition ans Physiology Symposium (ENPS Ed.). p. 36–41

Ordakowski, A.L., D.S. Kronfeld, J.L. Holland, B.J. Hargreaves, L.S. Gay, et al. 2001. Alkanes as internal markers to estimate digestibility of hay or hay plus

concentrate diets in horses. Journal of animal science 79(6): 1516–1522. doi: 10.2527/2001.7961516x.

Orton, R.K., I.D. Hume, and R.A. Leng. 1985. Effects of exercise and level of dietary protein on digestive function in horses. Equine Veterinary Journal 17(5): 386–390. doi: 10.1111/j.2042-3306.1985.tb02530.x.

Owens, F.N., and C.F. Hanson. 1992. External and Internal Markers for Appraising Site and Extent of Digestion in Ruminants1. Journal of Dairy Science 75(9): 2605–2617. doi: 10.3168/jds.S0022-0302(92)78023-0.

Pagan, J.D., P. Harris, T. Brewster-Barnes, S.E. Duren, and S.G. Jackson. 1998. Exercise Affects Digestibility and Rate of Passage of All-Forage and Mixed Diets in Thoroughbred Horses. J. Nutr. 128(12): 2704S-2707S.

Palmgren Karlsson, C., J.E. Lindberg, and M. Rundgren. 2000. Associative effects on total tract digestibility in horses fed different ratios of grass hay and whole oats. Livestock Production Science 65(1): 143–153. doi: 10.1016/S0301-6226(99)00178-5.

Palmonari, A., M. Fustini, G. Canestrari, E. Grilli, and A. Formigoni. 2014. Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content. Journal of Dairy Science 97(12): 7729–7734. doi: 10.3168/jds.2014-8123.

Parkins, J.J., D.H. Snow, and S. Adams. 1982. The apparent digestibility of "complete diet" cubes given to Thoroughbred horses and the use of chromic oxide as an inert faecal marker. Br. Vet. J. 138(4): 350–355.

Pearson, R.A., R.F. Archibald, and R.H. Muirhead. 2001. The effect of forage quality and level of feeding on digestibility and gastrointestinal transit time of oat straw and alfalfa given to ponies and donkeys. British Journal of Nutrition 85(5): 599–606. doi: 10.1079/BJN2001321.

Pearson, R.A., R.F. Archibald, and R.H. Muirhead. 2006. A comparison of the effect of forage type and level of feeding on the digestibility and gastrointestinal mean retention time of dry forages given to cattle, sheep, ponies and donkeys. British Journal of Nutrition 95(1): 88–98. doi: 10.1079/BJN20051617.

Pearson, R.A., and J.B. Merritt. 1991. Intake, digestion and gastrointestinal transit time in resting donkeys and ponies and exercised donkeys given ad libitum hay and straw diets. Equine Veterinary Journal 23(5): 339–343. doi: 10.1111/j.2042-3306.1991.tb03734.x.

Penning, P.D., and R.H. Johnson. 1983a. The use of internal markers to estimate herbage digestibility and intake: 1. Potentially indigestible cellulose and acid insoluble ash. The Journal of Agricultural Science 100(1): 127–131. doi: 10.1017/S0021859600032512.

Penning, P.D., and R.H. Johnson. 1983b. The use of internal markers to estimate herbage digestibility and intake: 2. Indigestible acid detergent fibre. The Journal of Agricultural Science 100(1): 133–138. doi: 10.1017/S0021859600032524.

Poore, M.H., J.A. Moore, and R.S. Swingle. 1990. Differential passage rates and digestion of neutral detergent fiber from grain and forages in 30, 60 and 90% concentrate diets fed to steers. Journal of animal science 68(9): 2965–2973. doi: 10.2527/1990.6892965x.

Potter, G.D., F.F. Arnold, D.D. Householder, D.H. Hansen, and K.M. Brown. 1992. Digestion of starch in the small or large intestine of the equine. Pferdeheilkunde 1(4): 107–111.

Potts, L., J. Hinkson, B. Graham, C. Löest, and J. Turner. 2010. Nitrogen Retention and Nutrient Digestibility in Geldings Fed Grass Hay, Alfalfa Hay, or Alfalfa Cubes. Journal of Equine Veterinary Science 30(6): 330–333. doi: 10.1016/j.jevs.2010.04.007.

Probst, A., and R. Smith. 2011. Harvest frequency effects on yield, persistence, and regrowth rate among new alfalfa cultivars. Forage and Grazinglands 9(1): 1–8. doi: 10.1094/FG-2011-0926-01-RS.

Putnam, D.H., S. Orloff, and L.R. Teuber. 2005. Strategies for balancing quality and yield using cutting schedules and varieties. Proceedings of the 35th California Alfalfa & Forage Symposium. University of California Cooperative Extension, Visalia, California. p. 237–252

Reddy, M.S.S., F. Chen, G. Shadle, L. Jackson, H. Aljoe, et al. 2005. Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (Medicago sativa L.). PNAS 102(46): 16573–16578. doi: 10.1073/pnas.0505749102.

Rosenfeld, I., and D. Austbø. 2009. Effect of type of grain and feed processing on gastrointestinal retention times in horses. Journal of Animal Science 87(12): 3991–3996. doi: 10.2527/jas.2008-1150.

Rosenfeld, I., D. Austbø, and H. Volden. 2006. Models for estimating digesta passage kinetics in the gastrointestinal tract of the horse. Journal of Animal Science 84(12): 3321–3328. doi: 10.2527/jas.2006-039.

Sales, J. 2012. A review on the use of indigestible dietary markers to determine total tract apparent digestibility of nutrients in horses. Animal Feed Science and Technology 174(3): 119–130. doi: 10.1016/j.anifeedsci.2012.03.005.

Sanderson, M.A., J.S. Hornstein, and W.F. Wedin. 1989. Alfalfa Morphological Stage and its Relation to in Situ Digestibility of Detergent Fiber Fractions of Stems. Crop Science 29(5): 1315. doi: 10.2135/cropsci1989.0011183X002900050045x.

Sanderson, M.A., and W.F. Wedin. 1988. Cell wall composition of alfalfa stems at similar morphological stages and chronological age during spring growth and summer regrowth. Crop Science 28(2): 342–347. doi: 10.2135/cropsci1988.0011183X002800020034x.

Schaafstra, F.J.W.C., D.A. van Doorn, J.T. Schonewille, F.C. Wartena, M.V. Zoon, et al. 2015. Evaluation of methodological aspects of digestibility measurements in ponies fed different grass hays. Journal of Animal Science 93(10): 4742. doi: 10.2527/jas.2015-9240.

Schneider, B.H., and W.P. Flatt. 1975. The evaluation of feeds through digestibility experiments. University of Georgia Press, Athens, GA.

Sellers, A.F., and J.E. Lowe. 1986. Review of large intestinal motility and mechanisms of impaction in the horse. Equine Veterinary Journal 18(4): 261–263. doi: 10.1111/j.2042-3306.1986.tb03621.x.

Sewalt, V.J.H., W. Ni, H.G. Jung, and R.A. Dixon. 1997. Lignin impact on fiber degradation: Increased enzymatic digestibility of genetically engineered tobacco (Nicotiana tabacum) stems reduced in lignin content. J. Agric. Food Chem. 45(5): 1977–1983. doi: 10.1021/jf9609690.

Sheaffer, C.C., G.D. Lacefield, and V.L. Marble. 1988. Cutting schedules and stands. Alfalfa and Alfalfa Improvement. ASA, CSSA, and SSSA, Madison, WI. p. 411–437

Sheaffer, C.C., N.P. Martin, J.F.S. Lamb, G.R. Cuomo, J.G. Jewett, et al. 2000. Leaf and stem properties of alfalfa entries. Agronomy Journal 92(4): 733–739. doi: 10.2134/agronj2000.924733x.

Smolders, E.A.A., A. Steg, and V.A. Hindle. 1990. Organic matter digestibility in horses and its prediction. Netherlands Journal of Agricultural Science 38(3B): 435–447.

Stevens, D.M., J.V. Ryssen, and J.P. Marais. 2002. The use of n-alkane markers to estimate the intake and apparent digestibility of ryegrass and Kikuyu by horses. South African Journal of Animal Science 32(1): 50–56. doi: 10.4314/sajas.v32i1.3791.

Sturgeon, L.S., L.A. Baker, J.L. Pipkin, J.C. Haliburton, and N.K. Chirase. 2000. The digestibility and mineral availability of Matua, Bermuda grass, and alfalfa hay in mature horses. Journal of Equine Veterinary Science 20(1): 45–48. doi: 10.1016/S0737-0806(00)80320-2.

Teeter, R.G., F.N. Owens, and T.L. Mader. 1984. Ytterbium Chloride as a Marker for Particulate Matter in the Rumen. Journal of Animal Science 58(2): 465–473. doi: 10.2527/jas1984.582465x.

Thielemans, M.-F., E. Francois, C. Bodart, and A. Thewis. 1978. Gastrointestinal transit in the pig: measurement using radioactive lanthanides and comparison with sheep. Ann. Biol. Anim. Biochim. Biophys 18(2A): 237–247.

Tilley, J.M.A., and R.A. Terry. 1963. A Two-Stage Technique for the in Vitro Digestion of Forage Crops. Grass and Forage Science 18(2): 104–111. doi: 10.1111/j.1365-2494.1963.tb00335.x.

Todd, L.K., W.C. Sauer, R.J. Christopherson, R.J. Coleman, and W.R. Caine. 1995. The effect of level of feed intake on nutrient and energy digestibilities and rate of feed passage in horses. Journal of Animal Physiology and Animal Nutrition 73(1–5): 140–148. doi: 10.1111/j.1439-0396.1995.tb00413.x.

Udén, P., P.E. Colucci, and P.J. Van Soest. 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. J. Sci. Food Agric. 31(7): 625–632. doi: 10.1002/jsfa.2740310702.

Undersander, D., M. McCaslin, C. Sheaffer, D. Whalen, D. Miller, et al. 2009. Low lignin alfalfa: Redefining the yield/quality tradeoff. UC Cooperative Extension, Reno, Nevada. p. 1–4

Van Soest, P.J., J.B. Robertson, and B.A. Lewis. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. Journal of Dairy Science 74(10): 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2.

Van Soest, P.J., R.H. Wine, and L.A. Moore. 1966. Estimation of the true digestibility of forages by the in vitro digestion of cell walls. Estimation of the true digestibility of forages by the in vitro digestion of cell walls. https://www.cabdirect.org/cabdirect/abstract/19670700081 (accessed 4 September 2017).

Van Weyenberg, S., J. Sales, and G.P.J. Janssens. 2006. Passage rate of digesta through the equine gastrointestinal tract: A review. Livestock Science 99(1): 3–12. doi: 10.1016/j.livprodsci.2005.04.008.

Weakley, D., D.R. Mertens, and M. McCaslin. 2008. Lactating cow responses to alfalfa hays with down-regulated lignin biosynthesis. Journal of Dairy Science. p. 170

Weir, W.C., L.G. Jones, and J.H. Meyer. 1960. Effect of cutting interval and stage of maturity on the digestibility and yield of alfalfa. Journal of Animal Science 19(1): 5–19. doi: 10.2527/jas1960.1915.

Wilman, D., and P.R. Moghaddam. 1998. In vitro digestibility and neutral detergent fibre and lignin contents of plant parts of nine forage species. The Journal of Agricultural Science 131(1): 51–58.

Woodward, A.D., B.D. Nielsen, J. Liesman, T. Lavin, and N.L. Trottier. 2011. Protein quality and utilization of timothy, oat-supplemented timothy, and alfalfa at differing harvest maturities in exercised Arabian horses. Journal of Animal Science 89(12): 4081–4092. doi: 10.2527/jas.2010-3825.

Yari, M., R. Valizadeh, A.A. Naserian, G.R. Ghorbani, P. Rezvani Moghaddam, et al. 2012. Botanical traits, protein and carbohydrate fractions, ruminal degradability and energy contents of alfalfa hay harvested at three stages of maturity and in the afternoon and morning. Animal Feed Science and Technology 172(3–4): 162–170. doi: 10.1016/j.anifeedsci.2012.01.004.

Yari, M., R. Valizadeh, A.A. Naserian, A. Jonker, A. Azarfar, et al. 2014. Effects of including alfalfa hay cut in the afternoon or morning at three stages of maturity in high concentrate rations on dairy cow performance, diet digestibility and feeding behavior. Animal Feed Science and Technology 192: 62–72. doi: 10.1016/j.anifeedsci.2014.04.001.

Yu, P., D.A. Christensen, J.J. McKinnon, and J.D. Markert. 2003. Effect of variety and maturity stage on chemical composition, carbohydrate and protein subfractions, in vitro rumen degradability and energy values of timothy and alfalfa. Can. J. Anim. Sci. 83(2): 279–290. doi: 10.4141/A02-053.

# CHAPTER 2

## Forage Accumulation and Nutritive Value of Reduced Lignin and Reference Alfalfa Cultivars

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### CHAPTER SUMMARY

Reduced lignin alfalfa (*Medicago sativa* L.) cultivars have the potential to increase the feeding value of alfalfa for livestock by improving forage fiber digestibility and to increase harvest management flexibility. The objectives were to compare the forage accumulation and nutritive value of reduced lignin and reference alfalfa cultivars when subject to diverse cutting treatments in the establishment and first production year. Research was established in 2015 at four locations in Minnesota. Reference alfalfa cultivars 54R02, DKA43-22RR, WL 355.RR, and the reduced lignin cultivar 54HVX41

were subject to cutting treatments with variable intervals between harvests. Cultivar by cutting treatment interactions were not significant ($P > 0.05$), but cultivar and cutting treatment effects were significant. Cultivars did not consistently differ in forage accumulation. Establishment year forage accumulation was greater when a fall harvest was taken, and first production year forage accumulation was generally greatest when alfalfa was harvested on a 40-d cutting schedule. Compared to reference alfalfa cultivars, 54HVX41 had an average of 8% less acid detergent lignin (ADL) and 10% greater neutral detergent fiber digestibility (NDFD) but was similar in crude protein (CP) and neutral detergent fiber (NDF) concentrations. Cutting treatments with shorter harvest intervals increased forage CP and NDFD and decreased NDF and ADL concentrations. With a 5-d harvest delay, 54HVX41 harvested on a 35-d harvest interval had a 21% gain in forage mass and a 3% reduction in relative forage quality (RFQ) compared to reference cultivars harvested on a 30-d harvest interval, which could allow for increased management flexibility.

## INTRODUCTION

Alfalfa is widely used as forage for livestock due to its high nutrient content (Marita et al., 2003; Yu et al., 2003). However, the digestibility and utilization of alfalfa by these animals is hampered by its lignin content (Sewalt et al., 1997; Casler et al., 2002). Lignin is a complex structural polymer that is the second most abundant component of secondary plant cells walls (Li et al., 2015b), providing the strength and rigidity necessary for the plant to stand upright (Inoue et al., 1998; Guo et al., 2001a). As a plant matures, lignin concentration also increases, filling the space between cellulose,

hemicellulose, and pectin molecules and forming cross-linkages with hemicellulose (Albrecht et al., 1987; Jung et al., 1997b; Inoue et al., 1998; Casler and Vogel, 1999). While it is essential for normal plant growth, the deposition of lignin into plant cell walls can reduce the feeding value of alfalfa by negatively affecting rumen microbial degradation and the digestion of feed by intestinal enzymes (Buxton and Hornstein, 1986; Liu and Yu, 2011). Lignification has been reported to be the major factor limiting both the *in vitro* digestibility of plant cell-wall polysaccharides (Morrison, 1979; Albrecht et al., 1987; Jung et al., 2012) and the *in vitro* dry matter digestibility (DMD) of whole plant forage (Casler, 1986, 1987; Reddy et al., 2005). These negative effects have primarily been associated with lignin concentration, as numerous studies have found strong negative correlations between lignin concentrations and forage digestibility (Albrecht et al., 1987; Casler, 1987; Jung et al., 1997b; a; Reddy et al., 2005).

With such a strong influence on forage digestibility, small decreases in the lignin concentration of forages can be expected to improve the fiber digestibility at any plant maturity stage (Casler, 1987; Undersander et al., 2009). Predictions by Casler (1987) estimated that a single unit decrease (g $kg^{-1}$) in the concentration of ADL of smooth bromegrass (*Bromus inermis* L.) would result in a 7.0 unit increase in *in vitro* DMD. Feeding and grazing studies have shown that small changes in forage digestibility can significantly impact animal performance. For a number of grass cultivars, Casler and Vogel (1999) reported a positive relationship between *in vitro* DMD improvement and animal daily gains, with a 1% increase in *in vitro* DMD resulting in a 3.2% increase in daily weight gains for beef cattle.

Several experimental lines of alfalfa have been developed with down-regulation of the caffeic acid 3-O-methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCOMT) lignin biosynthetic genes (Inoue et al., 1998; Guo et al., 2001a; Marita et al., 2003; Getachew et al., 2011). Experimental populations of COMT and/or CCOMT down-regulated alfalfa have shown a 4 to 29% decrease in stem lignin concentration and a 1 to 24% decrease in herbage lignin concentration compared to reference alfalfa cultivars (Guo et al., 2001a; b; Marita et al., 2003; Reddy et al., 2005; Undersander et al., 2009; Getachew et al., 2011). The wide variation in lignin reduction reported could be due to the specific down-regulated gene (Guo et al., 2001a; b; Marita et al., 2003; Undersander et al., 2009; Getachew et al., 2011), the methods used for lignin analysis (Guo et al., 2001a; b; Jung et al., 2012), or the plant growing conditions (Baucher et al., 1999).

Populations of reduced lignin alfalfa have shown an increase in *in vitro* DMD, *in situ* rumen digestibility, and *in vitro* NDFD (Guo et al., 2001b; Reddy et al., 2005; Mertens and McCaslin, 2008; Weakley et al., 2008; Undersander et al., 2009; Getachew et al., 2011). Reddy et al. (2005) reported a strong negative linear relationship between *in situ* digestibility and ADL levels across all reduced lignin lines. In addition to increased digestibility, reduced lignin alfalfa populations have also shown reduced NDF concentrations and greater non-fiber carbohydrate concentrations compared to control lines (Guo et al., 2001b; Reddy et al., 2005; Getachew et al., 2011; Li et al., 2015a), while CP concentrations remained similar for reduced lignin and reference alfalfa lines (Getachew et al., 2011; Li et al., 2015a).

Recently released reduced lignin alfalfa cultivars have potential to increase the digestibility of alfalfa forage compared to reference cultivars (Guo et al., 2001a; b; Marita et al., 2003; Reddy et al., 2005; Getachew et al., 2011). These improvements in alfalfa forage nutritive value may lengthen the time period when alfalfa has a forage nutritive value suitable for high-producing livestock. This could allow for a wider optimal harvest window, making it possible for alfalfa growers to achieve greater forage accumulation by delaying alfalfa harvest while still maintaining acceptable forage nutritive value (Undersander et al., 2009).

Research with experimental populations of reduced lignin alfalfa has shown their potential to improve forage digestibility. However, field evaluations under diverse conditions are needed to determine the performance of new commercial alfalfa cultivars containing the reduced lignin trait, especially with regard to forage accumulation and nutritive value under different harvest frequencies. The objectives of this study were to evaluate forage accumulation and nutritive values of reduced lignin and reference alfalfa cultivars when subject to diverse cutting treatments during the establishment and first production year.

## MATERIALS AND METHODS

Research was conducted at the University of Minnesota Agricultural Experiment Stations at St. Paul, Becker, Rosemount, and Rochester, MN in 2015, and continued at St. Paul, Becker, and Rosemount, MN in 2016. Similar establishment year results across locations combined with a lack of sufficient time and resources resulted in the exclusion of the Rochester location from the 2016 experiment. The soil was a Waukegan silt loam

(fine-silty over sandy or sandy-skeletal, mixed, superactive, mesic Typic Hapludolls) at St. Paul (44°59'14" N, 93°10'24" W, elevation 291 m), a Hubbard-Mosford complex (sandy, mixed, frigid Entic Hapludolls) at Becker, MN (45°23'13" N, 93°53'18" W, elevation 290 m), a Port Byron silt loam (fine-silty, mixed, superactive, mesic Typic Hapludolls) at Rosemount, MN (44°41'16" N, 93°04'21" W, elevation 288 m), and a Marshan silt loam (fine-loamy over sandy or sandy-skeletal, mixed, superactive, mesic Typic Endoaquolls) at Rochester, MN (44°00'46" N, 92°25'02" W, elevation 317 m).

Monthly mean air temperature and precipitation data were collected at each location and year. Mean daily air temperature for the 2015 and 2016 growing seasons (May through October) was similar to the 30-yr average except for September and October, which tended to be warmer than normal (Figure 2.1A-B). Total rainfall during the 2015 and 2016 growing seasons (May through October) fell between 56 and 78 cm and was greater compared to the 30-yr average, which ranged from 51 to 62 cm across locations (Figure 2.1A-B). Seasonal rainfall was not evenly distributed and varied greatly across location, month, and year.

All sites were planted between 27 and 30 Apr 2015. Inoculated seed was seeded into a prepared seedbed at a rate of 18.8 kg $ha^{-1}$ in plots measuring 0.9 × 6.1 m. Soil fertility at each site was amended to meet recommendations for alfalfa hay production according to University of Minnesota fertility guidelines (Kaiser et al., 2011). In the establishment year, weeds were controlled using a single application of glyphosate (N-(phosphonomethyl)glycine) applied at rate of 2.34 L a.i. $ha^{-1}$; additional weed control was not required during the first production year. Potato leafhoppers were controlled using Arctic 3.2 EC ((m-Phenoxybenzyl)-cis,trans-3-(2,2-dichlorovinyl)-2,2-

dimethylcyclopropanecarboxylate) as needed when potato leafhoppers exceeded threshold populations, which generally occurred each July (Cancelado and Radcliffe, 1979; Chasen et al., 2015).

At all sites, the experimental design was a randomized complete block with five replicates and a split-plot arrangement of treatments. Whole plots were four cutting treatments with varying harvest frequencies. In 2015, cutting treatments began a minimum of 60 days following seeding and included 'Standard' (60d + 30d + 30d), 'Standard + Fall' (60d + 30d + 30d + Fall), 'Standard + Delay' (60d + 37d + 37d), and 'Delay + Fall' (67d + 45d + Fall). A first harvest 60 days following seeding is a recommended practice to promote the establishment of alfalfa (Sheaffer et al., 1988). A 30-d cutting interval was chosen to represent the standard for high quality alfalfa production in the northern Midwest, while the 37- and 45-d cutting intervals were chosen to represent alfalfa production for maximum forage accumulation and persistence (Undersander et al., 2011), as well as to test the effects of reduced lignin alfalfa grown under a delayed harvest schedule. A fall cut refers to an alfalfa harvest taken around the first week of October in the northern Midwest (Undersander et al., 2011). Fall cuts are a common method used to increase seasonal forage accumulation in the northern Midwest; however, a fall cut will often reduce stand persistence and forage mass the following spring (Undersander et al., 2011; Wells et al., 2014). For these reasons, a fall cut was included for two of the 2015 cutting treatments to test the effects of a fall harvest on reduced lignin alfalfa cultivars. In 2016, the first cutting occurred on the same day for all treatments to determine the effect of establishment year cutting treatments on forage mass and to asses any potential winter injury. Results from the 2016 first cut showed a slight

reduction in forage mass for the more intensive Standard + Fall establishment year cutting treatment at some locations (see forage accumulation results for further details). As a result, the plots in the 2015 Standard + Fall cutting treatment continued to be harvested under a more intensive 30-d cutting schedule in 2016 in order to investigate the potential effects of a more intense harvest schedule. No differences in forage mass were found among the rest of the 2015 cutting treatments; therefore, the remaining 2016 cutting treatments were randomly assigned to plots. Cutting treatments in 2016 included '30-d' (30d + 30d + 30d + Fall), '35-d' (35d + 35d + 35d + Fall), '40-d' (40d + 40d + Fall), and '45-d' (45d + 45d + Fall). Similar to the establishment year, a range of cutting intervals was chosen to test the effects of reduced lignin alfalfa when grown for a variety of production goals. Harvest dates for cutting treatments within each location and year are shown in Table 2.1. Sub-plots were four alfalfa cultivars, which included the reference alfalfa cultivars 54R02, DKA43-22RR, WL 355.RR, and the reduced lignin cultivar 54HVX41. All alfalfa cultivars were marketed as Round-up Ready and were rated as Fall Dormancy 4 cultivars.

To determine plant maturity and forage nutritive value, random duplicate samples were hand-harvested from non-border rows within each plot to a stubble height of 5 cm. Samples were weighed to determine wet weight. One sample from each plot was used to evaluate alfalfa maturity using the mean stage by count (MSC) method developed by Kalu and Fick (1981), where vegetative growth included stages 0 through 2, budding plants included stages 3 and 4, and flowering plants included stages 5 and 6. The other sample from each plot was dried in forced-air ovens for 48 h at 60°C and weighed for dry matter (DM) determination. Dried samples were ground through a 6-mm screen in a

Wiley mill (Thomas Scientific, Swedesboro, NJ) followed by a 1-mm screen in a Cyclotec (Foss, Hillerød, Denmark). Samples were mixed thoroughly and scanned under near infrared reflectance spectroscopy (NIRS) using a Perten NIRS (Model DA 7200; Perten Instruments, Springfield, IL) with calibration equations developed in Minnesota to estimate forage nutritive value for CP, NDF, ADL, and NDFD. The standard error of cross validation was 0.98, 1.98, 1.52, and 2.64, respectively, for CP, ADL, NDF, and NDFD, while the $R^2$ was 0.98, 0.80, 0.86, and 0.87, respectively. Wet chemistry procedures were as follows: CP (AOAC 990.03, 2010); NDF and ADL (Goering and Van Soest, 1970; Van Soest et al., 1991); and NDFD (Hoffman et al., 1993). Forage nutritive value parameters from the NIRS analysis were used to calculate RFQ using the equations provided by Moore and Undersander (2002) to provide a relative measure of forage quality.

Following the hand-sampling, alfalfa plot forage masses were determined by mechanically harvesting a 0.9 × 5.2 m strip using a flail harvester (Carter Manufacturing Company Inc., Brookston, IN) set to leave a 5-cm stubble. Mechanically harvested samples were weighed, and hand-sample wet weights were added to calculate a total plot wet weight for DM forage mass determination. After each harvest was complete, stand density was assessed via stem counts, which were measured as the number of green stems (≥ 2.5 cm in length) along a 0.3-m section in non-border rows in two locations within each harvested plot (Smith et al., 1989). In the fall of each year, plant densities were measured in two locations within each plot using a frequency grid (Vogel and Masters, 2001).

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Individual plots comprised the experimental unit, and statistical significance was set at $P \leq 0.05$. Due to management differences (i.e. cutting treatments), analysis of the establishment year (2015) and first production year (2016) was completed separately. Differences among environments resulted in significant interactions between location and cutting treatment; therefore, locations were analyzed and reported separately. Replicate was considered a random effect; cutting treatment and cultivar were designated as fixed effects. Within each year and location, forage masses are reported as seasonal cumulative forage accumulation, and forage nutritive values are reported for the mid-season harvests (excluding the first and fall cut). The first harvest of each year was excluded because harvest for all plots occurred on the same date and initiated the different cutting treatments. The fall cut was excluded because it did not correspond to a specific harvest frequency or follow cutting treatment schedules. Means separations were performed on significant effects using Tukey's HSD test. Variables analyzed included maturity (MSC), forage accumulation, CP, NDF, ADL, and NDFD. To assess the relationship between plant maturity and forage nutritive value, Pearson correlation coefficients were calculated between MSC and the forage nutritive values for CP, NDF, ADL, and NDFD using the CORR procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC).

Cumulative growing degree days (GDD, $T_{base} = 5.6$ °C) were tracked and reset between each cutting interval for all sites during the 2016 growing season. The number of GDD accumulated at harvest in 2016 averaged across cuttings and locations were 832, 1035, and 1248 GDD for the 30-, 35-, and 40-d cutting treatments, respectively. Due to

the large decrease in RFQ for the 45-d cutting treatment, the 45-d cutting treatment was excluded to better reflect high quality alfalfa production management. Cumulative GDD were utilized to further explore the relationships between alfalfa harvest frequency (i.e. cutting treatments) and forage mass, as well as between alfalfa harvest frequency and RFQ. The REG procedure in SAS and the Akaike's Information Criterion (AIC) were used to aid in model selection comparing linear and quadratic models that best predict forage mass and RFQ (Burnham and Anderson, 2002); version 9.4; SAS Institute Inc., Cary, NC). Quadratic models with cumulative GDD as the predictor variable were selected to best predict forage mass. Forage nutritive value parameters from the NIRS analysis were applied to the RFQ equation and the RFQ estimates were regressed on cumulative GDD. The quadratic model with MSC as a covariate was determined to best predict RFQ.

## RESULTS AND DISCUSSION

For maturity, forage accumulation, and forage nutritive values, statistical analysis indicated no significant interactions ($P > 0.05$) between cutting treatment and cultivar; therefore, only the main effects of cutting treatment and cultivar were reported. Data for cutting treatments were averaged across cultivars, and data for cultivars were averaged across cutting treatments.

### Maturity

**Cultivar Response.** Maturities differed among cultivars (Table 2.2), although differences were minimal and inconsistent. In 2015, there were no differences in maturity among cultivars at Rosemount. At Becker, Rochester, and St. Paul, 54HVX41

was among the least mature (MSC ≤ 2.6), while reference cultivars were among the most mature. In 2016, 54HVX41 was less mature (MSC = 3.5) compared to DKA43-22RR (MSC = 3.9) at Rosemount. At Becker and St. Paul, cultivars had similar maturities. Maturities for all cultivars were within normal ranges, ranging from the late vegetative to early bud stage in 2015, and from the early bud to early flower stage in 2016 (Kalu and Fick, 1981).

**Cutting Treatment Response.** Maturities differed among cutting treatments (Table 2.2). In 2015, plants subject to the Standard and Standard + Fall cutting treatments were the least mature (MSC ≤ 2.1), while plants in the Delay + Fall cutting treatment were the most mature (MSC ≥ 3.1). In 2016, plants in the 30-d cutting treatment were the least mature (MSC ≤ 3.2), while plants in the 40- and 45-d cutting treatments were the most mature (MSC ≥ 4.4). The increase in maturity with increasingly delayed cutting treatments was expected, as a wider harvest interval allows for further growth and maturation.

## Forage Accumulation

**Cultivar Response.** Forage accumulation differed among alfalfa cultivars but was not consistent across locations (Table 2.3). Forage accumulation differences were more pronounced during the establishment year (2015) than during the first production year (2016). In 2015 at Becker and Rosemount, DKA43-22RR and WL 355.RR were among the cultivars with the greatest forage accumulation (≥ 7.3 Mg $ha^{-1}$), while 54HVX41 was among those with the least forage accumulation (≤ 7.0 Mg $ha^{-1}$). At Rochester, 54HVX41 had decreased forage accumulation (5.8 Mg $ha^{-1}$) compared to all reference cultivars (≥ 6.6 Mg $ha^{-1}$). At St. Paul, forage accumulation was similar for all

alfalfa cultivars. In 2016, only the Becker location resulted in seasonal cumulative forage accumulation differences. At Becker, 54HVX41 had a decreased forage accumulation (15.0 Mg $ha^{-1}$) compared to all reference cultivars (15.9 Mg $ha^{-1}$).

To the best of our knowledge, this is the first public study comparing forage accumulation between reduced lignin and reference alfalfa cultivars under diverse cutting schedules. Forage accumulation for 54HVX41 tended to be decreased compared to reference cultivars at some locations in the establishment year. However, fewer differences in forage accumulation were observed during the first production year, indicating that forage accumulation differences between 54HVX41 and reference alfalfa cultivars could be minimal following establishment. Forage accumulations ranged from 5.8 to 9.3 Mg $ha^{-1}$ in the establishment year and from 15.0 to 20.8 Mg $ha^{-1}$ in the first production year and are comparable to previously reported alfalfa forage accumulations. In Wisconsin, establishment year forage accumulations for University variety trials ranged from 2.7 to 16.1 Mg $ha^{-1}$ for alfalfa planted between 2013 and 2015 (Undersander, 2016). First production year forage accumulations ranged from 8.5 to 23.8 Mg $ha^{-1}$ and from 11.2 to 25.6 Mg $ha^{-1}$ in University variety trials planted between 2013 and 2015 in Minnesota and Wisconsin, respectively (Sheaffer et al., 2016; Undersander, 2016).

**Cutting Treatment Response.** Forage accumulation differed among cutting treatments (Table 2.3). In 2015, the Standard + Fall and Delay + Fall cutting treatments were among those with the greatest forage accumulation (≥ 6.7 Mg $ha^{-1}$), while the Standard cutting treatment was among those with the least forage accumulation. In 2016, the 40-d cutting treatment was among those with the greatest forage accumulation (≥ 16.3 Mg $ha^{-1}$), while the 30- and 45-d cutting treatments were among those with the least

forage accumulation. These results demonstrate that alfalfa has high forage accumulation potential in the both the establishment and first production year, and that forage accumulations were affected by harvest scheduling.

During the establishment year, forage accumulations were improved when a fall cut was added. During the first production year, alfalfa forage accumulations were greater with the 40-d cutting treatment compared to the 30-d cutting treatment. These results suggest that adding a fall harvest or using a cutting schedule with a longer interval between harvests can result in greater forage accumulation compared to a traditional 30-d cutting system or one without a fall harvest. Compared to the traditional 30-d cutting system, a 40-d harvest interval also offered the advantage of reducing the number of cuts per season from five to four while still producing greater forage accumulation.

Previous research has also concluded that as the interval between harvests increased, annual DM forage accumulation increased (Brink and Marten, 1989; Kallenbach et al., 2002; Putnam et al., 2005; Probst and Smith, 2011; Min, 2016). In Missouri, Kallenbach et al. (2002) reported that alfalfa harvested four times per year produced 7% and 28% more than when harvested five or six times per year, respectively. Similarly, Min (2016) reported increasing alfalfa forage accumulation as cutting intervals increased from 28 days to 42 days in Kansas. However, there is a point where increasing the interval between harvests no longer results in increased forage accumulation. Delayed harvests beyond early flowering can reduce forage accumulation as a result of leaf loss from lower portions of the canopy (Sheaffer et al., 1988). In the present study, the 45-d cutting treatment had the longest harvest interval but was among the cutting treatments with the least forage accumulation. Min (2016) also observed that alfalfa DM

forage accumulation decreased by approximately 16% when alfalfa was harvested every 49 days compared to every 42 days.

To clarify the effects of establishment year (2015) cutting treatments on first cut forage masses the following spring (2016), a uniform harvest date was applied over all cutting treatments for the first cut in 2016. Cultivar forage masses for the first cut in 2016 did not differ based on establishment year (2015) cutting treatments (data not shown). However, establishment year (2015) cutting treatments had minor effects on first cut forage masses within the first production year (2016; data not shown). At St. Paul, there was no effect of 2015 cutting treatments on first cut forage masses in 2016. At Becker, 2016 first cut forage masses were reduced following the Standard + Fall (4.1 Mg $ha^{-1}$) cutting treatment compared to the Standard cutting treatment (4.6 Mg $ha^{-1}$). At Rosemount, 2016 first cut forage masses were decreased following the Standard + Fall cutting treatment (5.0 Mg $ha^{-1}$) compared to all other cutting treatments (≥ 5.4 Mg $ha^{-1}$). These results suggest that the more intensive Standard + Fall establishment year cutting treatment may have had negative impacts on first cut forage masses in 2016; however, results were inconsistent across locations and further research is needed for definite conclusions to be drawn.

## Forage Nutritive Value

### *Crude Protein*

**Cultivar Response.** Crude protein concentrations differed among alfalfa cultivars only at the Becker location (Table 2.4). In 2015, CP concentrations were greater for 54HVX41 (230 g $kg^{-1}$) compared to 54R02 and DKA43-22RR (≤ 224 g $kg^{-1}$).

In 2016, CP concentrations were greater for 54HVX41 (187 g $kg^{-1}$) compared to WL 355.RR (178 g $kg^{-1}$).

Across locations and years, CP concentrations for all cultivars ranged from 175 to 234 g $kg^{-1}$ and are comparable to previously reported values for alfalfa (Hall et al., 2000; Kallenbach et al., 2002; Palmonari et al., 2014; Min, 2016). Previous studies examining reduced lignin alfalfa experimental lines also found similar CP concentrations for reduced lignin alfalfa compared to reference alfalfa cultivars (Weakley et al., 2008; Getachew et al., 2011; Li et al., 2015a).

**Cutting Treatment Response.** Crude protein concentrations differed among cutting treatments at all locations (Table 2.5). In 2015, CP concentrations were greatest for the Standard and Standard + Fall cutting treatments (≥ 230 g $kg^{-1}$) and least for the Standard + Delay and Delay + Fall cutting treatments (≤ 226 g $kg^{-1}$). In 2016, CP concentrations were greatest for the 30-d cutting treatment (≥ 215 g $kg^{-1}$) and least for the 40- and 45-d cutting treatments (≤ 163 g $kg^{-1}$).

More frequent cutting schedules with shorter intervals between harvests resulted in greater CP concentrations. This agrees with findings from previous studies examining the CP content of alfalfa under various harvest frequencies. It has been well-documented that CP content declines as harvest intervals are lengthened (Weir et al., 1960; Nordkvist and Åman, 1986; Hall et al., 2000; Kallenbach et al., 2002; Yu et al., 2003; Palmonari et al., 2014; Min, 2016). In Kansas, Min (2016) reported that delaying a 28-d harvest by 5 or 18 days reduced CP concentrations by 4 and 6%, respectively. In Missouri, Kallenbach et al. (2002) found that CP levels averaged 250 g $kg^{-1}$ when alfalfa was harvested six times per year compared to 227 g $kg^{-1}$ and 195 g $kg^{-1}$ when harvested five

or four times, respectively. Hall et al. (2000) and Yu et al. (Yu et al., 2003) both also reported declines in CP concentrations with advancing morphological development across multiple harvests.

In the present study, plant maturity (MSC) was negatively associated with CP concentrations at all locations in both the establishment (2015) and first production (2016) year. In the establishment year, correlation coefficients were -0.77, -0.77, -0.82, and -0.52 for Becker, Rochester, Rosemount, and St. Paul, respectively. In the first production year, correlation coefficients were -0.65, -0.78, and -0.68 for Becker, Rosemount, and St. Paul, respectively. This decline in CP concentration with progressing plant maturity can be attributed to the associated effects of increasing stem proportions and decreasing leaf proportions on forage nutritive value as the plant matures (Kalu and Fick, 1983; Nordkvist and Åman, 1986; Albrecht et al., 1987; Sanderson and Wedin, 1988; Sheaffer et al., 2000). As plants mature, defoliation increases as leaf senescence and abscission occurs in the lower, shaded portions of the plant, further contributing to the loss in CP (Albrecht et al., 1987; Sheaffer et al., 1988; Undersander et al., 2011). Although leaf loss was not measured in the present experiment, the research team did observe some leaf loss from the lower portions of alfalfa stems under the 40- and 45-d cutting treatments.

### *Neutral Detergent Fiber*

**Cultivar Response.** Concentrations of NDF differed among alfalfa cultivars only at the Becker location (Table 2.4). In 2015, NDF concentrations were less for 54HVX41 (387 g $kg^{-1}$) compared to 54R02 and DKA43-22RR (≥ 401 g $kg^{-1}$). In 2016, NDF

concentrations were less for 54HVX41 (441 g $kg^{-1}$) compared to DKA43-22RR (458 g $kg^{-1}$).

Across locations and years, NDF concentrations for all cultivars ranged from 346 to 458 g $kg^{-1}$ and are comparable to reports from previous studies (Hall et al., 2000; Kallenbach et al., 2002; Palmonari et al., 2014; Min, 2016). Studies investigating reduced lignin alfalfa experimental lines have also found similar or slightly reduced NDF concentrations for reduced lignin alfalfa compared to reference (Guo et al., 2001b; Getachew et al., 2011). Similarly, preliminary results evaluating reduced lignin alfalfa hay found either no change (Mertens and McCaslin, 2008) or decreased (Li et al., 2015a) NDF concentrations for reduced lignin cultivars compared to controls.

**Cutting Treatment Response.** Concentrations of NDF differed among cutting treatments at all locations (Table 2.5). In 2015, the Standard and Standard + Fall cutting treatments were among those with the least NDF concentrations (≤ 371 g $kg^{-1}$), while the Delay + Fall cutting treatment was among those with the greatest NDF concentrations (≥ 395 g $kg^{-1}$). In 2016, NDF concentrations were least for the 30-d cutting treatment (≤ 391 g $kg^{-1}$) and greatest for the 40- and 45-d cutting treatments (≥ 444 g $kg^{-1}$).

A more frequent cutting schedule with shorter intervals between harvests resulted in decreased NDF concentrations. These results were expected, and agree with findings from previous studies demonstrating an increase in NDF concentrations as harvest intervals are lengthened (Weir et al., 1960; Hall et al., 2000; Kallenbach et al., 2002; Brink et al., 2010; Min, 2016). As the cutting interval was increased from 28 to 49 days, Min (2016) reported increasing NDF concentrations from 277 g $kg^{-1}$ to 455 g $kg^{-1}$. Kallenbach et al. (2002) found that NDF concentrations were approximately 46 g $kg^{-1}$

greater when alfalfa was harvested four times per year compared to five, and 28 g $kg^{-1}$ greater when harvested five times per year compared to six. Hall et al. (2000) also reported increasing NDF concentrations across multiple alfalfa harvests. Similar to CP, plant maturity is likely the main attributing factor affecting NDF concentrations, with NDF concentrations increasing as cutting intervals lengthened. In the present study, plant maturity (MSC) was positively associated with NDF concentrations at all locations in both the establishment (2015) and first production (2016) year. In the establishment year, correlation coefficients were 0.80, 0.75, 0.79, and 0.49 for Becker, Rochester, Rosemount, and St. Paul, respectively. In the first production year, correlation coefficients were 0.71, 0.71, and 0.54 for Becker, Rosemount, and St. Paul, respectively. As plants mature, leaf proportions decrease, stem proportions increase, stem cell wall concentrations increase, and whole plant nutritive value decreases (Kalu and Fick, 1983; Nordkvist and Åman, 1986; Albrecht et al., 1987; Sanderson and Wedin, 1988; Sheaffer et al., 2000).

### *Acid Detergent Lignin*

**Cultivar Response.** Across all years and locations, 54HVX41 contained less ADL compared to reference alfalfa cultivars (Table 2.4). Acid detergent lignin concentrations for reference cultivars ranged from 71 to 88 g $kg^{-1}$, while ADL concentrations for 54HVX41 ranged from 65 to 81 g $kg^{-1}$.

Compared to reference alfalfa cultivars, 54HVX41 demonstrated a 7 to 12% reduction in ADL during the establishment year (2015) and a 6 to 8% reduction in ADL during the first production year (2016). Acid detergent lignin concentrations for reference cultivars are comparable to previous reports (Jung et al., 1997a; Palmonari et

al., 2014). Previous studies investigating reduced lignin alfalfa experimental lines have shown a 4 to 29% decrease in stem lignin concentration (Guo et al., 2001b; Marita et al., 2003; Reddy et al., 2005) and a 1 to 24% decrease in whole plant lignin concentration (Guo et al., 2001a; b; Getachew et al., 2011) compared to control lines. Preliminary results evaluating reduced lignin alfalfa hay also reported decreased lignin concentrations ranging from 4 to 12% compared to control cultivars (Mertens and McCaslin, 2008; Undersander et al., 2009).

The reduced lignin concentration for 54HVX41 compared to other cultivars could be due to a number of reasons, including a greater leaf:stem, a reduction in stem lignin, or a lower plant maturity. In the present study, the leaf:stem and the stem lignin content of the plants were not measured, but 54HVX41 did demonstrate a slight reduction in maturity at some of the locations. However, maturity differences among cultivars were minimal and inconsistent across locations and years, while the reduction in lignin was present across all locations and years, which suggests that the maturity differences had little impact on lignin concentrations. Further research is needed to pinpoint the cause of lignin reduction for 54HVX41.

**Cutting Treatment Response.** Concentrations of ADL differed among cutting treatments at all locations (Table 2.5). In 2015, the Delay + Fall cutting treatment contained greater ADL concentrations ($\geq$ 84 g $kg^{-1}$) compared to all other cutting treatments ($\leq$ 80 g $kg^{-1}$) at Becker, Rochester, and Rosemount. At St. Paul, the Delay + Fall cutting treatment contained greater ADL concentrations (76 g $kg^{-1}$) compared to the Standard and Standard + Fall cutting treatments ($\leq$ 69 g $kg^{-1}$). In 2016, the 30- and 35-d

cutting treatments contained the least ADL (≤ 82 g $kg^{-1}$), while the 40- and 45-d cutting treatments contained the most ADL (≥ 83 g $kg^{-1}$) across all locations.

A more frequent cutting schedule with shorter intervals between harvests generally resulted in decreased ADL concentrations. Findings from previous studies have also shown that lignin concentrations increase as harvest intervals are lengthened (Weir et al., 1960; Nordkvist and Åman, 1986; Palmonari et al., 2014). As harvest intervals increased from 21 to 35 days, Palmonari et al. (2014) reported an increase in lignin concentrations from 63 g $kg^{-1}$ to 73 g $kg^{-1}$. Similarly, Nordkvist and Åman (1986) reported lignin contents increasing from 43 g $kg^{-1}$ to 147 g $kg^{-1}$ across harvest intervals encompassing a range of alfalfa maturities. Increasing lignin concentrations with widening harvest intervals are a function of increasing plant maturity and the growth of secondary plant cell walls (Albrecht et al., 1987; Sanderson and Wedin, 1988). In the present study, plant maturity (MSC) was positively associated with ADL concentrations at all locations in both the establishment (2015) and first production (2016) year. In the establishment year, correlation coefficients were 0.58, 0.62, 0.77, and 0.43 for Becker, Rochester, Rosemount, and St. Paul, respectively. In the first production year, correlation coefficients were 0.55, 0.58, and 0.62 for Becker, Rosemount, and St. Paul, respectively. As a plant grows, the deposition of lignin is necessary to provide the strength and rigidity for a plant to stand upright (Inoue et al., 1998; Guo et al., 2001a).

### *Neutral Detergent Fiber Digestibility*

**Cultivar Response.** With the exception of Rosemount, NDFD for 54HVX41 were greater compared to all reference alfalfa cultivars (Table 2.4). Increases in NDFD ranged from 8 to 10% in the establishment year (2015) and 11 to 18% in the first

production year (2016). There were no differences in NDFD among alfalfa cultivars at Rosemount in either 2015 or 2016. The lack of differences detected among alfalfa cultivars at Rosemount could be related to a number of factors, including but not limited to a larger amount of variation among cultivars (indicated by greater standard errors) and a lower plant maturity at this particular location. Average plant maturities for alfalfa cultivars at Becker and St. Paul were ≥ 2.5 in 2015 and ≥ 4.0 in 2016, while maturity averages at Rosemount were ≤ 2.1 in 2015 and ≤ 3.9 in 2016. Alfalfa grown in Rosemount generally had less NDF and more NDFD compared to other locations; this, coupled with a lower maturity, could be indicative of a more digestible forage and might have masked some cultivar differences.

Previous studies investigating reduced lignin alfalfa experimental lines found similar results, reporting increases in DMD (Reddy et al., 2005; Getachew et al., 2011), *in situ* rumen digestibility (Guo et al., 2001b; Reddy et al., 2005), and NDFD (Guo et al., 2001b) for reduced lignin alfalfa compared to control cultivars. Preliminary results evaluating reduced lignin alfalfa hay also showed greater DMD and NDFD, with a 3 to 5% increase in DMD (Mertens and McCaslin, 2008) and a 3 to 26% increase in NDFD (Mertens and McCaslin, 2008; Weakley et al., 2008; Undersander et al., 2009; Li et al., 2015a) for reduced lignin alfalfa. Increases in NDFD for 54HVX41 can likely be attributed to reduced lignin concentrations, as the deposition of lignin into plant cell walls can negatively affect rumen microbial degradation and the digestion of feed by intestinal enzymes (Buxton and Hornstein, 1986; Liu and Yu, 2011). These results have potential biological significance, as feeding and grazing studies have shown that small changes in forage digestibility can impact animal performance. Casler and Vogel (1999) reported

that a 1% increase in *in vitro* DMD resulted in a 3.2% increase in daily animal weight gains. Similarly, a one-unit increase in NDFD has been associated with a 0.17-kg increase in dry matter intake and a 0.25-kg increase in 4% fat-corrected milk for dairy cows (Oba and Allen, 1999).

**Cutting Treatment Response.** Neutral detergent fiber digestibility differed among cutting treatments at all locations (Table 2.5). In 2015, the Standard and Standard + Fall cutting treatments had the greatest NDFD ($\geq$ 396 g $kg^{-1}$), while the Delay + Fall cutting treatment had the least ($\leq$ 385 g $kg^{-1}$). In 2016, the 30- and 35-d cutting treatments contained the greatest NDFD ($\geq$ 322 g $kg^{-1}$), while the 40- and 45-d cutting treatments contained the least ($\leq$ 275 g $kg^{-1}$).

A more frequent cutting schedule with shorter intervals between harvests resulted in increased NDFD. Previous studies have made similar conclusions, showing decreasing fiber or DMD digestibility as harvest intervals increased (Weir et al., 1960; Nordkvist and Åman, 1986; Hall et al., 2000; Brink et al., 2010; Palmonari et al., 2014). Hall et al. (2000) reported a drop in DMD by 43 g $kg^{-1}$ across four weekly sampling periods, and Palmonari et al. (2014) reported a reduction in NDFD levels from 440 g $kg^{-1}$ to 340 g $kg^{-1}$ as the harvest interval increased from 21 to 35 days. Similar to the other forage nutritive value components, the decrease in NDFD with increasing harvest intervals can be attributed to advancing plant maturity. In the present study, plant maturity (MSC) was negatively associated with NDFD at all locations in both the establishment (2015) and first production (2016) year. In the establishment year, correlation coefficients were -0.76, -0.76, -0.84, and -0.63 for Becker, Rochester, Rosemount, and St. Paul, respectively. In the first production year, correlation

coefficients were -0.55, -0.75, and -0.64 for Becker, Rosemount, and St. Paul, respectively. Research has shown that maturity influences the fiber digestibility of alfalfa through increased leaf:stem ratios and increased lignification of the stem portion of the plant (Weir et al., 1960; Mowat et al., 1965; Albrecht et al., 1987; Yu et al., 2003).

## Alfalfa Stand Density

Minor differences in alfalfa population densities and stem counts were detected across cutting treatments and cultivars (data not shown); however, differences were negligible and inconsistent. Compared to initial plant populations, plant densities were ≥ 88% at the end of the establishment year and ≥ 79% at the end of the first production year. Stem densities ranged from 743 to 942 stems $m^{-2}$ during the establishment year and from 520 to 795 stems $m^{-2}$ during the first production year and fall within the normal range suggested to maximize alfalfa forage accumulation potential (Undersander et al., 2011).

Differences in alfalfa population measurements were expected to be minor, as the present study contains only establishment and first production year data. Previous research has shown that along with increasing forage accumulation, a delayed cutting schedule could also benefit stand longevity. Frequent, repeated harvests of immature alfalfa have been shown to reduce stand persistence, vigor, and forage accumulation (Brink and Marten, 1989; Sheaffer and Marten, 1990; Probst and Smith, 2011). Continuation of this study is required to further evaluate the effects of cutting treatment and alfalfa cultivar on plant persistence over time.

## Relationships in Forage Mass and Relative Forage Quality

To examine the effect of cutting treatment on alfalfa forage mass and RFQ from the first production year (2016), values were regressed across GDD for 54HVX41 and the average of the reference cultivars (Figure 2.2 and 2.3). The number of GDD accumulated at harvest in 2016 averaged across cuttings and locations were 832, 1035, and 1248 GDD for the 30-, 35-, and 40-d cutting treatments, respectively (Figure 2.2 and 2.3).

As expected, alfalfa forage masses increased across GDD for both 54HVX41 and reference cultivars (Figure 2.2). No differences in forage mass were observed between 54HVX41 and the reference cultivars across GDD ranging from 700 to 1400. These results support the previously stated forage accumulation comparisons, which showed very minimal differences in forage accumulation among alfalfa cultivars during the first production year. With a 5-d harvest delay (e.g. delayed from 30 to 35 days), all cultivars produced an average of 0.83 kg DM $ha^{-1}$ in additional forage mass. Delaying the alfalfa harvest by 10 days (e.g. delayed from 30 to 40 days) resulted in a 1.3 kg DM $ha^{-1}$ increase in cultivar forage masses. Under these circumstances (i.e. 10-d delay), a delayed harvest can result in as much as a 28% increase in DM production; however, this increase in DM forage mass will traditionally be coupled with a reduction in alfalfa forage quality.

The relationship between alfalfa forage mass and quality is widely recognized (Kalu and Fick, 1983). As expected, alfalfa RFQ decreased with increasing GDD for both 54HVX41 and reference cultivars (Figure 2.3). Although a 5-d delay in harvest improved forage mass, RFQ for 54HVX41 and reference cultivars was reduced by 11 and 13%, respectively, with increasing GDD (Figure 2.3). While there were no differences between 54HVX41 and reference cultivars in forage mass, RFQ for 54HVX41 was 9, 10, and 12% greater compared to reference cultivars when cut under the same 30-, 35-, and

40-d treatment schedule, respectively (Figure 2.3). A significantly greater RFQ (i.e. non-overlapping 95% confidence intervals) for 54HVX41 compared to reference cultivars was observed from 772 to 1248 GDD and represented a range of cutting intervals from 28 to 40 days (Figure 2.3). This finding illustrates that the reduced lignin cultivar 54HVX41 has increased RFQ relative to reference cultivars under cutting schedules with increasing harvest intervals.

The greater RFQ observed for 54HVX41 across a wide range of GDD when compared to reference cultivars can offer flexibility for producers by reducing the forage digestibility penalty associated with a lengthened harvest window. Both 54HVX41 and reference cultivars produced a 22% gain in forage mass with a delayed harvest from 30- to 35-d. In addition, both showed similar reductions in RFQ across the same period. However, the value of the reduced lignin technology is most apparent when making cross comparisons. Reference cultivars cut at 30-d produced 3.74 kg DM $ha^{-1}$ with a RFQ of 144, whereas 54HVX41 cut at 35-d produced 4.53 kg DM $ha^{-1}$ with a RFQ of 139. This represents a 21% gain in forage mass with only a 3% reduction in RFQ. According to these results, a producer could successfully maintain RFQ by growing 54HVX41 instead of reference cultivars. Depending on production goals, this could allow for a wider optimal harvest window, making it possible for alfalfa growers to increase forage mass by delaying alfalfa harvest while still maintaining greater forage digestibility. A delayed harvest may also provide additional benefits in the form of increased plant persistence over time. Although outside the scope of this study, future research should investigate the long term effects of harvest delay for reduced lignin alfalfa cultivars.

## SUMMARY AND CONCLUSIONS

Forage accumulation differences among alfalfa cultivars were more pronounced during the establishment year than during the first production year. During the establishment year, forage accumulations for 54HVX41alfalfa were decreased compared to reference cultivars at some locations. During the first production year, forage accumulation differences were minimal, indicating that forage accumulation differences between 54HVX41 and reference alfalfa cultivars could be minimal following establishment. Forage accumulations were also affected by cutting treatment. During the establishment year, alfalfa forage accumulations were increased when a fall cut was added. During the first production year, alfalfa forage accumulations were greater with the 40-d cutting treatment compared to the 30-d cutting treatment. These results suggest that during a normal production year, adding a fall harvest or using a cutting schedule with a longer interval between harvests can maximize forage accumulation, and that a longer harvest interval can reduce the number of cuts per season while still producing greater forage accumulation. Alfalfa cultivars in this study were tolerant of a diversity of cutting treatments and maintained adequate population densities throughout the establishment and first production year.

Compared to reference alfalfa cultivars, 54HVX41 generally had reduced ADL concentrations, increased NDFD, and similar CP and NDF concentrations. Cutting treatments with shorter harvest intervals had decreased plant maturities and generally resulted in greater forage nutritive value, including increased CP concentration, decreased NDF and ADL concentrations, and increased NDFD.

When alfalfa forage mass and RFQ were regressed across GDD in the first production year, forage masses for 54HVX41 and reference cultivars were similar, but RFQ for 54HVX41 was greater compared to reference cultivars from 772 to 1248 GDD. This increase in RFQ observed for 54HVX41 across a wide range of GDD can offer increased flexibility for producers. If 54HVX41 were harvested at the same time as reference cultivars, it would allow producers to obtain a higher quality and more digestible forage. However, if 54HVX41 were harvested under a delayed cutting schedule, it would provide producers with an option to reduce the quality penalty associated with a lengthened harvest window. With a 5-d harvest delay, 54HVX41 harvested on a 35-d harvest interval showed a 21% gain in forage mass and a 3% reduction in RFQ compared to reference cultivars harvested on a 30-d harvest interval. This could allow for a wider optimal harvest window, making it possible for alfalfa growers to achieve greater forage mass by delaying alfalfa harvest while still maintaining higher forage nutritive value.

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Table 2.1. Alfalfa harvest schedule for 2015 and 2016 growing seasons in Becker, Rochester, Rosemount, and St. Paul, MN.

| Cutting Treatment | Cutting Interval | Cut 1 | Cut 2 | Cut 3 | Cut 4 | Fall Cut |
|---|---|---|---|---|---|---|
| | | | 2015 Harvest Dates | | | |
| Standard | 60, 30, 30 | 25 Jun-2 Jul | 23-30 Jul | 18-27 Aug | — | — |
| Standard + Fall | 60, 30, 30, Fall | 25 Jun-2 Jul | 23-30 Jul | 18-27 Aug | — | 2-7 Oct |
| Standard + Delay | 60, 37, 37 | 25 Jun-2 Jul | 31 Jul-5 Aug | 8-11 Sep | — | — |
| Delay + Fall | 67, 45, Fall | 1-10 Jul | 14-27 Aug | — | — | 2-7 Oct |
| | | | 2016 Harvest Dates | | | |
| 30-d | 30, 30, 30, Fall | 23-26 May | 20-23 Jun | 18-20 Jul | 15-17 Aug | 30 Sep-6 Oct |
| 35-d | 35, 35, 35, Fall | 23-26 May | 27-30 Jun | 1-3 Aug | 7-8 Sep | 30 Sep-6 Oct |
| 40-d | 40, 40, Fall | 23-26 May | 5-7 Jul | 15-17 Aug | — | 30 Sep-6 Oct |
| 45-d | 45, 45, Fall | 23-26 May | 8-14 Jul | 22-30 Aug | — | 30 Sep-6 Oct |

Table 2.2. Average stage of maturity across multiple cuts for alfalfa grown in Becker (BE), Rochester (ROC), Rosemount (ROS), and St. Paul (SP), MN in 2015 and 2016 as determined by cultivar and cutting treatment.

| Treatment | 2015 | | | | 2016 | | |
|---|---|---|---|---|---|---|---|
| | BE | ROC | ROS | SP | BE | ROS | SP |
| | Maturity index[†] | | | | | | |
| | Alfalfa cultivar | | | | | | |
| 54HVX41 | 2.5[b] | 1.7[b] | 1.9 | 2.6[b] | 4.0 | 3.5[b] | 4.6 |
| 54R02 | 2.9[a] | 2.1[a] | 2.1 | 2.8[ab] | 4.0 | 3.6[ab] | 4.5 |
| DKA43-22RR | 2.7[ab] | 2.1[a] | 2.1 | 3.0[a] | 4.0 | 3.9[a] | 5.0 |
| WL 355.RR | 2.9[a] | 2.2[a] | 2.1 | 2.8[ab] | 4.0 | 3.7[ab] | 4.9 |
| *SE* | *0.09* | *0.09* | *0.12* | *0.08* | *0.10* | *0.12* | *0.15* |
| | 2015 Cutting treatment | | | | | | |
| Standard[‡] | 2.1[c] | 1.5[c] | 1.4[c] | 2.0[c] | — | — | — |
| Standard + Fall | 2.1[c] | 1.5[c] | 1.4[c] | 2.0[c] | — | — | — |
| Standard + Delay | 2.7[b] | 2.0[b] | 1.9[b] | 2.8[b] | — | — | — |
| Delay + Fall | 4.1[a] | 3.1[a] | 3.5[a] | 4.3[a] | — | — | — |
| *SE* | *0.09* | *0.09* | *0.12* | *0.08* | — | — | — |
| | 2016 Cutting treatment | | | | | | |
| 30-d | — | — | — | — | 2.8[c] | 2.3[d] | 3.2[d] |
| 35-d | — | — | — | — | 3.5[b] | 3.0[c] | 4.4[c] |
| 40-d | — | — | — | — | 4.7[a] | 4.4[b] | 5.4[b] |
| 45-d | — | — | — | — | 4.9[a] | 5.1[a] | 6.0[a] |
| *SE* | — | — | — | — | *0.10* | *0.12* | *0.15* |

[†]Numerical index referring to stage of alfalfa development (Kalu and Fick, 1981). Vegetative growth includes stages 0 through 2, budding plants includes stages 3 and 4, and flowering plants includes stages 5 and 6.

[ab]Within column and section, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

[‡]2015 cutting treatments included 'Standard' (60d + 30d + 30d), 'Standard + Fall' (60d + 30d + 30d + Fall), 'Standard + Delay' (60d + 37d + 37d), and 'Delay + Fall' (67d + 45d + Fall)

Table 2.3. Seasonal cumulative forage accumulation for alfalfa grown in Becker (BE), Rochester (ROC), Rosemount (ROS), and St. Paul (SP), MN in 2015 and 2016 as determined by cultivar and cutting treatment.

| Treatment | 2015 | | | | 2016 | | |
|---|---|---|---|---|---|---|---|
| | BE | ROC | ROS | SP | BE | ROS | SP |
| | Mg ha$^{-1}$ | | | | | | |
| | Alfalfa cultivar | | | | | | |
| 54HVX41 | 7.0$^{b}$ | 5.8$^{b}$ | 6.9$^{b}$ | 8.6 | 15.0$^{b}$ | 16.2 | 20.1 |
| 54R02 | 7.2$^{b}$ | 6.8$^{a}$ | 7.2$^{ab}$ | 8.7 | 15.9$^{a}$ | 16.8 | 20.5 |
| DKA43-22RR | 7.6$^{ab}$ | 6.9$^{a}$ | 7.4$^{a}$ | 9.3 | 15.9$^{a}$ | 16.5 | 20.8 |
| WL 355.RR | 7.9$^{a}$ | 6.6$^{a}$ | 7.3$^{ab}$ | 8.8 | 15.9$^{a}$ | 16.5 | 20.6 |
| *SE* | *0.31* | *0.29* | *0.44* | *0.34* | *0.31* | *0.30* | *0.33* |
| | 2015 Cutting treatment | | | | | | |
| Standard$^{\dagger}$ | 6.5$^{b}$ | 5.8$^{b}$ | 6.3$^{b}$ | 7.6$^{b}$ | — | — | — |
| Standard + Fall | 7.7$^{a}$ | 7.1$^{a}$ | 7.1$^{ab}$ | 9.7$^{a}$ | — | — | — |
| Standard + Delay | 7.3$^{ab}$ | 6.5$^{ab}$ | 7.2$^{ab}$ | 8.2$^{b}$ | — | — | — |
| Delay + Fall | 8.2$^{a}$ | 6.7$^{a}$ | 8.1$^{a}$ | 9.8$^{a}$ | — | — | — |
| *SE* | *0.35* | *0.32* | *0.56* | *0.34* | — | — | — |
| | 2016 Cutting treatment | | | | | | |
| 30-d | — | — | — | — | 15.1$^{b}$ | 15.5$^{b}$ | 19.6$^{b}$ |
| 35-d | — | — | — | — | 16.6$^{a}$ | 16.7$^{ab}$ | 20.5$^{b}$ |
| 40-d | — | — | — | — | 16.3$^{a}$ | 17.5$^{a}$ | 21.9$^{a}$ |
| 45-d | — | — | — | — | 14.7$^{b}$ | 16.2$^{ab}$ | 20.1$^{b}$ |
| *SE* | — | — | — | — | *0.31* | *0.40* | *0.33* |

$^{ab}$Within column and section, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

$^{\dagger}$2015 cutting treatments included 'Standard' (60d + 30d + 30d), 'Standard + Fall' (60d + 30d + 30d + Fall), 'Standard + Delay' (60d + 37d + 37d), and 'Delay + Fall' (67d + 45d + Fall)

Table 2.4. Crude protein, neutral detergent fiber, acid detergent lignin, and neutral detergent fiber digestibility for alfalfa cultivars grown in Becker (BE), Rochester (ROC), Rosemount (ROS), and St. Paul (SP), MN in 2015 and 2016.

| Treatment | 2015 | | | | 2016 | | |
|---|---|---|---|---|---|---|---|
| | BE | ROC | ROS | SP | BE | ROS | SP |
| | g kg$^{-1}$ | | | | | | |
| | Crude protein | | | | | | |
| 54HVX41 | 230[a] | 218 | 226 | 234 | 187[a] | 186 | 182 |
| 54R02 | 220[b] | 211 | 221 | 226 | 180[ab] | 182 | 178 |
| DKA43-22RR | 224[b] | 212 | 224 | 228 | 179[ab] | 186 | 175 |
| WL 355.RR | 225[ab] | 215 | 227 | 230 | 178[b] | 184 | 178 |
| *SE* | *0.16* | *0.27* | *0.30* | *0.32* | *0.24* | *0.34* | *0.25* |
| | Neutral detergent fiber | | | | | | |
| 54HVX41 | 387[c] | 388 | 351 | 351 | 441[b] | 403 | 416 |
| 54R02 | 408[a] | 403 | 359 | 364 | 456[ab] | 414 | 424 |
| DKA43-22RR | 401[ab] | 399 | 357 | 363 | 458[a] | 405 | 430 |
| WL 355.RR | 396[bc] | 389 | 346 | 356 | 452[ab] | 407 | 419 |
| *SE* | *0.31* | *0.48* | *0.62* | *0.67* | *0.44* | *0.68* | *0.57* |
| | Acid detergent lignin | | | | | | |
| 54HVX41 | 74[c] | 74[b] | 67[b] | 65[b] | 81[b] | 79[b] | 77[b] |
| 54R02 | 86[a] | 83[a] | 73[a] | 74[a] | 87[a] | 85[a] | 81[a] |
| DKA43-22RR | 84[ab] | 83[a] | 71[a] | 73[a] | 88[a] | 84[a] | 83[a] |
| WL 355.RR | 83[b] | 81[a] | 71[a] | 73[a] | 88[a] | 85[a] | 82[a] |
| *SE* | *0.08* | *0.10* | *0.12* | *0.11* | *0.08* | *0.14* | *0.12* |
| | Neutral detergent fiber digestibility | | | | | | |
| 54HVX41 | 443[a] | 391[a] | 447 | 453[a] | 333[a] | 333 | 339[a] |
| 54R02 | 397[b] | 353[b] | 429 | 419[b] | 288[b] | 300 | 306[b] |
| DKA43-22RR | 404[b] | 359[b] | 428 | 418[b] | 282[b] | 305 | 305[b] |
| WL 355.RR | 402[b] | 365[b] | 436 | 419[b] | 278[b] | 301 | 306[b] |
| *SE* | *0.68* | *0.61* | *0.79* | *0.41* | *0.69* | *1.05* | *0.81* |

[ab]Within column and section, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

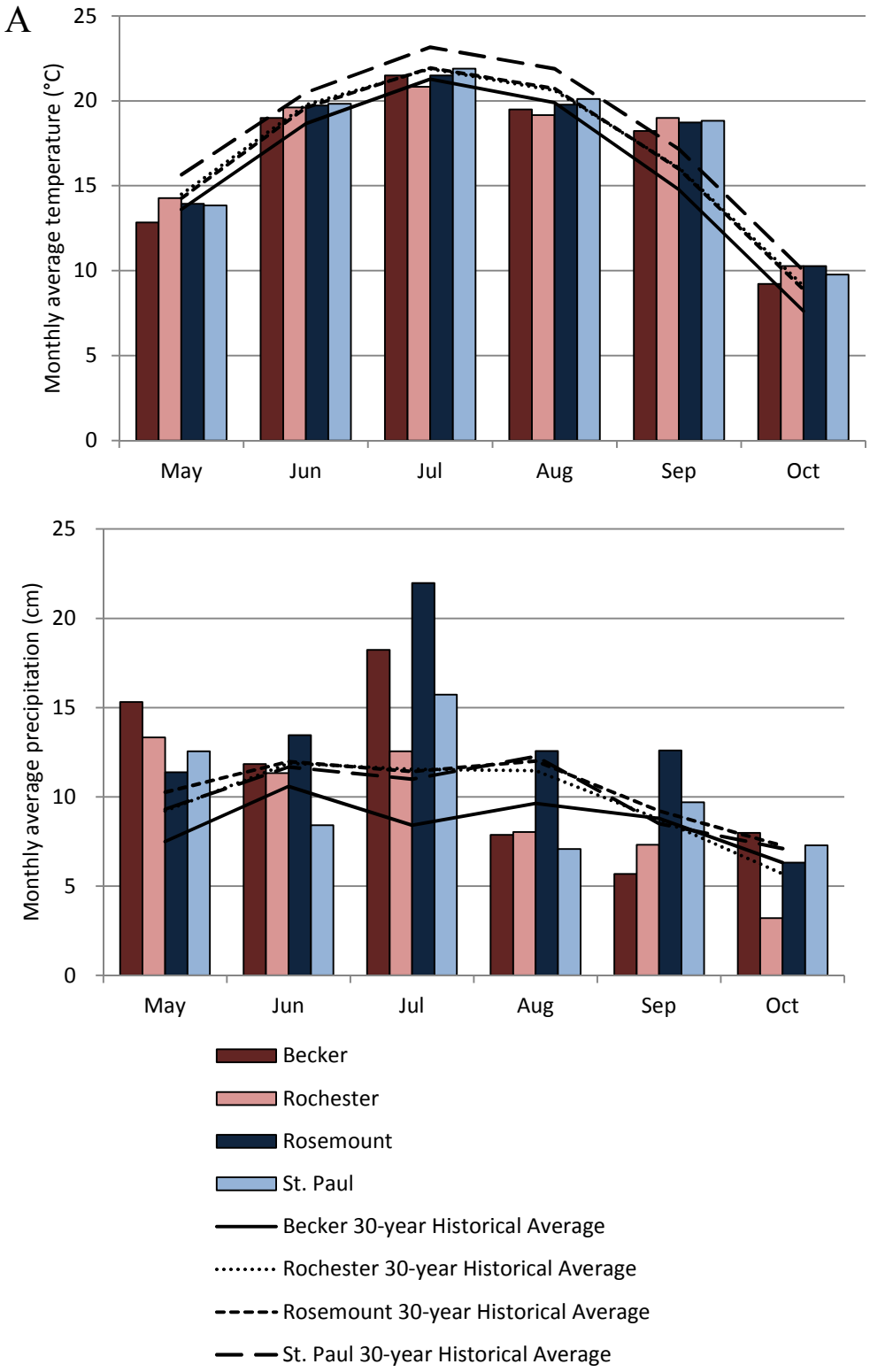
Table 2.5. Crude protein, acid detergent fiber, neutral detergent fiber, acid detergent lignin, and neutral detergent fiber digestibility for alfalfa grown in Becker (BE), Rochester (ROC), Rosemount (ROS), and St. Paul (SP), MN under various cutting treatments in 2015 and 2016.

| | 2015 | | | | | 2016 | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | BE | ROC | ROS | SP | Treatment | BE | ROS | SP |
| | g kg$^{-1}$ | | | | | g kg$^{-1}$ | | |
| | Crude protein | | | | | Crude protein | | |
| Standard[†] | 242$^{a}$ | 230$^{a}$ | 239$^{a}$ | 253$^{a}$ | 30-d | 215$^{a}$ | 221$^{a}$ | 215$^{a}$ |
| Standard + Fall | 242$^{a}$ | 238$^{a}$ | 236$^{a}$ | 248$^{a}$ | 35-d | 188$^{b}$ | 199$^{b}$ | 187$^{b}$ |
| Standard + Delay | 219$^{b}$ | 215$^{b}$ | 226$^{b}$ | 209$^{b}$ | 40-d | 163$^{c}$ | 161$^{c}$ | 157$^{c}$ |
| Delay + Fall | 195$^{c}$ | 173$^{c}$ | 197$^{c}$ | 208$^{b}$ | 45-d | 160$^{c}$ | 158$^{c}$ | 153$^{c}$ |
| *SE* | *0.16* | *0.30* | *0.30* | *0.32* | *SE* | *0.24* | *0.34* | *0.27* |
| | Neutral detergent fiber | | | | | Neutral detergent fiber | | |
| Standard | 367$^{c}$ | 369$^{bc}$ | 321$^{c}$ | 331$^{b}$ | 30-d | 391$^{d}$ | 343$^{d}$ | 366$^{c}$ |
| Standard + Fall | 371$^{c}$ | 352$^{c}$ | 324$^{c}$ | 336$^{b}$ | 35-d | 426$^{c}$ | 368$^{c}$ | 408$^{b}$ |
| Standard + Delay | 390$^{b}$ | 373$^{b}$ | 359$^{b}$ | 373$^{a}$ | 40-d | 483$^{b}$ | 444$^{b}$ | 446$^{a}$ |
| Delay + Fall | 464$^{a}$ | 486$^{a}$ | 408$^{a}$ | 395$^{a}$ | 45-d | 507$^{a}$ | 473$^{a}$ | 469$^{a}$ |
| *SE* | *0.31* | *0.48* | *0.61* | *0.69* | *SE* | *0.44* | *0.68* | *0.65* |
| | Acid detergent lignin | | | | | Acid detergent lignin | | |
| Standard | 80$^{b}$ | 77$^{b}$ | 64$^{c}$ | 68$^{c}$ | 30-d | 79$^{b}$ | 76$^{b}$ | 75$^{b}$ |
| Standard + Fall | 78$^{b}$ | 76$^{b}$ | 65$^{c}$ | 69$^{bc}$ | 35-d | 82$^{b}$ | 73$^{b}$ | 77$^{b}$ |
| Standard + Delay | 80$^{b}$ | 71$^{c}$ | 71$^{b}$ | 72$^{ab}$ | 40-d | 92$^{a}$ | 90$^{a}$ | 83$^{a}$ |
| Delay + Fall | 90$^{a}$ | 95$^{a}$ | 84$^{a}$ | 76$^{a}$ | 45-d | 93$^{a}$ | 94$^{a}$ | 87$^{a}$ |
| *SE* | *0.08* | *0.11* | *0.14* | *0.12* | *SE* | *0.08* | *0.14* | *0.15* |
| | Neutral detergent fiber digestibility | | | | | Neutral detergent fiber digestibility | | |
| Standard | 436$^{a}$ | 396$^{ab}$ | 476$^{a}$ | 454$^{a}$ | 30-d | 362$^{a}$ | 389$^{a}$ | 373$^{a}$ |
| Standard + Fall | 440$^{a}$ | 424$^{a}$ | 470$^{a}$ | 451$^{a}$ | 35-d | 322$^{b}$ | 367$^{a}$ | 347$^{b}$ |
| Standard + Delay | 414$^{b}$ | 380$^{b}$ | 440$^{b}$ | 418$^{b}$ | 40-d | 257$^{c}$ | 242$^{b}$ | 262$^{c}$ |
| Delay + Fall | 357$^{c}$ | 267$^{c}$ | 355$^{c}$ | 385$^{c}$ | 45-d | 241$^{c}$ | 241$^{b}$ | 275$^{c}$ |
| *SE* | *0.68* | *0.72* | *0.78* | *0.54* | *SE* | *0.69* | *1.04* | *0.81* |

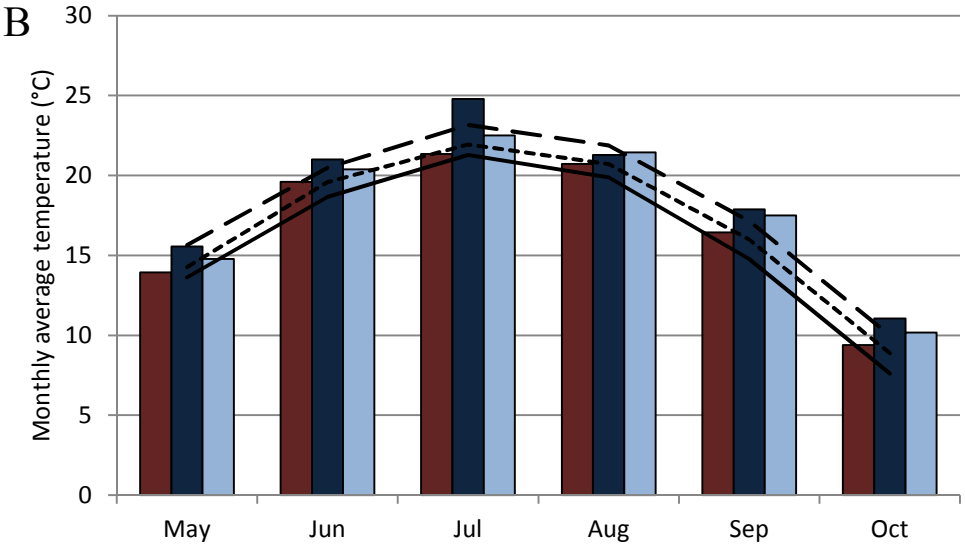
[†]2015 cutting treatments included ‘Standard’ (60d + 30d + 30d), ‘Standard + Fall’ (60d + 30d + 30d + Fall), ‘Standard + Delay’ (60d + 37d + 37d), and ‘Delay + Fall’ (67d + 45d + Fall)

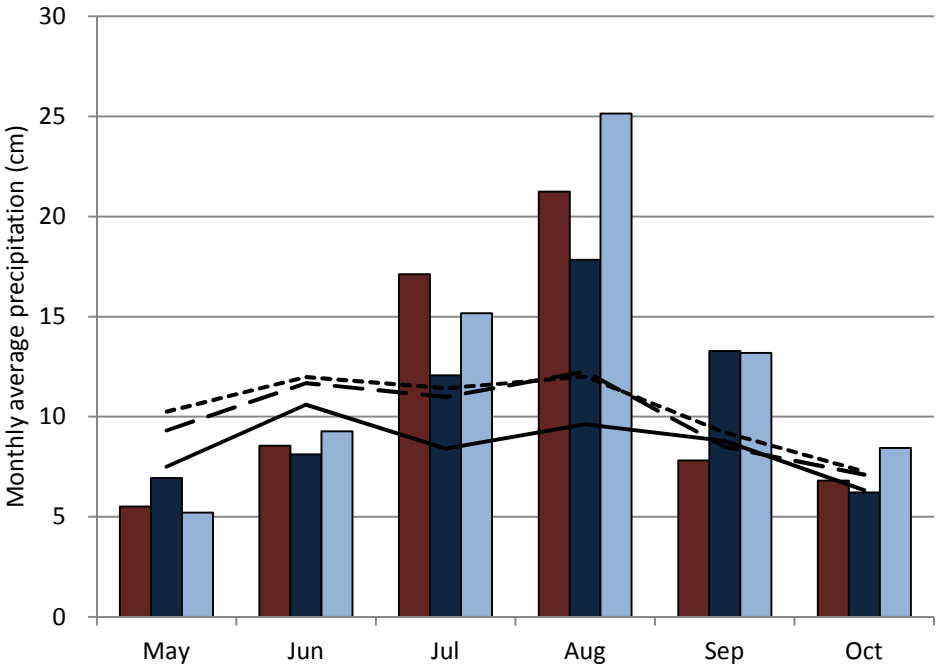
[ab]Within column and section, means without a common letter differ based on a Tukey’s HSD test ($P \leq 0.05$)

Figure 2.1A-B. Monthly air temperature (°C), precipitation (cm), and 30-year historical average for Becker, Rochester, Rosemount, and St. Paul, MN during the 2015 (A) and 2016 (B) growing season. Weather data was obtained from http://mrcc.isws.illinois.edu/.



B


Monthly average temperature (°C)
30
25
20
15
10
5
0
May
Jun
Jul
Aug
Sep
Oct


Monthly average precipitation (cm)
30
25
20
15
10
5
0
May
Jun
Jul
Aug
Sep
Oct

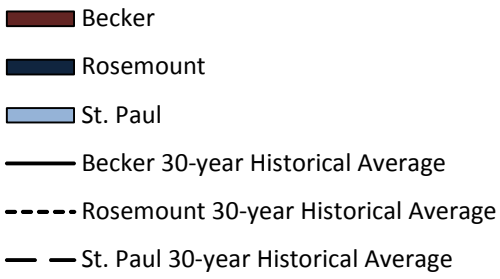

Becker
Rosemount
St. Paul
Becker 30-year Historical Average
Rosemount 30-year Historical Average
St. Paul 30-year Historical Average

Figure 2.2. Predicted alfalfa forage dry matter mass and 95% confidence intervals (shaded area) for reduced lignin 54HVX41 ($y_1$) and reference alfalfa cultivars ($y_2$) in response to average cumulative growing degree days. Vertical dashed lines correspond to treatment prescribed cutting intervals and their direct relationship to cumulative growing degree days during the 2016 growing season.

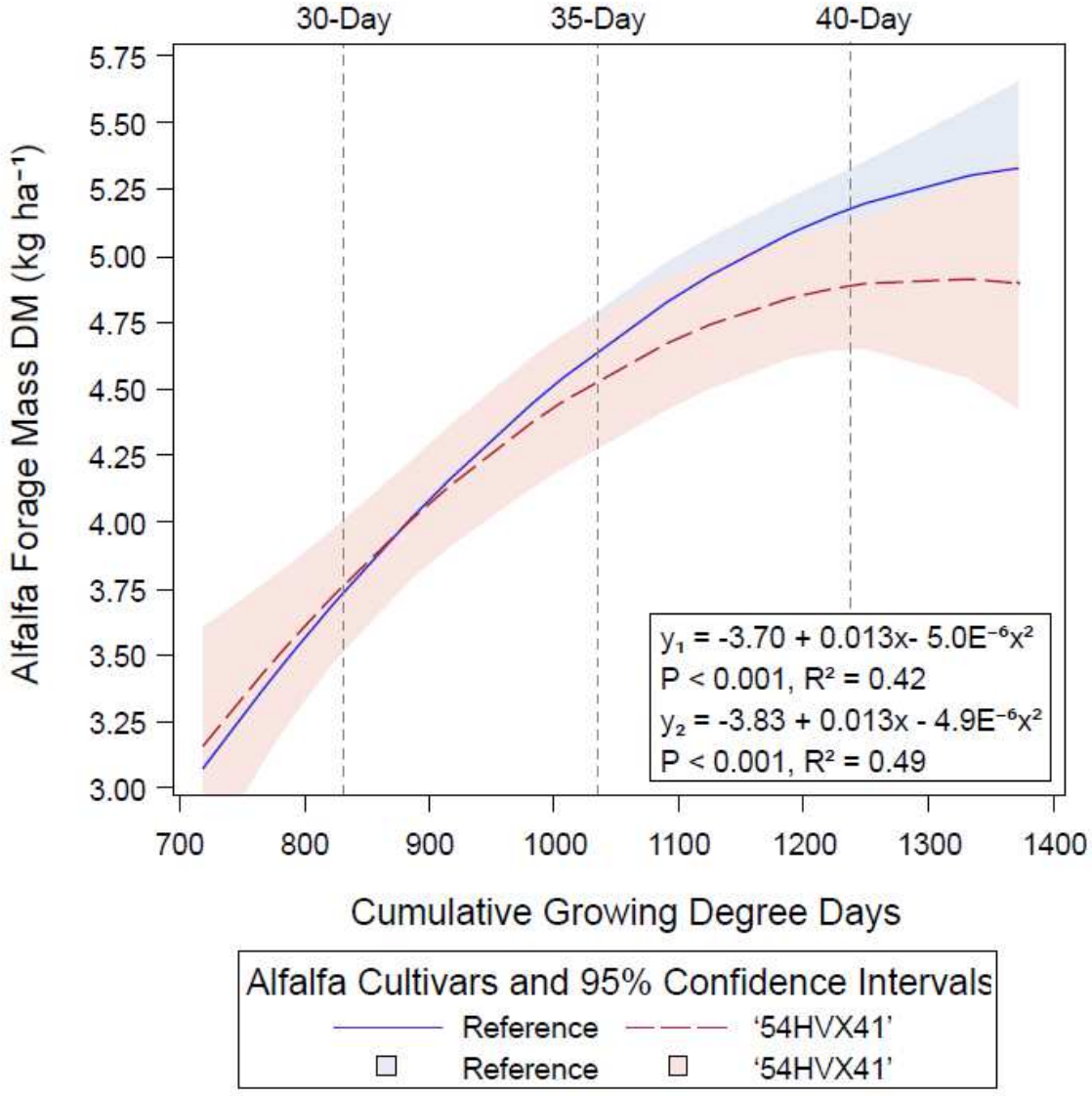
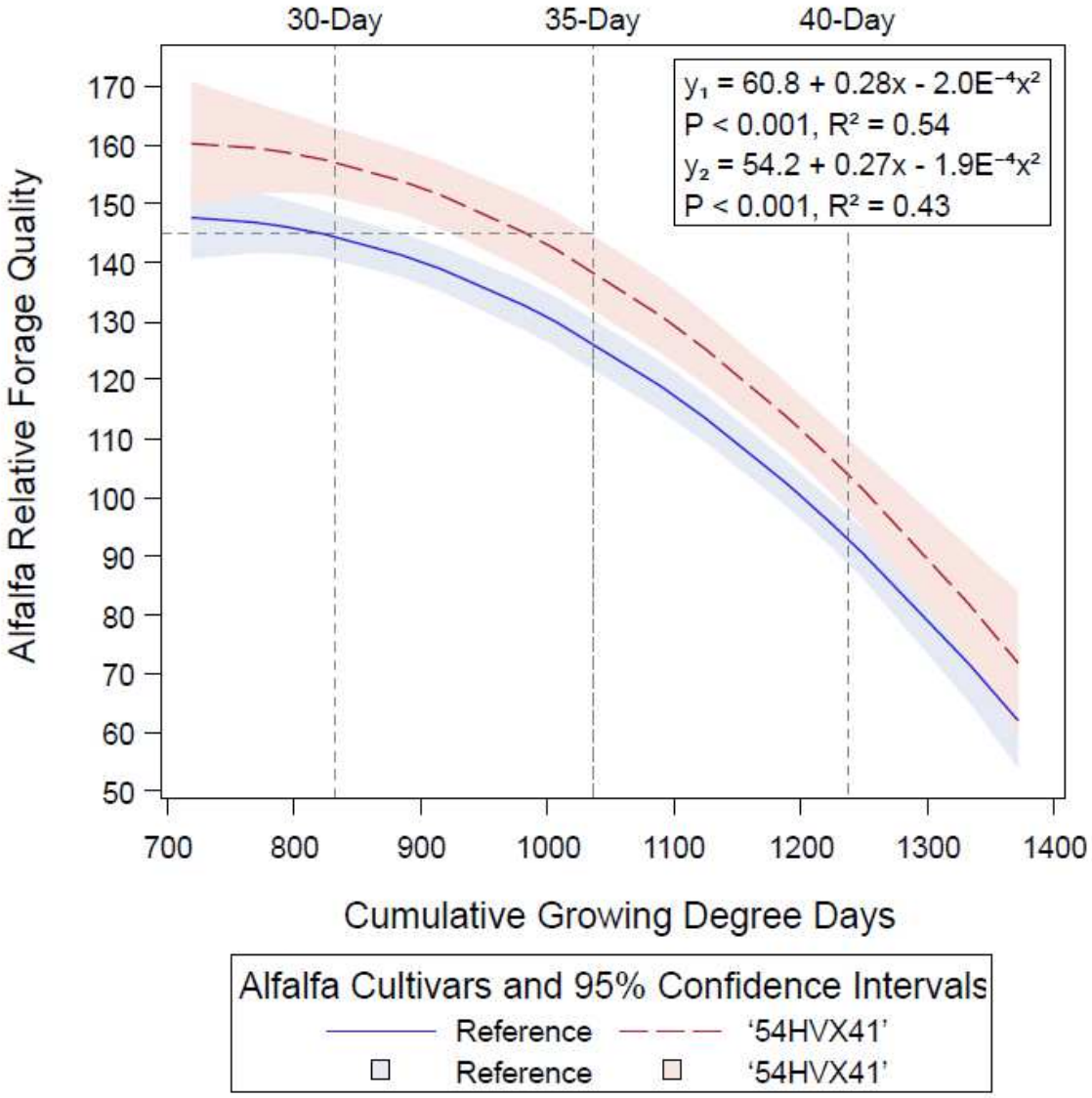


Figure 2.3. Predicted alfalfa relative forage quality and 95% confidence intervals (shaded area) for reduced lignin 54HVX41 ($y_1$) and reference alfalfa cultivars ($y_2$) in response to average cumulative growing degree days. Vertical dashed lines correspond to treatment prescribed cutting intervals and their direct relationship to cumulative growing degree days during the 2016 growing season. Horizontal dashed lines correspond to the RFQ for reference alfalfa cultivars harvested under a 30-d cutting interval.



## LITERATURE CITED


Albrecht, K.A., W.F. Wedin, and D.R. Buxton. 1987. Cell-wall composition and digestibility of alfalfa stems and leaves. Crop Sci. 27(4): 735–741. doi: 10.2135/cropsci1987.0011183X002700040027x.

AOAC 990.03. 2010. Protein (Crude) in Animal Feed: Combustion Method. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD.

Baucher, M., M.A. Bernard-vailhé, B. Chabbert, J.-M. Besle, C. Opsomer, et al. 1999. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (Medicago sativa L.) and the effect on lignin composition and digestibility. Plant Mol. Biol. 39(3): 437–447. doi: 10.1023/A:1006182925584.

Brink, G., M. Hall, G. Shewmaker, D. Undersander, N. Martin, et al. 2010. Changes in alfalfa yield and nutritive value within individual harvest periods. Agron. J. 102(4): 1274–1282. doi: 10.2134/agronj2010.0080.

Brink, G.E., and G.C. Marten. 1989. Harvest management of alfalfa—Nutrient yield vs. forage quality, and relationship to persistence. J. Prod. Agric. 2(1): 32–36. doi: 10.2134/jpa1989.0032.

Burnham, K.P., and D.R. Anderson. 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. Springer Science & Business Media, New York, NY.

Buxton, D.R., and J.S. Hornstein. 1986. Cell-wall concentration and components in stratified canopies of alfalfa, birdsfoot trefoil, and red clover. Crop Sci. 26(1): 180–184. doi: 10.2135/cropsci1986.0011183X002600010043x.

Cancelado, R.E., and E.B. Radcliffe. 1979. Action Thresholds for Potato Leafhopper on Potatoes in Minnesota. J. Econ. Entomol. 72(4): 566–569. doi: 10.1093/jee/72.4.566.

Casler, M.D. 1986. Causal effects among forage yield and quality measures of smooth bromegrass. Can. J. Plant Sci. 66(3): 591–600. doi: 10.4141/cjps86-079.

Casler, M.D. 1987. In vitro digestibility of dry matter and cell wall constituents of smooth bromegrass forage. Crop Sci. 27(5): 931–934. doi: 10.2135/cropsci1987.0011183X002700050021x.

Casler, M.D., D.R. Buxton, and K.P. Vogel. 2002. Genetic modification of lignin concentration affects fitness of perennial herbaceous plants. Theor. Appl. Genet. 104(1): 127–131. doi: 10.1007/s001220200015.

Casler, M.D., and K.P. Vogel. 1999. Accomplishments and impact from breeding for increased forage nutritional value. Crop Sci. 39(1): 12–20. doi: 10.2135/cropsci1999.0011183X003900010003x.

Chasen, E.M., D.J. Undersander, and E.M. Cullen. 2015. Revisiting the economic injury level and economic threshold model for potato leafhopper (Hemiptera: Cicadellidae) in alfalfa. J. Econ. Entomol. 108(4): 1748–1756. doi: 10.1093/jee/tov120.

Getachew, G., A.M. Ibáñez, W. Pittroff, A.M. Dandekar, M. McCaslin, et al. 2011. A comparative study between lignin down regulated alfalfa lines and their respective unmodified controls on the nutritional characteristics of hay. Anim. Feed Sci. Technol. 170(3–4): 192–200. doi: 10.1016/j.anifeedsci.2011.09.009.

Goering, H.K., and P.J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, prcedures, and some applications). USDA Agr Handb. http://agris.fao.org/agris-search/search.do?recordID=US201301156229 (accessed 26 April 2017).

Guo, D., F. Chen, K. Inoue, J.W. Blount, and R.A. Dixon. 2001a. Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: Impacts on lignin structure and implications for the biosynthesis of G and S lignin. Plant Cell 13(1): 73–88. doi: 10.2307/3871154.

Guo, D., F. Chen, J. Wheeler, J. Winder, S. Selman, et al. 2001b. Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. Transgenic Res. 10(5): 457–464. doi: 10.1023/A:1012278106147.

Hall, M.H., W.S. Smiles, and R.A. Dickerson. 2000. Morphological development of alfalfa cultivars selected for higher quality. Agron. J. 92(6): 1077–1080. doi: 10.2134/agronj2000.9261077x.

Hoffman, P.C., S.J. Sievert, R.D. Shaver, D.A. Welch, and D.K. Combs. 1993. In Situ Dry Matter, Protein, and Fiber Degradation of Perennial Forages. J. Dairy Sci. 76(9): 2632–2643. doi: 10.3168/jds.S0022-0302(93)77599-2.

Inoue, K., V.J.H. Sewalt, G.M. Ballance, W. Ni, C. Stürzer, et al. 1998. Developmental expression and substrate specificities of alfalfa caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase in relation to lignification. Plant Physiol. 117(3): 761–770. doi: 10.1104/pp.117.3.761.

Jung, H.G., D.R. Mertens, and A.J. Payne. 1997a. Correlation of acid detergent lignin and klason lignin with digestibility of forage dry matter and neutral detergent fiber. J. Dairy Sci. 80(8): 1622–1628. doi: 10.3168/jds.S0022-0302(97)76093-4.

Jung, H.-J.G., D.A. Samac, and G. Sarath. 2012. Modifying crops to increase cell wall digestibility. Plant Sci. 185–186: 65–77. doi: 10.1016/j.plantsci.2011.10.014.

Jung, H.G., C.C. Sheaffer, D.K. Barnes, and J.L. Halgerson. 1997b. Forage quality variation in the U.S. alfalfa core collection. Crop Sci. 37(4): 1361–1366. doi: 10.2135/cropsci1997.0011183X003700040054x.

Kaiser, D.E., J.A. Lamb, and R. Eliason. 2011. Fertilizer Recommendations for Agronomic Crops in Minnesota: Alfalfa. https://www.extension.umn.edu/agriculture/nutrient-management/nutrient-lime-guidelines/fertilizer-recommendations-for-agronomic-crops-in-minnesota/alfalfa/ (accessed 13 March 2017).

Kallenbach, R.L., C.J. Nelson, and J.H. Coutts. 2002. Yield, quality, and persistence of grazing- and hay-type alfalfa under three harvest frequencies. Agron. J. 94(5): 1094–1103. doi: 10.2134/agronj2002.1094.

Kalu, B.A., and G.W. Fick. 1981. Quantifying morphological development of alfalfa for studies of herbage quality. Crop Sci. 21(2): 267–271. doi: 10.2135/cropsci1981.0011183X002100020016x.

Kalu, B.A., and G.W. Fick. 1983. Morphological stage of development as a predictor of alfalfa herbage quality. Crop Sci. 23(6): 1167–1172. doi: 10.2135/cropsci1983.0011183X002300060033x.

Li, Z., Z. Li, D. Combs, and D. Undersander. 2015a. Effect of reduced lignin alfalfa on forage quality at three harvest intervals. Journal of Animal Science/Journal of Dairy Science, Orlando, FL. p. 675

Li, X., Y. Zhang, A. Hannoufa, and P. Yu. 2015b. Transformation with TT8 and HB12 RNAi constructs in model forage (Medicago sativa, alfalfa) affects carbohydrate structure and metabolic characteristics in ruminant livestock systems. J. Agric. Food Chem. 63(43): 9590–9600. doi: 10.1021/acs.jafc.5b03717.

Liu, N., and P. Yu. 2011. Molecular clustering, interrelationships and carbohydrate conformation in hull and seeds among barley cultivars. J. Cereal Sci. 53(3): 379–383. doi: 10.1016/j.jcs.2011.02.011.

Marita, J.M., J. Ralph, R.D. Hatfield, D. Guo, F. Chen, et al. 2003. Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase. Phytochemistry 62(1): 53–65. doi: 10.1016/S0031-9422(02)00434-X.

Mertens, D.R., and M. McCaslin. 2008. Evaluation of alfalfa hays with down-regulated lignin biosynthesis. Journal of Dairy Science. p. 170

Min, D. 2016. Effects of cutting interval between harvests on dry matter yield and nutritive value in alfalfa. Am. J. Plant Sci. 07(08): 1226–1231. doi: 10.4236/ajps.2016.78118.

Moore, J.E., and D.J. Undersander. 2002. Relative forage quality: An alternative to relative feed value and quality index. Proceedings of the 13th Annual Florida Ruminant Nutrition Symposium. University of Florida Extension, Gainesville, Florida. p. 16–32

Morrison, I.M. 1979. Carbohydrate chemistry and rumen digestion. Proc. Nutr. Soc. 38(03): 269–274. doi: 10.1079/PNS19790048.

Mowat, D.N., R.S. Fulkerson, W.E. Tossell, and J.E. Winch. 1965. The in vitro digestibility and protein content of leaf and stem portions of forages. Can. J. Plant Sci. 45(4): 321–331. doi: 10.4141/cjps65-065.

Nordkvist, E., and P. Åman. 1986. Changes during growth in anatomical and chemical composition and in-vitro degradability of lucerne. J. Sci. Food Agric. 37(1): 1–7. doi: 10.1002/jsfa.2740370102.

Oba, M., and M.S. Allen. 1999. Evaluation of the importance of the digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. J. Dairy Sci. 82(3): 589–596. doi: 10.3168/jds.S0022-0302(99)75271-9.

Palmonari, A., M. Fustini, G. Canestrari, E. Grilli, and A. Formigoni. 2014. Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content. J. Dairy Sci. 97(12): 7729–7734. doi: 10.3168/jds.2014-8123.

Probst, A., and R. Smith. 2011. Harvest frequency effects on yield, persistence, and regrowth rate among new alfalfa cultivars. Forage Grazinglands 9(1): 1–8. doi: 10.1094/FG-2011-0926-01-RS.

Putnam, D.H., S. Orloff, and L.R. Teuber. 2005. Strategies for balancing quality and yield using cutting schedules and varieties. Proceedings of the 35th California Alfalfa & Forage Symposium. University of California Cooperative Extension, Visalia, California. p. 237–252

Reddy, M.S.S., F. Chen, G. Shadle, L. Jackson, H. Aljoe, et al. 2005. Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (Medicago sativa L.). Proc. Natl. Acad. Sci. U. S. A. 102(46): 16573–16578. doi: 10.1073/pnas.0505749102.

Sanderson, M.A., and W.F. Wedin. 1988. Cell wall composition of alfalfa stems at similar morphological stages and chronological age during spring growth and summer regrowth. Crop Sci. 28(2): 342–347. doi: 10.2135/cropsci1988.0011183X002800020034x.

Sewalt, V.J.H., W. Ni, H.G. Jung, and R.A. Dixon. 1997. Lignin impact on fiber degradation: Increased enzymatic digestibility of genetically engineered tobacco (Nicotiana tabacum) stems reduced in lignin content. J. Agric. Food Chem. 45(5): 1977–1983. doi: 10.1021/jf9609690.

Sheaffer, C.C., G.D. Lacefield, and V.L. Marble. 1988. Cutting schedules and stands. Alfalfa and Alfalfa Improvement. ASA, CSSA, and SSSA, Madison, WI. p. 411–437

Sheaffer, C.C., and G.C. Marten. 1990. Alfalfa cutting frequency and date of fall cutting. J. Prod. Agric. 3(4): 486–491. doi: 10.2134/jpa1990.0486.

Sheaffer, C.C., N.P. Martin, J.F.S. Lamb, G.R. Cuomo, J.G. Jewett, et al. 2000. Leaf and stem properties of alfalfa entries. Agron. J. 92(4): 733–739. doi: 10.2134/agronj2000.924733x.

Sheaffer, C.C., M.S. Wells, and J. Larson. 2016. Alfalfa Field Crop Trials. Minn. Agric. Exp. Stn. https://www.maes.umn.edu/publications/field-crop-trials/alfalfa (accessed 3 March 2017).

Smith, S.R., J.H. Bouton, and C.S. Hoveland. 1989. Alfalfa Persistence and Regrowth Potential under Continuous Grazing. Agron. J. 81(6): 960–965. doi: 10.2134/agronj1989.00021962008100060023x.

Undersander, D. 2016. 2016 Alfalfa Trial Results. 2016 Alfalfa Trial Results. http://fyi.uwex.edu/forage/alfalfa-trial-results-2016/ (accessed 5 April 2017).

Undersander, D., D. Cosgrove, E. Cullen, C. Grau, M. Rice, et al. 2011. Alfalfa Management Guide. https://www.agronomy.org/files/publications/alfalfa-management-guide.pdf (accessed 23 February 2017).

Undersander, D., M. McCaslin, C. Sheaffer, D. Whalen, D. Miller, et al. 2009. Low lignin alfalfa: Redefining the yield/quality tradeoff. UC Cooperative Extension, Reno, Nevada. p. 1–4

Van Soest, P.J., J.B. Robertson, and B.A. Lewis. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. J. Dairy Sci. 74(10): 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2.

Vogel, K.P., and R.A. Masters. 2001. Frequency grid: A simple tool for measuring grassland establishment. J. Range Manag. 54(6): 653–655. doi: 10.2307/4003666.

Weakley, D., D.R. Mertens, and M. McCaslin. 2008. Lactating cow responses to alfalfa hays with down-regulated lignin biosynthesis. Journal of Dairy Science. p. 170

Weir, W.C., L.G. Jones, and J.H. Meyer. 1960. Effect of cutting interval and stage of maturity on the digestibility and yield of alfalfa. J. Anim. Sci. 19(1): 5–19. doi: 10.2527/jas1960.1915.

Wells, M.S., K.L. Martinson, D.J. Undersander, and C.C. Sheaffer. 2014. A survey investigating alfalfa winter injury in Minnesota and Wisconsin from the winter of 2012-2013. Forage Grazinglands 12(1): 1–7. doi: 10.2134/FG-2013-0051-RS.

Yu, P., D.A. Christensen, J.J. McKinnon, and J.D. Markert. 2003. Effect of variety and maturity stage on chemical composition, carbohydrate and protein subfractions, in

vitro rumen degradability and energy values of timothy and alfalfa. Can. J. Anim. Sci. 83(2): 279–290. doi: 10.4141/A02-053.

# CHAPTER 3

## Morphology and Stem and Leaf Forage Nutritive Value of Reduced Lignin Alfalfa

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### CHAPTER SUMMARY

Reduced lignin alfalfa (*Medicago sativa* L.) has the potential to improve alfalfa forage quality, yet forage morphology, biomass allocation, and stem and leaf nutritive value remains undetermined. The objectives were to characterize changes in morphological development and forage nutritive value within stem and leaf fractions for reduced lignin alfalfa. Reduced lignin (54HVX41) and reference (DKA43-22RR) alfalfa cultivars were sampled weekly from the mid-vegetative stage through full flower during the seeding (2015) and first production (2016) year at two locations in Minnesota. Samples were staged to determine maturity, divided into stem and leaf fractions, weighed, and analyzed for forage nutritive value. Alfalfa cultivars were similar in stem and leaf yield, leaf to stem ratio (L:S), leaf forage nutritive value, stem crude protein

(CP), and stem neutral detergent fiber (NDF). On average, 54HVX41 showed a 13 to 14% decrease in stem acid detergent lignin (ADL) and an 8 to 15% increase in stem NDF digestibility (NDFD) compared to DKA43-22RR. For both cultivars, increases in forage maturity resulted in increasing stem and leaf yields and decreasing L:S. Forage maturity affected both stem and leaf forage nutritive value; as maturity increased, NDF and ADL increased while CP and NDFD decreased. These results indicate that changes in forage nutritive value for reduced lignin alfalfa are largely a result of changes in ADL and NDFD within the stem fraction of the plant, and that forage nutritive value within stem and leaf fractions is affected by forage maturity, particularly within the stem portion of the plant.

## INTRODUCTION

The maturity stage at which alfalfa is harvested is often one of the most critical factors affecting agronomic characteristics such as yield and forage nutritive value (Kallenbach et al., 2002; Lamb et al., 2007, 2012; Brink et al., 2010; Probst and Smith, 2011). In the North Central region, producers typically initiate harvest when alfalfa maturity reaches the bud to early flowering stage (Sheaffer et al., 2000). These harvest maturity choices are determined by producer needs to optimize either herbage yield, nutritive value, or nutrient yield (Sheaffer et al., 2000). It has been well established that increasing forage maturity results in increasing fiber, declining CP, and decreasing digestibility within alfalfa herbage (Albrecht et al., 1987; Sanderson and Wedin, 1988; Sanderson et al., 1989; Griffin et al., 1994; Hall et al., 2000; Kallenbach et al., 2002; Lamb et al., 2007, 2012; Brink et al., 2010; Palmonari et al., 2014; Grev et al., 2017).

Therefore, highest herbage nutritive value and intake potential usually occur with pre-flowering alfalfa, while alfalfa harvested at later maturity stages has been shown to have a lower nutritive value (Sheaffer et al., 1988, 2000; Kallenbach et al., 2002; Lamb et al., 2007, 2012; Brink et al., 2010).

The decline in the quality of alfalfa herbage with advancing maturity can be attributed to a decrease in leaf and increase in stem proportion as the plant matures (Buxton et al., 1985; Albrecht et al., 1987; Sanderson and Wedin, 1988; Julier and Huyghe, 1997; Sheaffer et al., 2000; Lamb et al., 2003, 2012; Milić et al., 2011; Yari et al., 2012, 2014). Increasing forage maturity and canopy height results in defoliation through leaf senescence and abscission from the lower portions of the plant due to shading and disease (Buxton et al., 1985; Albrecht et al., 1987; Sheaffer et al., 1988). This increase in leaf loss is coupled with increases in stem growth, resulting in an increased contribution of the stem to the total herbage amount and a decreased leaf to stem ratio.

Shifts in the proportions of leaf and stem material result in significant changes in herbage quality largely because of the differences in forage nutritive value within the stem and leaf fractions of the plant. Alfalfa leaves are protein-rich and low in cell wall concentration, and therefore have a high nutritive value and are highly digestible; in contrast, alfalfa stems exhibit low digestibility as a result of high concentrations of cell wall polysaccharides and lignin (Buxton et al., 1985; Buxton and Hornstein, 1986; Albrecht et al., 1987; Buxton and Russell, 1988; Julier and Huyghe, 1997; Milić et al., 2011; Marković et al., 2012; Yari et al., 2012; Lamb et al., 2012). As maturity advances, herbage digestibility declines and cell wall concentrations increase at much slower rates

in leaves compared to stems (Kilcher and Heinrichs, 1974; Buxton et al., 1985; Buxton and Hornstein, 1986; Albrecht et al., 1987; Sheaffer et al., 2000; Marković et al., 2012). Therefore, because stems not only increase in proportion but also decrease in digestibility faster with advancing maturity compared to leaves, they exert a larger influence and have a greater detrimental impact on total herbage quality compared to leaves (Buxton et al., 1985; Buxton and Hornstein, 1986; Sanderson et al., 1989; Sheaffer et al., 2000; Jung and Lamb, 2006; Lamb et al., 2012).

Major improvements in the digestibility and quality of alfalfa may be possible if plants can be selected that exhibit a slower decline in loss of digestibility, particularly within the stem portion of the plant (Buxton et al., 1985; Sanderson et al., 1989; Jung and Lamb, 2006; Lamb et al., 2014). Poor stem digestibility can result in major losses in feeding value, not only because of the greater impact stems have on forage quality compared to leaves, but also because alfalfa stems typically represent 45 to 70% of the total forage biomass (Sheaffer et al., 2000; Lamb et al., 2003). One strategy for improving whole plant and stem digestibility is to alter the quantity and/or composition of lignin within the plant (Baucher et al., 1999; Guo et al., 2001a). Lignification has been reported to be the major factor limiting the *in vitro* dry matter digestibility (IVDMD) of whole plant forage (Casler, 1987; Reddy et al., 2005; Jung et al., 2012), and numerous studies have reported a strong inverse relationship between forage digestibility and lignification (Buxton and Hornstein, 1986; Albrecht et al., 1987; Casler, 1987; Buxton and Russell, 1988; Sanderson et al., 1989; Jung et al., 1997; Reddy et al., 2005).

Populations of reduced lignin alfalfa are now commercially available and have potential to increase the digestibility of alfalfa forage compared to reference cultivars

(Guo et al., 2001a; b; Marita et al., 2003; Reddy et al., 2005; Getachew et al., 2011). Field research evaluating the performance of reduced lignin alfalfa under different harvest frequencies has demonstrated a reduction in total herbage ADL and an increase in NDFD and relative forage quality (RFQ) for reduced lignin alfalfa compared to reference cultivars (Grev et al., 2017; Getachew et al., 2018). However, forage nutritive value changes within individual stem and leaf fractions have yet to be evaluated. Additionally, the effects of this reduction in total herbage lignin on forage morphology and nutritive value across a range of forage maturities is unknown. Therefore, the objectives of this study were to characterize changes in morphological development and forage nutritive value within leaf and stem fractions for reduced lignin and reference alfalfa cultivars over time.

## MATERIALS AND METHODS

Research was conducted at the University of Minnesota Agricultural Experiment Stations at Becker and St. Paul, MN in 2015 and 2016. Research plots were established on 27 Apr 2015 on a Hubbard-Mosford complex (sandy, mixed, frigid Entic Hapludolls) at Becker, MN (45°23’13” N, 93°53’18” W, elevation 290 m) and on 28 Apr 2015 on a Waukegan silt loam (fine-silty over sandy or sandy-skeletal, mixed, superactive, mesic Typic Hapludolls) at St. Paul (44°59’14” N, 93°10’24” W, elevation 291 m). At each site, inoculated seed was seeded into a prepared seedbed at a rate of 18.8 kg $ha^{-1}$ in plots measuring 0.9 × 6.1 m. Soil fertility was amended to meet recommendations for alfalfa hay production according to University of Minnesota fertility guidelines (Kaiser et al., 2011).

At each site, reduced lignin ('54HVX41') and reference ('DKA43-22RR') alfalfa cultivars were established in a randomized complete block design with four replicates. Both cultivars were marketed as Round-up Ready and were rated as Fall Dormancy 4 cultivars. In the establishment year, weeds were controlled using a single application of glyphosate (N-(phosphonomethyl)glycine) applied at rate of 2.34 L a.i. $ha^{-1}$; additional weed control was not required during the first production year. Potato leafhoppers were controlled using Arctic 3.2 EC ((m-Phenoxybenzyl)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) as needed when potato leafhoppers exceeded threshold populations, which generally occurred each July (Cancelado and Radcliffe, 1979; Chasen et al., 2015).

To determine plant morphology and forage nutritive value, all plots were hand-sampled at weekly intervals for a period of five to six weeks during the summer of 2015 and the spring and summer of 2016. Within each season (summer 2015, spring 2016, and summer 2016), hand-sampling began on 15 Jul 2015, 21 Apr 2016, and 10 Jun 2016 at Becker, and on 14 Jul 2015, 21 Apr 2016, and 8 Jun 2016 at St. Paul. Each week, a 0.9 × 0.5 m section consisting of non-border rows was clipped from each plot down to a stubble height of 5 cm. Samples were staged for forage maturity using the mean stage by weight (MSW) method developed by Kalu and Fick (1981), where vegetative growth included stages 0 through 2, budding plants included stages 3 and 4, and flowering plants included stages 5 and 6. Samples were then dried in forced-air ovens for 48 h at 60°C, and dried samples were divided by hand into stem and leaf fractions and weighed for dry matter (DM) determination. Stem and leaf components for each sample were ground separately through a 6-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ)

followed by a 1-mm screen in a Cyclotec (Foss, Hillerød, Denmark). Individual samples were mixed thoroughly and scanned using near infrared reflectance spectroscopy (NIRS; Model DA 7200; Perten Instruments, Springfield, IL) with calibration equations developed in Minnesota to estimate forage nutritive value for CP, NDF, ADL, and NDFD. The standard error of cross validation was 0.98, 1.98, 1.52, and 2.64, respectively, for CP, ADL, NDF, and NDFD, while the $R^2$ was 0.98, 0.80, 0.86, and 0.87, respectively. Wet chemistry procedures were as follows: CP (N x 6.25; AOAC 990.03, 2010); NDF and ADL (Goering and Van Soest, 1970; Van Soest et al., 1991); and NDFD (Hoffman et al., 1993).

Data were analyzed as a randomized complete block design using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Response variables included stem yield, leaf yield, L:S, stem and leaf CP, stem and leaf NDF, stem and leaf ADL, and stem and leaf NDFD. Individual plots comprised the experimental unit, and statistical significance was set at $P \leq 0.05$. Analysis of the seeding year (2015) and first production year (2016) was completed separately. For all response variables, models included alfalfa maturity (MSW), alfalfa cultivar, and the MSW × cultivar interaction as fixed effects. Models were fit with MSW as a linear and quadratic predictor, and the REG procedure in SAS and the Akaike's Information Criterion (AIC) were used to compare linear and quadratic models to determine the superior fit for each response variable (Burnham and Anderson, 2002; version 9.4; SAS Institute Inc., Cary, NC). Random effects included replicate, location, and season. For categorical effects (e.g. alfalfa cultivar), means separations were performed on significant effects using Tukey's HSD test. To further assess the relationship between alfalfa maturity, forage morphology

characteristics, and forage nutritive value, Pearson correlation coefficients were calculated between MSW and all response variables using the CORR procedure of SAS.

## RESULTS AND DISCUSSION

With the exception of stem NDFD in 2015, statistical analysis for all forage morphology and forage nutritive value parameters indicated no interactions ($P > 0.05$) between MSW and alfalfa cultivar (Table 3.1). Therefore, for all response variables except for 2015 stem NDFD, the main effects of alfalfa cultivar and MSW are reported.

### Weather

Monthly mean air temperature and precipitation data were collected for each location throughout the 2015 and 2016 growing seasons (April through October; Figure 3.1). At Becker, mean daily air temperature throughout the 2015 and 2016 growing seasons was similar to the 30-yr average except for September and October, which tended to be warmer than normal. Total rainfall during the 2015 and 2016 growing seasons was 71 cm and was higher than the 30-yr average of 58 cm. Seasonal rainfall was not evenly distributed, with higher than average rainfall occurring in May 2015, July 2015, July 2016, and August 2016.

At St. Paul, mean daily air temperature throughout the 2015 and 2016 growing seasons was similar to the 30-yr average except for May, which tended to be cooler than normal, and September, which tended to be warmer than normal. Total rainfall during the 2015 growing season was 66 cm and was similar to the 30-yr average of 67 cm. Total rainfall during the 2016 growing season was 86 cm and was higher than the 30-yr average of 67 cm. Again, seasonal rainfall was not evenly distributed, with higher than

average rainfall occurring in July 2015, July 2016, August 2016, and September 2016 and lower than average rainfall occurring in August 2015 and May 2016.

## Forage Morphology

**Cultivar Response.** During the seeding year, alfalfa cultivars did not differ in stem yield; however, leaf yields were greater for 54HVX41 (161 g $m^{-2}$) compared to DKA43-22RR (143 g $m^{-2}$; Table 3.2). Alfalfa cultivars had similar stem and leaf yield during the first production year (Table 3.2). Across cultivars, stem yields averaged 151 g $m^{-2}$ during the seeding year and 164 g $m^{-2}$ during the first production year, while leaf yields averaged 152 g $m^{-2}$ during the seeding year and 147 g $m^{-2}$ during the first production year. These results are comparable to previously reported alfalfa stem and leaf yields (Kilcher and Heinrichs, 1974; Sheaffer et al., 2000; Lamb et al., 2003). To the authors' knowledge, this is the first study comparing stem and leaf yields between reduced lignin and reference alfalfa cultivars. Although leaf yields were greater for 54HVX41 during the seeding year, the lack of differences in both stem and leaf yield between cultivars during the first production year indicates that morphological differences between cultivars are likely minimal following establishment. These findings agree with previous research which found minimal differences in total herbage yield between reduced lignin and reference alfalfa cultivars following establishment (Grev et al., 2017; Getachew et al., 2018).

Alfalfa cultivars did not differ in L:S in either the seeding or first production year (Table 3.2). Across cultivars, L:S averaged 1.08 during the seeding year and 1.01 during the first production year and is comparable to previously reported alfalfa L:S values (Buxton et al., 1985; Albrecht et al., 1987; Sanderson and Wedin, 1988; Julier and

Huyghe, 1997; Sheaffer et al., 2000; Yari et al., 2012, 2014). In Iowa, Sanderson and Wedin (1988) reported an average L:S of 1.04 for alfalfa grown across multiple seasons and years, and Sheaffer et al. (2000) reported an average L:S of 0.96 across six alfalfa cultivars grown at three locations in Minnesota. The lack of differences between reduced lignin and reference alfalfa cultivars in stem yield, leaf yield, and L:S in the present study supports the idea that forage morphology and growth patterns between these cultivars are similar.

**Maturity Response.** During both the seeding and first production year, stem and leaf yields increased with increasing forage maturity (Table 3.3). This increase in stem and leaf yield with increasing maturity was expected, as it has been well documented that increasing the interval between alfalfa harvests (and subsequently, increasing forage maturity) results in greater forage biomass accumulation (Kallenbach et al., 2002; Probst and Smith, 2011; Min, 2016).

Although both stem and leaf yields increased with increasing forage maturity, yield increases for leaf material occurred to a lesser extent than stem material. This is likely due to the fact that initial increases in leaf yield were often followed by a plateau or decrease in leaf yield at the later maturity stages. Kilcher and Heinrichs (1974) reported similar findings, noting that while stem yields continued to increase throughout an 8-week growing period, leaf yield increased until flowering but showed no appreciable increases after that. These results also agree with previous work demonstrating that a decline in leaf yield at a later harvest maturity stage is offset by a gain in stem yields (Luckett and Klopfenstein, 1970; Fick and Holthausen, 1975; Sheaffer et al., 2000; Lamb et al., 2003). This plateau or decline in leaf material at greater forage maturities is likely

a result of leaf senescence and abscission occurring within the lower portions of the canopy due to shading or disease (Albrecht et al., 1987; Sheaffer et al., 1988; Lamb et al., 2003; Undersander et al., 2011).

Changes in weekly stem and leaf yields resulted in a declining L:S with increasing forage maturity during both the seeding and first production year (Table 3.3). Leaf to stem ratios observed in the present study are comparable to previous reports (Buxton et al., 1985; Albrecht et al., 1987; Sanderson and Wedin, 1988; Sheaffer et al., 2000; Yari et al., 2012, 2014). As alfalfa matured from the vegetative to the early pod stage, Albrecht et al. (1987) and Sanderson and Wedin (1988) reported decreases in L:S from 1.45 to 0.50 and from 3.16 to 0.39, respectively, across two seasons in Iowa. Similarly, average L:S in Minnesota decreased from 1.15 to 0.66 for alfalfa harvested at mid-bud versus late flower, respectively (Sheaffer et al., 2000). This decline in L:S with increasing forage maturity has been well documented and can be attributed to the associated effects of increasing stem proportions and decreasing leaf proportions as the plant matures (Luckett and Klopfenstein, 1970; Kilcher and Heinrichs, 1974; Buxton et al., 1985; Julier and Huyghe, 1997; Wilman and Moghaddam, 1998; Yari et al., 2012, 2014).

## Forage Nutritive Value

### *Stem and Leaf Crude Protein*

**Cultivar Response.** Seeding year stem and leaf CP concentrations were similar for alfalfa cultivars, averaging 145 and 329 g $kg^{-1}$, respectively, across cultivars (Table 3.2). During the first production year, alfalfa cultivars were similar in stem CP (average 182 g $kg^{-1}$), but leaf CP was greater for DKA43-22RR (338 g $kg^{-1}$) compared to

54HVX41 (331 g $kg^{-1}$; Table 3.2). Crude protein concentrations for both cultivars were within normal ranges and are comparable to previously reported values for alfalfa stem and leaf CP content (Kalu and Fick, 1983; Buxton et al., 1985; Juan et al., 1993; Sheaffer et al., 2000; Lamb et al., 2007; Schnurr et al., 2007; Milić et al., 2011; Marković et al., 2012). Although this is the first report of stem and leaf CP content for reduced lignin alfalfa, previous research comparing total herbage CP concentrations between reduced lignin and reference alfalfa cultivars has also reported similar CP content between cultivars (Getachew et al., 2011, 2018; Grev et al., 2017).

**Maturity Response.** Stem and leaf CP concentrations decreased with increasing forage maturity, although leaf CP concentrations were greater than stem CP across all maturities (Table 3.3). The higher stem CP concentrations for the initial sampling dates during the first production year are reflective of the sampling that occurred during the spring season. Previous research has shown that seasonal changes can significantly affect alfalfa forage nutritive value, with spring growth producing a higher quality herbage compared to summer growth, even when harvested at the same maturity or chronological age (Griffin et al., 1994; Sheaffer et al., 1998; Hall et al., 2000; Tremblay et al., 2002; Brink et al., 2010).

Stem and leaf CP concentrations were negatively associated with MSW. Correlation coefficients for stem CP were -0.83 and -0.88 during the seeding and first production year, respectively. Correlation coefficients for leaf CP were -0.70 and -0.86 during the seeding and first production year, respectively. This decrease in stem and leaf CP with increasing forage maturity was expected, and agrees with findings from previous studies evaluating CP content within alfalfa stem and leaf fractions across various forage

maturities (Kalu and Fick, 1983; Buxton et al., 1985; Juan et al., 1993; Sheaffer et al., 2000; Lamb et al., 2007; Schnurr et al., 2007; Marković et al., 2012). Lamb et al. (2007) reported higher leaf CP concentrations when alfalfa was harvested at early bud compared to green pod, and Marković et al. (2012) reported leaf CP declining from 351 to 283 g $kg^{-1}$ and stem CP declining from 148 to 138 g $kg^{-1}$ when alfalfa was harvested across maturities ranging from bud to full bloom. In the present study, although CP concentrations declined within both stem and leaf fractions, the extent of this decrease was greater within the stem portion of the plant. This is also consistent with previous studies, as several other researchers have reported that stem CP concentrations decline at a faster rate with increasing forage maturity compared to leaves (Kilcher and Heinrichs, 1974; Fick and Holthausen, 1975; Kalu and Fick, 1983; Sheaffer et al., 2000).

### *Stem and Leaf Neutral Detergent Fiber*

**Cultivar Response.** Stem and leaf NDF concentrations did not differ between alfalfa cultivars in either the seeding or first production year (Table 3.2). Across cultivars, seeding year stem and leaf NDF concentrations averaged 479 and 171 g $kg^{-1}$, respectively. First production year stem and leaf NDF concentrations averaged 410 and 189 g $kg^{-1}$, respectively. These results are comparable to reports from previous research evaluating alfalfa stem and leaf NDF concentrations across a number of cultivars and locations (Kalu and Fick, 1983; Sanderson and Wedin, 1988; Juan et al., 1993; Sheaffer et al., 2000; Lamb et al., 2007, 2012; Schnurr et al., 2007; Milić et al., 2011; Marković et al., 2012). No prior studies have investigated NDF concentrations within stem and leaf fractions for reduced lignin alfalfa cultivars; however, total herbage NDF concentrations

have been shown to be similar between reduced lignin and reference alfalfa cultivars (Getachew et al., 2011, 2018; Grev et al., 2017).

**Maturity Response.** Concentrations of NDF within alfalfa stems and leaves increased with increasing forage maturation, although leaf NDF concentrations were lower than stem NDF across all maturity stages (Table 3.3). The lower stem NDF concentrations for the initial sampling dates during the first production year are reflective of the sampling that occurred during the spring season. Previous research has shown that seasonal changes can affect alfalfa forage nutritive value, with spring growth producing a higher quality herbage compared to summer growth, even when harvested at the same maturity or chronological age (Griffin et al., 1994; Sheaffer et al., 1998; Hall et al., 2000; Tremblay et al., 2002; Brink et al., 2010).

Stem and leaf NDF concentrations were positively associated with MSW. Correlation coefficients for stem NDF were 0.75 and 0.90 during the seeding and first production year, respectively. Correlation coefficients for leaf NDF were 0.72 and 0.80 during the seeding and first production year, respectively. Increases in NDF concentration within stem and leaf fractions with increasing forage maturity was expected, and agrees with previous research demonstrating an increase in NDF content within alfalfa stem and leaf fractions as forage maturity increased (Kalu and Fick, 1983; Sanderson and Wedin, 1988; Juan et al., 1993; Sheaffer et al., 2000; Lamb et al., 2007, 2012; Marković et al., 2012). Juan et al. (1993) reported an average of 507 g $kg^{-1}$ stem NDF and 229 g $kg^{-1}$ leaf NDF at the bud stage compared to 583 g $kg^{-1}$ stem NDF and 294 g $kg^{-1}$ leaf NDF at flowering. Similarly, Sheaffer et al. (2000) reported stem and leaf

NDF concentrations averaging 575 and 205 g $kg^{-1}$, respectively, at mid-bud compared to 664 and 229 g $kg^{-1}$ at late flower.

In the present study, although stem and leaf fractions both showed increases in NDF content with increasing forage maturity, the extent of this increase was greater within the stem portion of the plant. Several other researchers have reported similar findings, noting that herbage cell wall concentrations increased at a faster rate in stems compared to leaves (Kalu and Fick, 1983; Buxton and Hornstein, 1986; Sheaffer et al., 2000; Marković et al., 2012). This rapid accumulation in stem cell wall concentration, coupled with increasing stem proportions, demonstrates the significant impact forage maturity has on herbage nutritive value, particularly within the stem portion of the plant.

### *Stem and Leaf Acid Detergent Lignin*

**Cultivar Response.** Stem ADL concentrations differed between alfalfa cultivars during both the seeding and first production year (Table 3.2). Seeding year stem ADL averaged 67 g $kg^{-1}$ for 54HVX41 compared to 78 g $kg^{-1}$ for DKA43-22RR. First production year stem ADL averaged 48 g $kg^{-1}$ for 54HVX41 compared to 55 g $kg^{-1}$ for DKA43-22RR. Leaf ADL concentrations did not differ between alfalfa cultivars during the seeding year, but first production year leaf ADL was decreased for 54HVX41 (30 g $kg^{-1}$) compared to DKA43-22RR (31 g $kg^{-1}$; Table 3.2). Stem and leaf ADL concentrations observed in the present study are comparable to previous research evaluating ADL within alfalfa stem and leaf fractions (Kalu and Fick, 1983; Buxton and Hornstein, 1986; Albrecht et al., 1987; Buxton and Russell, 1988; Sanderson and Wedin, 1988; Jung and Lamb, 2006; Milić et al., 2011; Marković et al., 2012).

In the present study, 54HVX41 demonstrated a 13 to 14% reduction in stem ADL concentrations across the seeding and first production year. Previous studies investigating experimental lines for reduced lignin alfalfa have shown a 4 to 29% decrease in stem lignin concentration compared to control lines (Guo et al., 2001b; Marita et al., 2003; Reddy et al., 2005). Although ADL concentrations within leaf fractions for reduced lignin alfalfa cultivars have not yet been reported, reduced lignin alfalfa grown in Minnesota, Wisconsin, and Idaho has shown a 6 to 24% decrease in total herbage ADL concentrations compared to reference alfalfa cultivars (Getachew et al., 2011, 2018; Grev et al., 2017). The present study provides evidence that this reduction in total herbage lignin is primarily a result of reductions in lignin within the stem fraction of the plant, rather than a change in leaf lignin content or a greater L:S for the reduced lignin cultivar.

**Maturity Response.** Acid detergent lignin concentrations within alfalfa stems increased with increasing forage maturity (Table 3.3; Figure 3.2A-B). Similar to NDF, the lower stem ADL concentrations for the initial sampling dates during the first production year are reflective of the sampling that occurred during the spring season. Leaf ADL concentrations remained fairly constant over time and were lower than stem ADL across all maturities (Table 3.3).

Stem ADL concentrations were positively associated with MSW. Correlation coefficients for stem ADL were 0.69 and 0.75 during the seeding and first production year, respectively. The increasing stem ADL concentrations resulting from increased forage maturity were expected, as it has been well established that the proportion of lignin in alfalfa stem cell walls increases steadily with increasing forage maturation (Kalu

and Fick, 1983; Albrecht et al., 1987; Buxton and Russell, 1988; Sanderson and Wedin, 1988; Lamb et al., 2007, 2012; Marković et al., 2012). When alfalfa was harvested across increasing forage maturities, Sanderson and Wedin (1988) and Marković et al. (2012) reported increasing stem ADL concentrations ranging from 84 to 201 g $kg^{-1}$. In contrast to stem ADL, leaf ADL concentrations were positively associated with MSW only during the seeding year. Correlation coefficients for leaf ADL were 0.32 and 0.01 during the seeding and first production year, respectively. The weak association, or lack thereof, between forage maturity and leaf ADL is comparable to results from previous studies which have also found little to no change in leaf ADL concentrations over time (Kalu and Fick, 1983; Buxton and Hornstein, 1986; Albrecht et al., 1987). Increases in lignin concentration with increasing plant maturity are a result of growth within secondary plant cell walls, which provides the strength and rigidity a plant needs to remain upright (Albrecht et al., 1987; Sanderson and Wedin, 1988; Inoue et al., 1998; Guo et al., 2001a). Therefore, much of the change in herbage lignin occurs as a result of increases within the stem portion of the plant, while leaf lignin concentrations vary little over time.

### *Stem and Leaf Neutral Detergent Fiber Digestibility*

**Cultivar Response.** Stem NDFD differed between alfalfa cultivars during both the seeding and first production year (Table 3.2). Seeding year stem NDFD averaged 312 g $kg^{-1}$ for 54HVX41 compared to 271 g $kg^{-1}$ for DKA43-22RR. First production year stem NDFD averaged 443 g $kg^{-1}$ for 54HVX41 compared to 412 g $kg^{-1}$ for DKA43-22RR. Leaf NDFD did not differ between alfalfa cultivars, averaging 648 g $kg^{-1}$ during the seeding year and 641 g $kg^{-1}$ during the first production year (Table 3.2). Stem and

leaf NDFD for both alfalfa cultivars in the present study are comparable to previously reported values for alfalfa stem and leaf NDFD (Sanderson et al., 1989; Jung and Lamb, 2003, 2006; Lamb et al., 2012).

In the present study, 54HVX41 demonstrated an 8 to 15% improvement in stem NDFD across the seeding and first production year. Previous studies investigating reduced lignin alfalfa experimental lines found similar results, showing improvements in stem IVDMD (Reddy et al., 2005), stem NDFD (Guo et al., 2001b), and stem *in situ* rumen digestibility (Reddy et al., 2005) for reduced lignin alfalfa compared to control lines. To the authors' knowledge, previous research investigating reduced lignin alfalfa cultivars has not reported NDFD within the leaf fraction of the forage. However, field studies have shown improvements in total herbage IVDMD (Getachew et al., 2011, 2018) and NDFD (Grev et al., 2017; Getachew et al., 2018) for reduced lignin alfalfa compared to reference alfalfa cultivars. Increases in total herbage NDFD ranged from 8 to 18% in Minnesota (Grev et al., 2017) and 7 to 17% in Wisconsin (Getachew et al., 2018). The present study further confirms an improvement in NDFD for reduced lignin alfalfa and provides evidence that the improvement in total herbage NDFD is primarily a result of NDFD improvements within the stem fraction of the plant. Increases in stem NDFD for 54HVX41 can likely be attributed to the reduction in lignin concentration for this cultivar, as the deposition of lignin into plant cell walls can negatively affect rumen microbial degradation and the digestion of feed by intestinal enzymes (Buxton and Hornstein, 1986; Liu and Yu, 2011). These results have potential biological significance, as improvements in stem and total herbage digestibility can improve the value of alfalfa as a forage source for livestock.

**Maturity Response.** Stem and leaf NDFD decreased with increasing forage maturity (Table 3.3; Figure 3.3A-B). Similar to CP, the higher stem NDFD for the initial sampling dates during the first production year are reflective of the sampling that occurred during the spring season. Leaf NDFD also decreased with increasing forage maturity, and was greater than stem NDFD across all maturities (Table 3.3).

Stem and leaf NDFD were negatively associated with MSW. Correlation coefficients for stem NDFD were -0.84 and -0.90 during the seeding and first production year, respectively. Correlation coefficients for leaf NDFD were -0.64 and -0.75 during the seeding and first production year, respectively. The decrease in stem and leaf NDFD with increasing forage maturity was expected, and agrees with previous research demonstrating declining stem and leaf IVDMD (Fick and Holthausen, 1975; Buxton et al., 1985; Albrecht et al., 1987) and NDFD (Sanderson et al., 1989; Lamb et al., 2012) with increasing forage maturity. Sanderson et al. (1989) reported stem NDFD decreasing from 883 to 336 g $kg^{-1}$ for alfalfa harvested across a wide range of forage maturities, and Lamb et al. (2012) reported greater stem NDFD for alfalfa harvested at early bud compared to late flower. In the present study, although decreases in NDFD occurred within both leaf and stem fractions, the extent of NDFD decrease with increasing forage maturity was greater within the stem portion of the plant. Previous studies have made similar conclusions, showing a greater decline in stem digestibility with increasing forage maturity compared to leaf digestibility (Fick and Holthausen, 1975; Kalu and Fick, 1983; Buxton et al., 1985; Albrecht et al., 1987; Marković et al., 2012). This decline in digestibility with increasing forage maturity is likely a result of the increased lignification

within the plant cell walls, particularly within the stem portion of the plant (Albrecht et al., 1987; Yu et al., 2003).

## SUMMARY AND CONCLUSIONS

With the exception of seeding year leaf yields, reduced lignin (54HVX41) and reference (DKA43-22RR) alfalfa cultivars were not different in morphological characteristics, including stem yield, leaf yield, and L:S. Likewise, these morphological characteristics changed with maturity similarly across cultivars. The lack of differences in forage morphology between reduced lignin and reference alfalfa cultivars indicates that growth patterns between these cultivars are similar. Therefore, any changes in forage nutritive value between reduced lignin and reference alfalfa cultivars are likely a direct result of quality changes occurring within the plant, rather than differences in growth characteristics.

Across the seeding and first production year, 54HVX41 demonstrated a 13 to 14% reduction in stem ADL and an 8 to 15% increase in stem NDFD compared to DKA43-22RR. Stem and leaf CP, stem and leaf NDF, leaf ADL, and leaf NDFD were similar among cultivars. These results indicate that much of the change in forage nutritive value for the reduced lignin alfalfa cultivar 54HVX41 is a result of quality changes occurring within the stem portion of the plant.

Forage maturity had a strong influence on alfalfa stem yield, leaf yield, and L:S. As forage maturity increased, stem and leaf yields increased and L:S decreased. Yield increases were greater for stem fractions, while initial increases in leaf yield were often followed by a plateau at later maturity stages. As a result, declines in the L:S with

increasing alfalfa forage maturity can be attributed to increasing stem proportions and decreasing leaf proportions as the plant matures.

As expected, CP, NDF, ADL, and NDFD within alfalfa stem and leaf fractions were all directly impacted by forage maturation. Harvesting at a lesser plant maturity resulted in greater forage nutritive value for both stem and leaf components, including increased CP concentrations, decreased NDF and ADL concentration, and increased NDFD. Leaf fractions maintained a higher forage nutritive value compared to stem fractions across all maturities, and increasing forage maturity had a greater impact on alfalfa stem fractions compared to leaf fractions, pointing to the significance of stem nutritive value on total herbage quality.

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Table 3.1. Test of fixed effects for forage morphological characteristics and stem and leaf forage nutritive values, including stem yield, leaf yield, leaf to stem ratio (L:S), stem and leaf crude protein (CP), stem and leaf neutral detergent fiber (NDF), stem and leaf acid detergent lignin (ADL), and stem and leaf neutral detergent fiber digestibility (NDFD) for alfalfa cultivars grown in Becker and St. Paul, MN during the seeding (2015) and first production (2016) year.

| | Forage Morphology | | | Stem Forage Nutritive Value | | | | Leaf Forage Nutritive Value | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Source | Stem Yield | Leaf Yield | L:S | CP | NDF | ADL | NDFD | CP | NDF | ADL | NDFD |
| | *P > F* | | | | | | | | | | |
| | Seeding Year (2015) | | | | | | | | | | |
| MSW[†] (M) | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Cultivar (C) | 0.41 | 0.36 | 0.88 | 0.73 | 0.95 | <0.01 | <0.01 | 0.84 | 0.40 | 0.76 | 0.14 |
| M × C | 0.84 | 0.60 | 0.78 | 0.62 | 0.78 | 0.61 | 0.02 | 0.24 | 0.69 | 0.42 | 0.21 |
| | First Production Year (2016) | | | | | | | | | | |
| MSW (M) | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | 0.03 | <0.01 |
| Cultivar (C) | 0.63 | 0.23 | 0.73 | 0.53 | 0.26 | 0.02 | 0.01 | 0.11 | 0.20 | 0.15 | 0.94 |
| M × C | 0.96 | 0.91 | 0.78 | 0.51 | 0.44 | 0.70 | 0.91 | 0.99 | 0.93 | 0.84 | 0.76 |

[†]Numerical index referring to stage of alfalfa development (Kalu and Fick, 1981). Vegetative growth includes stages 0 through 2, budding plants includes stages 3 and 4, and flowering plants includes stages 5 and 6.

Table 3.2. Average stem yield, leaf yield, leaf to stem ratio (L:S), stem and leaf crude protein (CP), stem and leaf neutral detergent fiber (NDF), stem and leaf acid detergent lignin (ADL), and stem and leaf neutral detergent fiber digestibility (NDFD) for alfalfa cultivars grown in Becker and St. Paul, MN during the seeding (2015) and first production (2016) year.

| | Forage Morphology | | | Stem Forage Nutritive Value | | | | Leaf Forage Nutritive Value | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Source | Stem Yield | Leaf Yield | L:S | CP | NDF | ADL | NDFD | CP | NDF | ADL | NDFD |
| | —— g $m^{-2}$ —— | | | ———— g $kg^{-1}$ ———— | | | | ———— g $kg^{-1}$ ———— | | | |
| | | | | Seeding Year (2015) | | | | | | | |
| 54HVX41 | 155.6 | 160.8[a] | 1.08 | 145 | 477 | 67[b] | 312[a] | 326 | 173 | 27 | 651 |
| DKA43-22RR | 145.6 | 143.2[b] | 1.07 | 145 | 481 | 78[a] | 271[b] | 332 | 169 | 30 | 644 |
| *SE* | *15.5* | *30.1* | *0.12* | *5.1* | *17.1* | *4.8* | *7.4* | *13.8* | *3.4* | *7.5* | *11.4* |
| | | | | First Production Year (2016) | | | | | | | |
| 54HVX41 | 166.2 | 150.4 | 1.02 | 182 | 408 | 48[b] | 443[a] | 331[b] | 192 | 30[b] | 641 |
| DKA43-22RR | 161.2 | 143.2 | 1.01 | 181 | 413 | 55[a] | 412[b] | 338[a] | 187 | 31[a] | 643 |
| *SE* | *16.3* | *6.4* | *0.07* | *6.6* | *25.3* | *6.2* | *55.8* | *7.9* | *11.8* | *4.9* | *18.0* |

[ab]Within column and section, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

Table 3.3. Regression coefficients, associated $r^2$ values, and parameter estimates at a mid-vegetative (MSW 1) and late flower (MSW 6) maturity for models describing the responses of alfalfa stem yield, leaf yield, leaf to stem ratio (L:S), stem and leaf crude protein (CP), stem and leaf neutral detergent fiber (NDF), stem and leaf acid detergent lignin (ADL), and stem and leaf neutral detergent fiber digestibility (NDFD) to forage mean stage by weight (MSW) for alfalfa cultivars grown in Becker and St. Paul, MN during the seeding and first production year.

| Source | Model† | $\beta_0$ | $\beta_1$ | $\beta_2$ | $r^2$ | MSW 1‡ | MSW 6‡ |
|---|---|---|---|---|---|---|---|
| | | | Seeding Year (2015) | | | | |
| | | | g $m^{-2}$ | | | | |
| Stem Yield | Q | -79.3 | 105.7 | -9.4 | 0.8 | 42.8 | 215.0 |
| Leaf Yield | Q | -2.1 | 74.0 | -7.1 | 0.5 | 71.1 | 181.7 |
| L:S | Q | 2.3 | -0.6 | 0.1 | 0.7 | 1.50 | 0.82 |
| | | | g $kg^{-1}$ | | | | |
| Stem CP | Q | 23.6 | -4.4 | 0.4 | 0.8 | 189 | 122 |
| Leaf CP | L | 38.3 | -1.6 | — | 0.5 | 372 | 292 |
| Stem NDF | Q | 32.8 | 7.2 | -0.7 | 0.6 | 411 | 524 |
| Leaf NDF | Q | 13.8 | — | 0.3 | 0.6 | 147 | 221 |
| Stem ADL | Q | 3.4 | 1.7 | -0.2 | 0.5 | 53 | 87 |
| Leaf ADL | Q | 2.4 | — | 0.0 | 0.1 | 29 | 34 |
| Stem NDFD | | | | | | | |
| 54HVX41 | Q | 60.9 | -13.0 | 1.1 | 0.9 | 462 | 231 |
| DKA43-22RR | Q | 55.2 | -14.1 | 1.4 | 0.8 | 425 | 204 |
| Leaf NDFD | Q | 69.0 | — | -0.3 | 0.4 | 710 | 584 |
| | | | First Production Year (2016) | | | | |
| | | | g $m^{-2}$ | | | | |
| Stem Yield | Q | -1.9 | 94.8 | -9.1 | 0.7 | 68.0 | 223.6 |
| Leaf Yield | Q | 71.1 | 44.0 | -4.3 | 0.5 | 106.9 | 162.6 |
| L:S | Q | 2.0 | -0.6 | 0.1 | 0.6 | 1.56 | 0.74 |
| | | | g $kg^{-1}$ | | | | |
| Stem CP | Q | 29.4 | -6.1 | 0.5 | 0.9 | 246 | 122 |
| Leaf CP | L | 39.1 | -2.0 | — | 0.7 | 375 | 276 |
| Stem NDF | Q | 21.8 | 9.9 | -0.8 | 0.9 | 303 | 534 |
| Leaf NDF | Q | 16.3 | — | 0.2 | 0.7 | 166 | 257 |
| Stem ADL | Q | 0.4 | 2.7 | -0.3 | 0.7 | 26 | 74 |
| Leaf ADL | Q | 3.4 | -0.3 | 0.0 | 0.0 | 32 | 32 |
| Stem NDFD | Q | 72.1 | -14.2 | 1.0 | 0.9 | 591 | 231 |
| Leaf NDFD | L | 70.7 | -2.3 | — | 0.6 | 693 | 573 |

†L, linear; Q, quadratic

‡Numerical index referring to stage of alfalfa development (Kalu and Fick, 1981). Vegetative growth includes stages 0 through 2, budding plants includes stages 3 and 4, and flowering plants includes stages 5 and 6.

Figure 3.1. Monthly air temperature (°C), precipitation (cm), and 30-year historical average for Becker and St. Paul, MN during the 2015 and 2016 growing season. Weather data was obtained from http://mrcc.isws.illinois.edu/.

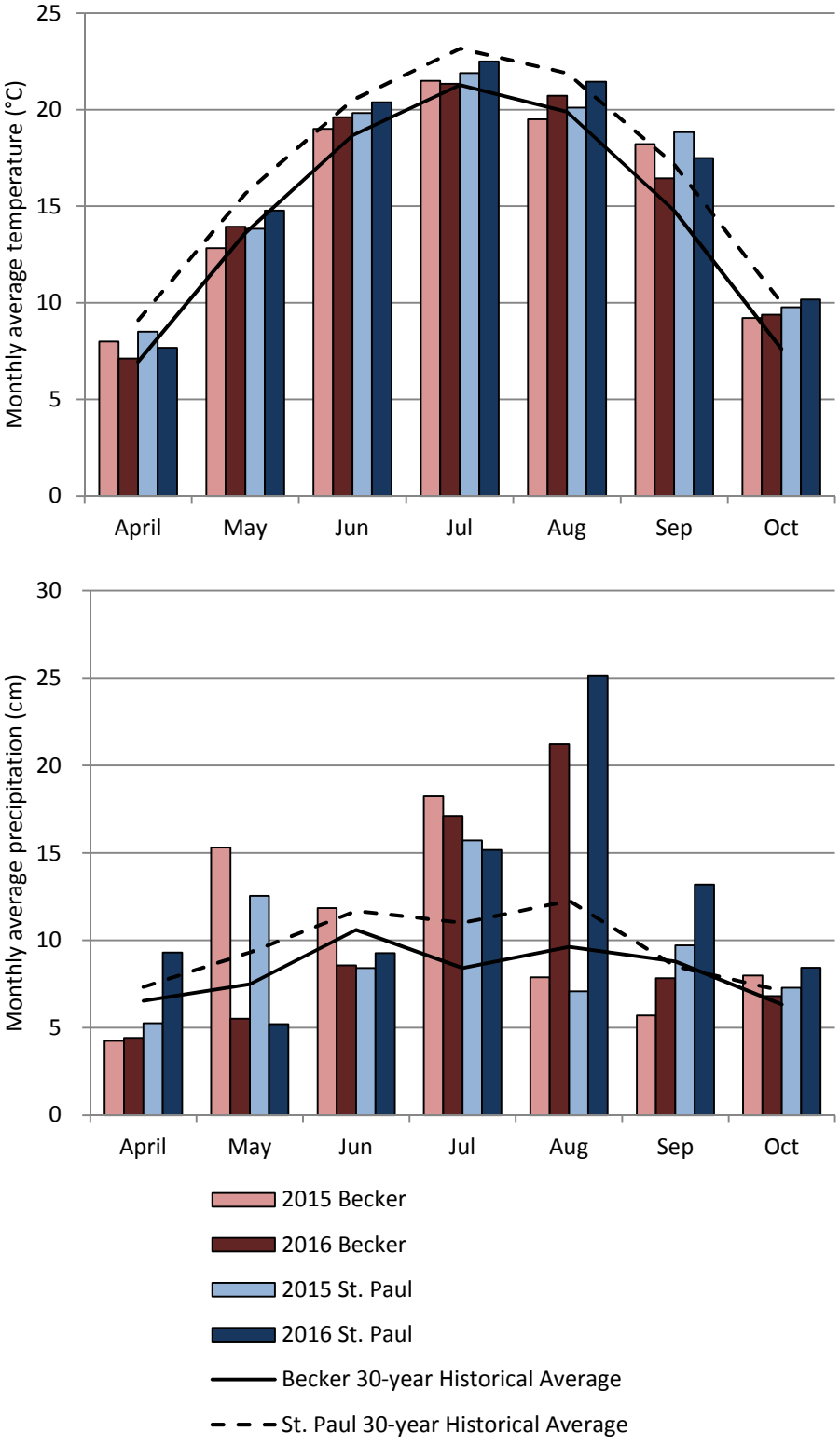
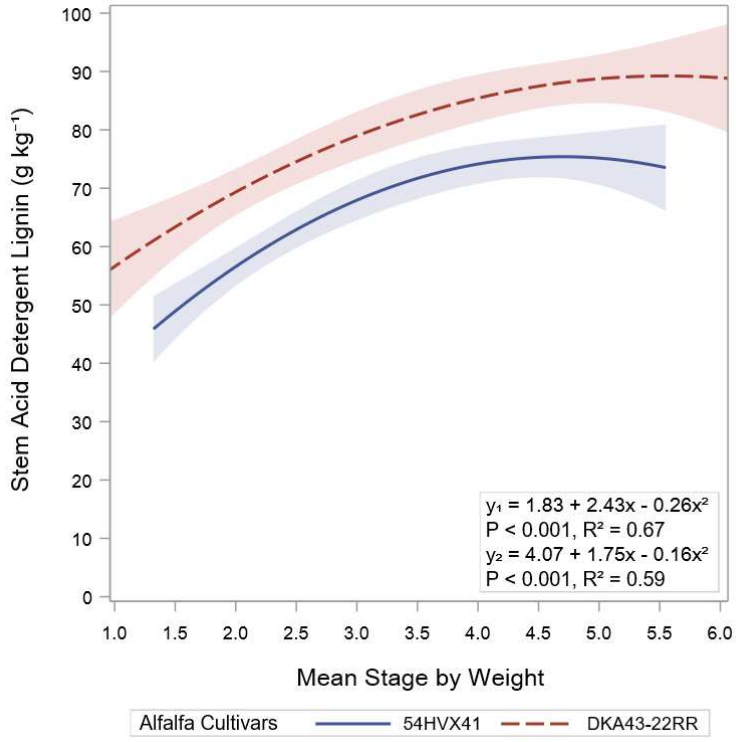


Figure 3.2A-B. Predicted alfalfa stem acid detergent lignin and 95% confidence intervals (shaded area) for reduced lignin cultivar 54HVX41 ($y_1$) and reference alfalfa cultivar DKA43-22RR ($y_2$) in response to forage mean stage by weight during the seeding (A) and first production (B) year.

A




B

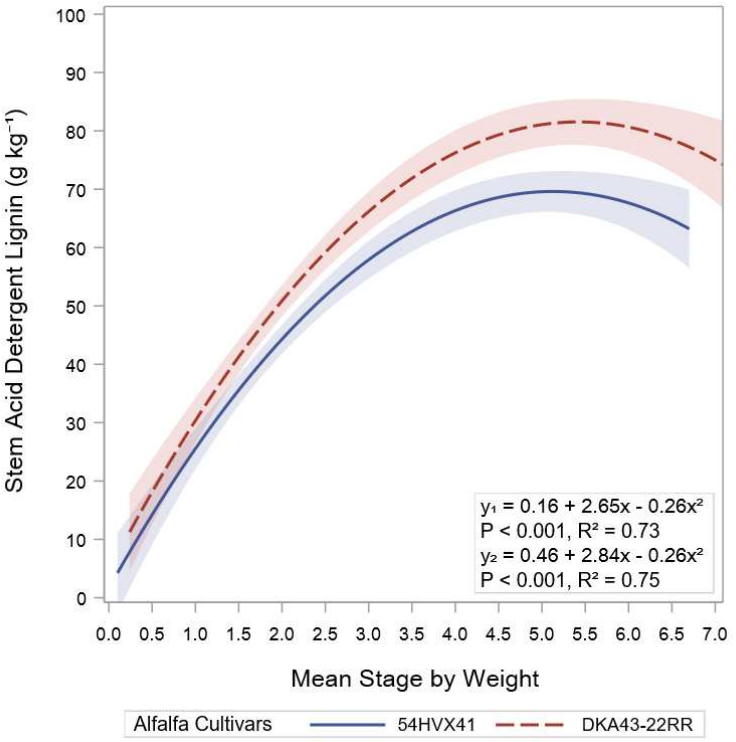
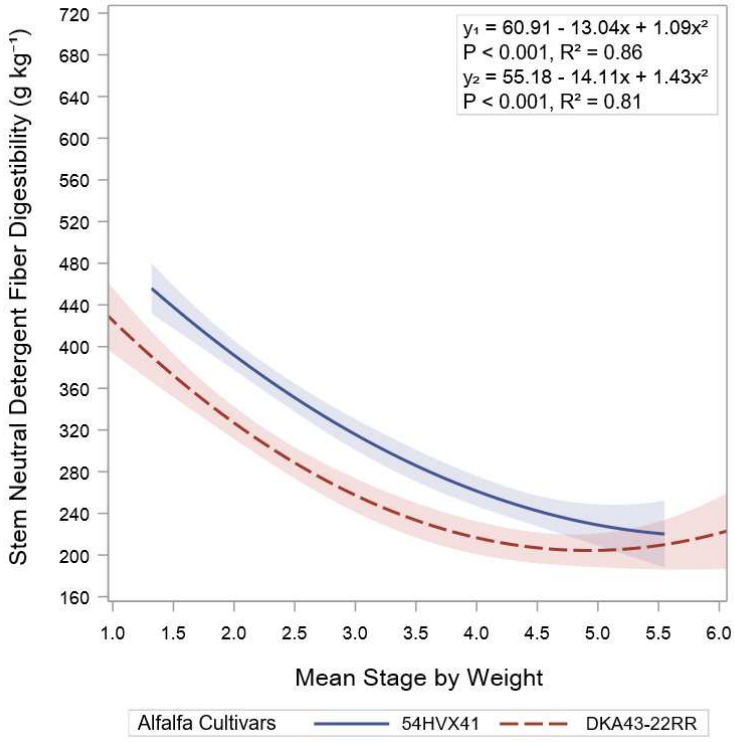
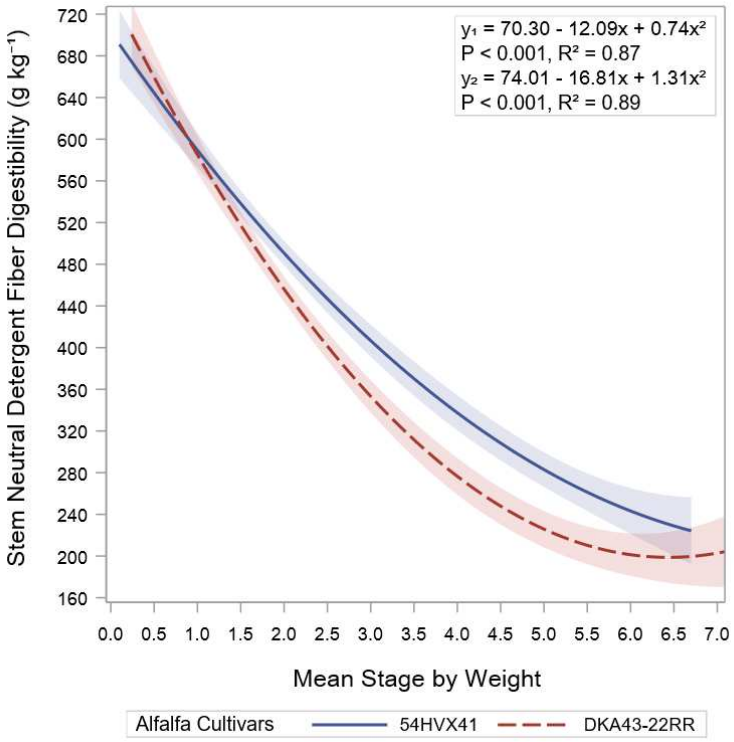


Figure 3.3A-B. Predicted alfalfa stem neutral detergent fiber digestibility and 95% confidence intervals (shaded area) for reduced lignin cultivar 54HVX41 ($y_1$) and reference alfalfa cultivar DKA43-22RR ($y_2$) in response to forage mean stage by weight during the seeding (A) and first production (B) year.

A




B



## LITERATURE CITED


Albrecht, K.A., W.F. Wedin, and D.R. Buxton. 1987. Cell-wall composition and digestibility of alfalfa stems and leaves. Crop Sci. 27(4): 735–741. doi: 10.2135/cropsci1987.0011183X002700040027x.

AOAC 990.03. 2010. Protein (Crude) in Animal Feed: Combustion Method. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD.

Baucher, M., M.A. Bernard-vailhé, B. Chabbert, J.-M. Besle, C. Opsomer, et al. 1999. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (Medicago sativa L.) and the effect on lignin composition and digestibility. Plant Mol. Biol. 39(3): 437–447. doi: 10.1023/A:1006182925584.

Brink, G., M. Hall, G. Shewmaker, D. Undersander, N. Martin, et al. 2010. Changes in alfalfa yield and nutritive value within individual harvest periods. Agron. J. 102(4): 1274–1282. doi: 10.2134/agronj2010.0080.

Burnham, K.P., and D.R. Anderson. 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. Springer Science & Business Media, New York, NY.

Buxton, D.R., and J.S. Hornstein. 1986. Cell-wall concentration and components in stratified canopies of alfalfa, birdsfoot trefoil, and red clover. Crop Sci. 26(1): 180–184. doi: 10.2135/cropsci1986.0011183X002600010043x.

Buxton, D.R., J.S. Hornstein, W.F. Wedin, and G.C. Marten. 1985. Forage Quality in Stratified Canopies of Alfalfa, Birdsfoot Trefoil, and Red Clover. Crop Sci. 25(2): 273–279. doi: 10.2135/cropsci1985.0011183X002500020016x.

Buxton, D.R., and J.R. Russell. 1988. Lignin Constituents and Cell-Wall Digestibility of Grass and Legume Stems. Crop Sci. 28(3): 553. doi: 10.2135/cropsci1988.0011183X002800030026x.

Cancelado, R.E., and E.B. Radcliffe. 1979. Action Thresholds for Potato Leafhopper on Potatoes in Minnesota. J. Econ. Entomol. 72(4): 566–569. doi: 10.1093/jee/72.4.566.

Casler, M.D. 1987. In vitro digestibility of dry matter and cell wall constituents of smooth bromegrass forage. Crop Sci. 27(5): 931–934. doi: 10.2135/cropsci1987.0011183X002700050021x.

Chasen, E.M., D.J. Undersander, and E.M. Cullen. 2015. Revisiting the economic injury level and economic threshold model for potato leafhopper (Hemiptera: Cicadellidae) in alfalfa. J. Econ. Entomol. 108(4): 1748–1756. doi: 10.1093/jee/tov120.

Fick, G.W., and R.S. Holthausen. 1975. Significance of Parts Other Than Blades and Stems in Leaf-stem Separations of Alfalfa Herbage 1. Crop Sci. 15(2): 259–262. doi: 10.2135/cropsci1975.0011183X001500020035x.

Getachew, G., A.M. Ibáñez, W. Pittroff, A.M. Dandekar, M. McCaslin, et al. 2011. A comparative study between lignin down regulated alfalfa lines and their respective unmodified controls on the nutritional characteristics of hay. Anim. Feed Sci. Technol. 170(3–4): 192–200. doi: 10.1016/j.anifeedsci.2011.09.009.

Getachew, G., E.A. Laca, D.H. Putnam, D. Witte, M. McCaslin, et al. 2018. The impact of lignin downregulation on alfalfa yield, chemical composition, and in vitro gas production. J. Sci. Food Agric. 98(11): 4205–4215. doi: 10.1002/jsfa.8942.

Goering, H.K., and P.J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, prcedures, and some applications). USDA Agr Handb. http://agris.fao.org/agris-search/search.do?recordID=US201301156229 (accessed 26 April 2017).

Grev, A.M., M.S. Wells, D.A. Samac, K.L. Martinson, and C.C. Sheaffer. 2017. Forage Accumulation and Nutritive Value of Reduced Lignin and Reference Alfalfa Cultivars. Agron. J. 109(6): 2749–2761. doi: 10.2134/agronj2017.04.0237.

Griffin, T.S., K.A. Cassida, O.B. Hesterman, and S.R. Rust. 1994. Alfalfa Maturity and Cultivar Effects on Chemical and In Situ Estimates of Protein Degradability. Crop Sci. 34(6): 1654–1661. doi: 10.2135/cropsci1994.0011183X003400060043x.

Guo, D., F. Chen, K. Inoue, J.W. Blount, and R.A. Dixon. 2001a. Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: Impacts on lignin structure and implications for the biosynthesis of G and S lignin. Plant Cell 13(1): 73–88. doi: 10.2307/3871154.

Guo, D., F. Chen, J. Wheeler, J. Winder, S. Selman, et al. 2001b. Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. Transgenic Res. 10(5): 457–464. doi: 10.1023/A:1012278106147.

Hall, M.H., W.S. Smiles, and R.A. Dickerson. 2000. Morphological development of alfalfa cultivars selected for higher quality. Agron. J. 92(6): 1077–1080. doi: 10.2134/agronj2000.9261077x.

Hoffman, P.C., S.J. Sievert, R.D. Shaver, D.A. Welch, and D.K. Combs. 1993. In Situ Dry Matter, Protein, and Fiber Degradation of Perennial Forages. J. Dairy Sci. 76(9): 2632–2643. doi: 10.3168/jds.S0022-0302(93)77599-2.

Inoue, K., V.J.H. Sewalt, G.M. Ballance, W. Ni, C. Stürzer, et al. 1998. Developmental expression and substrate specificities of alfalfa caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase in relation to lignification. Plant Physiol. 117(3): 761–770. doi: 10.1104/pp.117.3.761.

Juan, N.A., C.C. Sheaffer, D.K. Barnes, D.R. Swanson, and J.H. Halgerson. 1993. Leaf and Stem Traits and Herbage Quality of Multifoliolate Alfalfa. Agron. J. 85(6): 1121–1127. doi: 10.2134/agronj1993.00021962008500060005x.

Julier, B., and C. Huyghe. 1997. Effect of growth and cultivar on alfalfa digestibility in a multi-site trial. Agronomie 17(9–10): 481–489.

Jung, H.-J.G., and J.F.S. Lamb. 2003. Identification of lucerne stem cell wall traits related to in vitro neutral detergent fibre digestibility. Anim. Feed Sci. Technol. 110(1): 17–29. doi: 10.1016/j.anifeedsci.2003.08.003.

Jung, H.G., and J.F.S. Lamb. 2006. Stem Morphological and Cell Wall Traits Associated with Divergent In Vitro Neutral Detergent Fiber Digestibility in Alfalfa Clones. Crop Sci. 46(5): 2054–2061. doi: 10.2135/cropsci2005.12.0470.

Jung, H.G., D.R. Mertens, and A.J. Payne. 1997. Correlation of acid detergent lignin and klason lignin with digestibility of forage dry matter and neutral detergent fiber. J. Dairy Sci. 80(8): 1622–1628. doi: 10.3168/jds.S0022-0302(97)76093-4.

Jung, H.-J.G., D.A. Samac, and G. Sarath. 2012. Modifying crops to increase cell wall digestibility. Plant Sci. 185–186: 65–77. doi: 10.1016/j.plantsci.2011.10.014.

Kaiser, D.E., J.A. Lamb, and R. Eliason. 2011. Fertilizer Recommendations for Agronomic Crops in Minnesota: Alfalfa. https://www.extension.umn.edu/agriculture/nutrient-management/nutrient-lime-guidelines/fertilizer-recommendations-for-agronomic-crops-in-minnesota/alfalfa/ (accessed 13 March 2017).

Kallenbach, R.L., C.J. Nelson, and J.H. Coutts. 2002. Yield, quality, and persistence of grazing- and hay-type alfalfa under three harvest frequencies. Agron. J. 94(5): 1094–1103. doi: 10.2134/agronj2002.1094.

Kalu, B.A., and G.W. Fick. 1981. Quantifying morphological development of alfalfa for studies of herbage quality. Crop Sci. 21(2): 267–271. doi: 10.2135/cropsci1981.0011183X002100020016x.

Kalu, B.A., and G.W. Fick. 1983. Morphological stage of development as a predictor of alfalfa herbage quality. Crop Sci. 23(6): 1167–1172. doi: 10.2135/cropsci1983.0011183X002300060033x.

Kilcher, M.R., and D.H. Heinrichs. 1974. Contribution of Stems and Leaves to the Yield and Nutrient Level of Irrigated Alfalfa at Different Stages of Development. Can. J. Plant Sci. 54(4): 739–742. doi: 10.4141/cjps74-125.

Lamb, J.F.S., G.J. Hans-Joachim, and H. Riday. 2012. Harvest Impacts on Alfalfa Stem Neutral Detergent Fiber Concentration and Digestibility and Cell Wall Concentration and Composition. Crop Sci. 52(5): 2402–2412. doi: 10.2135/cropsci2012.01.0027.

Lamb, J.F.S., H.-J.G. Jung, and D.A. Samac. 2014. Environmental Variability and/or Stability of Stem Fiber Content and Digestibility in Alfalfa. Crop Sci. 54(6): 2854–2863. doi: 10.2135/cropsci2014.04.0323.

Lamb, J.F.S., H.-J.G. Jung, C.C. Sheaffer, and D.A. Samac. 2007. Alfalfa Leaf Protein and Stem Cell Wall Polysaccharide Yields under Hay and Biomass Management Systems. Crop Sci. 47(4): 1407–1415. doi: 10.2135/cropsci2006.10.0665.

Lamb, J.F.S., C.C. Sheaffer, and D.A. Samac. 2003. Population Density and Harvest Maturity Effects on Leaf and Stem Yield in Alfalfa. Agron. J. 95(3): 635–641. doi: 10.2134/agronj2003.6350.

Liu, N., and P. Yu. 2011. Molecular clustering, interrelationships and carbohydrate conformation in hull and seeds among barley cultivars. J. Cereal Sci. 53(3): 379–383. doi: 10.1016/j.jcs.2011.02.011.

Luckett, C.R., and T.J. Klopfenstein. 1970. Leaf-To-Stem Ratio and Composition of Alfalfa from Five Harvesting Systems. J. Anim. Sci. 31(1): 126–129. doi: 10.2527/jas1970.311126x.

Marita, J.M., J. Ralph, R.D. Hatfield, D. Guo, F. Chen, et al. 2003. Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase. Phytochemistry 62(1): 53–65. doi: 10.1016/S0031-9422(02)00434-X.

Marković, J.P., R.T. Scaron, trbanović, D.V. Terzić, D.J. Djokić, et al. 2012. Changes in lignin structure with maturation of alfalfa leaf and stem in relation to ruminants nutrition. Afr. J. Agric. Res. 7(2): 257–264. doi: 10.5897/AJAR11.1485.

Milić, D., D. Karagić, S. Vasiljević, A. Mikić, B. Mijić, et al. 2011. Leaf and stem chemical composition of divergent alfalfa cultivars. Biotechnol. Anim. Husb. 27(4): 1505–1511.

Min, D. 2016. Effects of cutting interval between harvests on dry matter yield and nutritive value in alfalfa. Am. J. Plant Sci. 07(08): 1226–1231. doi: 10.4236/ajps.2016.78118.

Palmonari, A., M. Fustini, G. Canestrari, E. Grilli, and A. Formigoni. 2014. Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content. J. Dairy Sci. 97(12): 7729–7734. doi: 10.3168/jds.2014-8123.

Probst, A., and R. Smith. 2011. Harvest frequency effects on yield, persistence, and regrowth rate among new alfalfa cultivars. Forage Grazinglands 9(1): 1–8. doi: 10.1094/FG-2011-0926-01-RS.

Reddy, M.S.S., F. Chen, G. Shadle, L. Jackson, H. Aljoe, et al. 2005. Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa

(Medicago sativa L.). Proc. Natl. Acad. Sci. U. S. A. 102(46): 16573–16578. doi: 10.1073/pnas.0505749102.

Sanderson, M.A., J.S. Hornstein, and W.F. Wedin. 1989. Alfalfa Morphological Stage and its Relation to in Situ Digestibility of Detergent Fiber Fractions of Stems. Crop Sci. 29(5): 1315. doi: 10.2135/cropsci1989.0011183X002900050045x.

Sanderson, M.A., and W.F. Wedin. 1988. Cell wall composition of alfalfa stems at similar morphological stages and chronological age during spring growth and summer regrowth. Crop Sci. 28(2): 342–347. doi: 10.2135/cropsci1988.0011183X002800020034x.

Schnurr, J.A., H.-J.G. Jung, and D.A. Samac. 2007. A Comparative Study of Alfalfa and Stem Traits: Morphology, Chemical Composition, and Ruminal Digestibility. Crop Sci. 47(4): 1672–1680. doi: 10.2135/cropsci2006.12.0762.

Sheaffer, C.C., D. Cash, N.J. Ehlke, J.C. Henning, J.G. Jewett, et al. 1998. Entry × Environment Interactions for Alfalfa Forage Quality. Agron. J. 90(6): 774–780. doi: 10.2134/agronj1998.00021962009000060010x.

Sheaffer, C.C., G.D. Lacefield, and V.L. Marble. 1988. Cutting schedules and stands. Alfalfa and Alfalfa Improvement. ASA, CSSA, and SSSA, Madison, WI. p. 411–437

Sheaffer, C.C., N.P. Martin, J.F.S. Lamb, G.R. Cuomo, J.G. Jewett, et al. 2000. Leaf and stem properties of alfalfa entries. Agron. J. 92(4): 733–739. doi: 10.2134/agronj2000.924733x.

Tremblay, G.F., G. Bélanger, K.B. McRae, and R. Michaud. 2002. Leaf and stem dry matter digestibility and ruminal undegradable proteins of alfalfa cultivars. Can. J. Plant Sci. 82(2): 383–393. doi: 10.4141/P01-122.

Undersander, D., D. Cosgrove, E. Cullen, C. Grau, M. Rice, et al. 2011. Alfalfa Management Guide. https://www.agronomy.org/files/publications/alfalfa-management-guide.pdf (accessed 23 February 2017).

Van Soest, P.J., J.B. Robertson, and B.A. Lewis. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. J. Dairy Sci. 74(10): 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2.

Wilman, D., and P.R. Moghaddam. 1998. In vitro digestibility and neutral detergent fibre and lignin contents of plant parts of nine forage species. J. Agric. Sci. 131(1): 51–58.

Yari, M., R. Valizadeh, A.A. Naserian, G.R. Ghorbani, P. Rezvani Moghaddam, et al. 2012. Botanical traits, protein and carbohydrate fractions, ruminal degradability and energy contents of alfalfa hay harvested at three stages of maturity and in the

afternoon and morning. Anim. Feed Sci. Technol. 172(3–4): 162–170. doi: 10.1016/j.anifeedsci.2012.01.004.

Yari, M., R. Valizadeh, A.A. Naserian, A. Jonker, A. Azarfar, et al. 2014. Effects of including alfalfa hay cut in the afternoon or morning at three stages of maturity in high concentrate rations on dairy cows performance, diet digestibility and feeding behavior. Anim. Feed Sci. Technol. 192: 62–72. doi: 10.1016/j.anifeedsci.2014.04.001.

Yu, P., D.A. Christensen, J.J. McKinnon, and J.D. Markert. 2003. Effect of variety and maturity stage on chemical composition, carbohydrate and protein subfractions, in vitro rumen degradability and energy values of timothy and alfalfa. Can. J. Anim. Sci. 83(2): 279–290. doi: 10.4141/A02-053.

# CHAPTER 4

## Apparent Digestibility, Fecal Particle Size, and Mean Retention Time of Reduced Lignin Alfalfa Hay Fed to Horses

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### CHAPTER SUMMARY

Reduced lignin alfalfa (*Medicago sativa*) has the potential to provide a higher quality forage source for livestock by improving forage digestibility. This study was conducted to evaluate apparent digestibility when feeding reduced lignin and reference alfalfa hays to adult horses, and to examine differences in mean fecal particle size (MFPS) and mean retention time (MRT) between alfalfa forage types. In 2017, reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hays were harvested in Minnesota at the late-bud stage. Alfalfa varieties were similar in crude protein (CP; 199 g $kg^{-1}$), neutral detergent fiber (NDF; 433 g $kg^{-1}$), and digestible energy (DE; 2.4 Mcal $kg^{-1}$) content. Acid detergent lignin (ADL) concentrations were lower for 54HVX41 (74 g $kg^{-}$

$^{1}$) compared to WL355.RR (81 g kg$^{-1}$). Dietary treatments were fed to six adult, stock-type horses in a crossover study. Experimental periods consisted of a 9-d dietary adaptation phase followed by a 5-d total fecal collection phase, during which horses were housed in individual boxstalls and manure was removed on a continuous 24-h basis. At 12-h intervals, feces were thoroughly mixed, subsampled in duplicate, and used for apparent digestibility and MFPS analysis. Additional samples were taken at 2-h intervals following marker dosing to evaluate MRT. Dietary treatments were similar in dry matter intake (DMI; 1.6% BW) and time to consumption (TTC; 7.6 h). Apparent dry matter digestibility (DMD) was greater for reduced lignin alfalfa (64.4%) compared to reference alfalfa (61.7%). Apparent CP digestibility (CPD), NDF digestibility (NDFD), MFPS, liquid phase MRT, and particulate phase MRT did not differ between dietary treatments, averaging 78.4%, 45.2%, 0.89 mm, 24.0 h, and 28.5 h, respectively. These results indicate an improvement in DMD for reduced lignin alfalfa hay when fed to adult horses, with no change in forage consumption, fecal particle size, or retention time.

## INTRODUCTION

Alfalfa is widely used as forage for horses due to its high nutrient content. Compared to grasses, legumes such as alfalfa are typically lower in NDF and contain greater concentrations of protein, energy, and essential vitamins and minerals like calcium (Gibbs et al., 1988; Cuddeford et al., 1992, 1995; Crozier et al., 1997; Wilman and Moghaddam, 1998; LaCasha et al., 1999; Sturgeon et al., 2000; Edouard et al., 2008; Potts et al., 2010; Earing et al., 2010; Woodward et al., 2011). As a result of these differences, alfalfa is generally more digestible compared to other grass forages.

Researchers comparing forage digestibility between legumes and grasses have reported average DMD ranging from 44 to 59% for grass hays and 58 to 73% for alfalfa hays (Cuddeford et al., 1995; Crozier et al., 1997; LaCasha et al., 1999; Sturgeon et al., 2000; Pearson et al., 2006; Edouard et al., 2008; Potts et al., 2010).

Although alfalfa is a preferred forage source for horses with advanced dietary requirements, the digestibility and utilization of alfalfa by these animals is hampered by its lignin content (Sewalt et al., 1997; Casler et al., 2002). Classified as one of the structural carbohydrates, lignin is a complex structural polymer and the second most abundant component of secondary plant cell walls (Li et al., 2015). While it is essential for providing the strength and rigidity necessary for normal plant growth, the deposition of lignin into plant cell walls can reduce the feeding value of alfalfa by negatively affecting microbial degradation and the digestion of feed by intestinal enzymes (Buxton and Hornstein, 1986; Liu and Yu, 2011). Lignification has been reported to be the major factor limiting the *in vitro* DMD of whole plant forage (Casler, 1986, 1987; Reddy et al., 2005; Jung et al., 2012), and numerous studies have reported a strong inverse relationship between lignin concentrations and forage digestibility (Albrecht et al., 1987; Casler, 1987; Buxton and Russell, 1988; Sanderson et al., 1989; Jung et al., 1997; Reddy et al., 2005).

Populations of reduced lignin alfalfa are now available and have potential to increase the digestibility of alfalfa forage compared to reference cultivars (Guo et al., 2001; Marita et al., 2003; Reddy et al., 2005; Getachew et al., 2011). Field research evaluating the performance of reduced lignin alfalfa under different harvest frequencies has demonstrated a reduction in total herbage ADL and an increase in NDFD and relative

forage quality (RFQ) for reduced lignin alfalfa compared to reference cultivars (Grev et al., 2017; Getachew et al., 2018). However, it remains to be seen if these increases in forage NDFD will translate into greater *in vivo* digestibility when fed to the animal directly. Preliminary results evaluating reduced lignin alfalfa hay found that when reduced lignin and reference alfalfa hays were fed to lambs, DMD and NDFD were greater for the reduced lignin hays (Mertens and McCaslin, 2008). Similarly, when reduced lignin alfalfa hays were included as 50% of the ration for lactating dairy cows, NDFD was significantly increased and the additional forage digestibility resulted in 1.3 kg more milk production per head per day compared to the control diet (Weakley et al., 2008). While this information is promising, information on forage digestibility for current reduced lignin alfalfa cultivars is not yet available, and digestibility changes have not yet been evaluated in the equine model. Therefore, the objectives for this study were to evaluate apparent digestibility when feeding reduced lignin and reference alfalfa hays to adult horses. To further explore potential changes in digestive parameters, MRT and MFPS between alfalfa forage types were also assessed.

## MATERIALS AND METHODS

All experimental procedures were conducted according to those approved by the University of Minnesota Institutional Animal Care and Use Committee.

### *Dietary Treatments*

Dietary treatments were two commercially available alfalfa cultivars, including one reduced lignin alfalfa cultivar (54HVX41) and one non-reduced lignin cultivar (WL355.RR). The 54HVX41 cultivar is available from Forage Genetics (Napa, ID) and

was produced via down-regulation of the lignin biosynthetic genes. The WL355.RR cultivar is available from W-L Alfalfa (Ozark, MO) and was selected because it is a high-performing cultivar with a similar fall dormancy; this cultivar is not marketed as reduced lignin and served as a reference cultivar for this experiment.

In April 2016, reduced lignin and reference alfalfa cultivars were seeded into a prepared seedbed at a rate of 18.7 kg $ha^{-1}$ on an established commercial hay production field in Minnesota. Soil was a combination of an Angus-Le Sueur complex (1 to 6 percent slopes) and a Cordova loam (0 to 2 percent slopes), and soil fertility was amended to meet recommendations for alfalfa hay production according to University of Minnesota fertility guidelines (Kaiser et al., 2011). Hay was baled from third cutting alfalfa, which was harvested on September 2, 2017 at the late bud stage (Kalu and Fick, 1981). All forage was cut, raked, and baled at recommended moisture levels using best management practices designed to minimize leaf loss and optimize forage quality and yield (Digman et al., 2011). Hay was baled into small-square bales, and treatments were individually identified using color-coded zip ties which were manually attached to each bale at the time of baling.

Immediately following baling, representative hay bales from each treatment were randomly selected and cored to determine forage nutritive value (Sheaffer et al., 2000). Hay samples were dried in a forced-air oven at 60°C for 48 h and ground to pass through a 1-mm screen in a Cyclotec (Foss, Hillerod, Denmark). Ground samples were analyzed for forage nutritive value by a commercial forage testing laboratory (Equi-Analytical, Ithaca, NY) using the following methods. Crude protein was calculated as the percentage of nitrogen multiplied by 6.25 (AOAC, 2010). Neutral and acid detergent fibers were

measured using filter bag techniques (Ankom Technology, 2013a; b). Starch, water soluble-carbohydrates (WSC), and ethanol-soluble carbohydrates (ESC) were measured using techniques described by Hall et al. (1999). Mineral concentrations were determined (Thermo Jarrell Ash IRIS Advantage HX Inductively Coupled Plasma Radial Spectrometer; Thermo Instrument Systems Inc., Waltham, MA) after microwave digestion (Microwave Accelerated Reaction System; CEM, Mathews, NC). Equine DE was calculated using an equation developed by Pagan (1998). This preliminary hay sampling and analysis was done to allow for confirmation of forage nutritive value differences due to alfalfa cultivar.

In addition to the forage nutritive value analysis, stem and leaf separations and stem length measurements were also taken to compare forage characteristics between reduced lignin and reference alfalfa hays. Twelve randomly selected samples from each forage type consisting of approximately 0.25 kg were divided by hand into stem and leaf fractions and weighed to determine leaf to stem ratios (L:S). In addition, 12 randomly selected samples from each forage type consisting of one hay flake (approximately 1.0 kg) were used for stem length measurements. For each sample, stem length was measured on 100 randomly selected stems, which were averaged to estimate the mean stem length. Hay bales were then consolidated by treatment and stored indoors throughout the duration of the study.

### *Experimental Design and Sample Collection*

The experiment was completed using a crossover design with two treatments (alfalfa forage type) and two periods. Six adult, stock-type horses with an average bodyweight (BW) of 544 kg (SE ± 36 kg) and body condition score (BCS; Henneke et

al., 1983) of 5.7 (SE ± 1.0) were divided into two similar herds with three horses each. Prior to the start of the study, horses were acclimated to their herd and paddock and given free-choice access to legume-grass mixed hay and water. Herds remained together for the duration of the study. Each experimental period consisted of a 9-d dietary adaptation phase (d 1 to 9) followed by a 5-d total fecal collection phase (d 10 to 14).

At the beginning of each adaptation phase (d 1), horses were weighed using a livestock platform scale and BCS was assessed (Henneke et al., 1983). For the duration of the adaptation phase, horse herds were housed in individual dry lots with access to shelter and water. Horses received their experimental diet *ad libitum*; hay was divided into two equal portions and fed twice daily at 0800 and 1900 h. With each morning feeding, horses were also given 0.9 kg of a commercially prepared ration balancer (vitamin and mineral mix) to ensure that all nutritional requirements were met for adult horses at maintenance (NRC, 2007).

At the beginning of each fecal collection phase (d 10), horses were moved to individual rubber-matted boxstalls (3.6 × 3.6 m), where they were housed for the duration of the fecal collection phase. Each morning of the fecal collection phase, representative forage samples were obtained by randomly sampling hay bales from each dietary treatment using a core-sampler (Penn State Forage Sampler, University Park, PA). Hay cores for each dietary treatment were combined by day and stored at -20°C for later analysis. Hay was offered at 2% BW from hay nets and was fed in two equal portions at 0800 and 2000 h. The time when horses began eating their hay meal and the time when horses finished that same hay meal was recorded in order to calculate total TTC for each hay meal. Prior to each feeding, any orts remaining in the stalls were removed, weighed,

and subtracted from the daily amount of hay offered for determination of total daily hay intake. Horses had free-choice access to water throughout the fecal collection phase. The amount of water provided to each horse was recorded in order to calculate total daily water intake.

For the duration of the fecal collection phase, horses were hand walked twice daily for 15 min immediately before receiving their meal. Manure was removed from the stalls continuously on a 24-h basis to allow for determination of total daily fecal output and to reduce any possible contamination with hay or urine. Feces for each horse were collected individually into large plastic containers lined with plastic bags that remained closed throughout the day to retain moisture. Cumulative feces weight was recorded every 12 h, at which time the collected feces was thoroughly mixed and subsampled in duplicate. Subsamples contained approximately 10% of the total fecal mass each and were placed in sealed collection bags and stored at -20°C for subsequent apparent digestibility and fecal particle size analysis. Total daily fecal output was calculated as the summed fecal weight from each 12-h period.

### *Marker Preparation*

Indigestible markers were used to measure MRT of solute- and particulate-phase digesta through the entire digestive tract. Solute MRT was measured using Cobalt-EDTA (Co-EDTA) as a solute-phase marker, and particulate MRT was measured using Ytterbium-labeled (Yb-labeled) NDF residue as a particulate-phase marker. The Co-EDTA marker was prepared according to the methods of Udén et al. (1980). A solution containing 25 g of Co(II) acetate $4H_2O$, 29.2 g EDTA, 4.3 g LiOH $H_2O$, and 200 mL distilled water was prepared and heated until solutes were dissolved. The mixture was

cooled and 20 mL of 30% hydrogen peroxide were added. After standing for 2 to 3 h at room temperature, 300 mL 95% ethanol were added and the mixture was stored overnight under refrigeration. The following day, the mixture was filtered, washed with 80% ethanol, and dried to a constant weight in a 60°C forced-air oven.

The Yb-labeled NDF residue markers were prepared using the immersion method described by Earing (2011). Markers were prepared separately with both reduced lignin and reference alfalfa hays so that horses were given Yb-labeled NDF residue that was consistent with their dietary treatment. Hays were chopped by passing through a Wiley Mill (Thomas Scientific, Swedesboro, NJ) with no screen four times. To prevent particle loss during the marker preparation process, hays used for marker attachment were kept in cloth bags throughout the labeling procedure. Hays were first prepped for marker attachment through the removal of soluble particles. Hays were soaked in boiling NDF solution for 1 h at a rate of 60 g chopped forage per 1 L NDF solution and then thoroughly rinsed with hot water to ensure soluble particle removal. Marker attachment to the resulting NDF residue was accomplished by soaking the residue at a rate of 100 g of NDF residue per 1 L of 0.007 M Yb solution for 24 h. The Yb solution was prepared by dissolving 2.96 g of Yb (III) acetate tetrahydrate in 1 L of distilled water. Following the 24 h soak, the NDF residue was soaked in tap water for 1 h and thoroughly rinsed. To ensure removal of any loosely bound Yb, the NDF residue was washed in 0.01 M acetic acid for 1.5 h and rinsed thoroughly. The final product was dried in a forced air oven at 60°C for 24 h or until a constant weight was reached.

### *Marker Administration and Sample Collection*

The morning of the second day of each total fecal collection period (d 11), horses received the prepared Co-EDTA and Yb-labeled NDF residue markers in a single dose immediately prior to their morning hay meal. Marker dosage was provided according to horse BW, with each horse receiving 9 mg of Co and Yb per kg $BW^{0.75}$. To encourage complete consumption, the marked feed was mixed with 0.9 kg of commercially prepared ration balancer and top dressed with a diluted molasses-water mixture. All horses readily consumed the marked feed in its entirety, after which the usual morning hay allotment was provided.

In addition to the fecal collection procedures outlined previously, additional fecal samples were collected for marker concentration analysis. Following the marker ingestion, excreted feces were collected from each horse immediately following defecation every 2 h from 0 to 96 h post-marker ingestion. At each time point, the collected fecal sample was weighed, the time of excretion and fecal weight were recorded, and the sample was thoroughly mixed by hand. A sub-sample consisting of at least 400 g of wet feces was placed in a sealed collection bag and stored at -20°C for subsequent marker concentration measurement. Following sub-sampling, any remaining fecal material was added to the ongoing 12-h cumulative fecal collection described previously. Weights for the collected marker sub-samples were added to the summed total daily fecal output amount.

### *Sample Analysis and Calculations*

Upon completion of the experiment, all hay and fecal samples were thawed and dried in a forced-air oven at 60°C for 48 h. Dry matter was determined by dividing the weight of the hay or feces after drying by the wet weight of the hay or feces as sampled.

Dry matter intake was calculated by multiplying the hay DM by the total daily hay intake (hay delivered minus orts). Hay DMI was expressed as a percentage of BW by dividing hay DMI by horse BW at the beginning of each period. Dry matter intake rate (DMIR) was determined by dividing the total amount of hay consumed (kg DM) by the TTC (h). Dry matter output (DMO) was calculated by multiplying the fecal DM by the total daily fecal output (summation of 12-h cumulative collection plus marker sub-samples).

Hay and fecal samples for apparent digestibility analysis were ground to pass through a 1-mm screen in a Cyclotec (Foss, Hillerod, Denmark) and analyzed for forage nutritive value via wet chemistry by a commercial forage testing laboratory (Equi-Analytical, Ithaca, NY). Samples were analyzed for CP, acid detergent fiber (ADF), NDF, and ADL using the methods described previously. Apparent DMD was calculated based on the mean daily DMI and the mean daily fecal DMO using the following equation:

$$DMD = \frac{DMI - fecal\ DMO}{DMI}$$

Calculations for individual nutrient digestibility followed the same format and were calculated using the following equation:

$$Digestibility = \frac{Nutrient\ intake - nutrient\ excreted\ in\ feces}{Nutrient\ intake}$$

A particle size distribution analysis was performed on dried fecal particle size samples using a sieve shaker with 4, 2, 1, 0.5, and 0.25 mm stainless steel sieves (Gilson

Co. Inc., Lewis Center, OH). A sub-sample of 50 g of dried feces was placed on the top screen and the stack of sieves was shaken at 300 RPM on an agitator (New Brunswick Scientific, Edison, NJ) for 4 minutes. At this point, any fecal lumps remaining in the top sieve were broken up via manual processing and the sample was sieved for an additional 8 minutes. The material retained within each sieve was weighed, and MFPS was calculated based on a weighted average of the particle distribution within each sieve size.

Fecal samples for marker concentration determination were ground to pass through a 1-mm screen in a Cyclotec (Foss, Hillerod, Denmark) and sent to a commercial laboratory (University of Kentucky ERTL, Lexington, KY) for analysis. Samples were digested in concentrated nitric acid and analyzed for Co and Yb concentrations using inductively-coupled plasma spectrophotometry with wavelengths set at 236.379 and 222.447 nm for Co and Yb, respectively. Fecal recovery (R, %) of each marker was calculated based on marker intake and fecal marker excretion using the following equation: R = (fecal marker excretion/marker intake) × 100. Total tract MRT for both fluid (Co) and particulate (Yb) phases were calculated algebraically according to Blaxter et al. (1956) as:

$$MRT = \frac{\sum m_i t_i}{\sum m_i}$$

where $m_i$ = the amount (mg) of marker at the *i*th sample and $t_i$ = the time (h) elapsed between marker ingestion and the time the *i*th sample was collected, and also according to Thielemans et al. (1978) as:

$$MRT = \frac{\sum t_i C_i \Delta t_i}{\sum C_i \Delta t_i}$$

where $t_i$ = time (h) elapsed between marker ingestion and the time the *i*th sample was collected, $C_i$ = concentration (mg $kg^{-1}$) of marker in the *i*th sample, and $\Delta t_i$ = time interval (h) between two consecutive samples.

### *Statistical Analysis*

Data for all response variables were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Mean comparisons are reported as least square means ± SE, with statistical significance set at $P \leq 0.05$. Means separations were performed on significant effects using Tukey's HSD test. For hay nutrient composition and forage characteristic response variables, individual forage samples comprised the experimental unit. The model included dietary treatment (i.e. alfalfa cultivar) as a fixed effect. Experimental period and replicate were included as random effects.

For intake, apparent digestibility, and fecal particle size response variables, individual horse within dietary treatment was the experimental unit. The model included dietary treatment and treatment by time as fixed effects. Experimental period, horse, and the period by horse interaction were included as random effects. Time was included as a repeated measures according to the methods of Littell et al. (1998). For TTC and DMIR variables, data where horses did not finish their full hay meal prior to receiving the subsequent hay meal (i.e. within a 12-h time window) were excluded from the analysis.

For marker concentration and MRT response variables, individual horse within dietary treatment was the experimental unit. Area under the curve for Yb and Co markers were calculated using the trapezoidal method. The model included dietary treatment as a fixed effect. Experimental period, horse, and the period by horse interaction were included as random effects.

## RESULTS AND DISCUSSION

### *Nutrient Composition and Forage Characteristics*

Nutrient composition, L:S, and average stem length for the reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hays are shown in Table 4.1. With the exception of ADL, nutrient content was similar between alfalfa hay types, and concentrations for all nutrients fell within the normal range for alfalfa hay (Cuddeford et al., 1992, 1995; Crozier et al., 1997; LaCasha et al., 1999; Sturgeon et al., 2000; Pearson et al., 2001; Potts et al., 2010; Earing et al., 2010; Woodward et al., 2011). The nutrient profile for both alfalfa hays in the present study was sufficient to meet the daily DE and CP requirements for adult horses at maintenance. At the intakes measured in this study, horses were consuming an average of 116 and 252% of their daily DE and CP requirements, respectively.

In the present study, reduced lignin alfalfa hay demonstrated a 9% reduction in ADL compared to reference alfalfa hay. Previous research comparing reduced lignin and reference alfalfa hays has also reported a reduction in ADL with little to no change in other nutrient components (Mertens and McCaslin, 2008). Similarly, reduced lignin alfalfa forage has shown a 6 to 24% decrease in total herbage ADL concentrations compared to reference alfalfa cultivars (Getachew et al., 2011, 2018; Grev et al., 2017). This reduction in total herbage lignin is likely a result of reductions in lignin within the stem fraction of the plant, as research has reported a 13 to 14% decrease in stem ADL concentrations and minimal differences in leaf ADL concentrations for reduced lignin alfalfa (Grev et al., 2019).

Stem length did not differ between alfalfa hay types, but the reduced lignin alfalfa hay contained a lower percentage of stems and a higher percentage of leaves compared to the reference alfalfa hay. As a result, L:S was greater for reduced lignin alfalfa hay (1.4) compared to reference alfalfa hay (0.9). To the authors' knowledge, this is the first study comparing stem length and L:S between reduced lignin and reference alfalfa hays. Previous research evaluating L:S on fresh forage found no difference in L:S between reduced lignin and reference alfalfa (Grev et al., 2019). While the higher L:S for reduced lignin alfalfa hay could be indicative of greater leaf retention during hay production for the reduced lignin cultivar, further research comparing reduced lignin and reference alfalfa hay production is needed to investigate this theory.

### *Intake and Time to Consumption*

Intake for hay and water was similar across dietary treatments when expressed on a DM basis as kilograms per day, percentage of BW, and grams per kilogram of $BW^{0.75}$ (Table 4.2). Across both treatments, DMI for alfalfa hay in the present study averaged 1.6% of BW and 78.0 g $kg^{-1}$ $BW^{0.75}$. This intake level is within the expected range for horses and corresponds with other studies reporting DMI for horses fed legume or grass hay at maintenance (Martin-Rosset et al., 1990; Palmgren Karlsson et al., 2000; Ordakowski et al., 2001; Pearson et al., 2006; Miyaji et al., 2008, 2014; Staniar et al., 2010; Woodward et al., 2011; Clauss et al., 2014). Although this is the first study reporting intake for reduced lignin and reference alfalfa hays fed to horses, previous research with other species found no difference in DMI when reduced lignin and reference alfalfa hays were fed to dairy cows as part of a total mixed ration (Weakley et al., 2008). Water intake in the present study averaged 34.3 kg $d^{-1}$ across both hay types

and is comparable to previous studies reporting daily water intake for horses (Cuddeford et al., 1995; Pearson et al., 2001, 2006).

Time to consumption and DMIR averaged 7.6 h and 0.7 kg $h^{-1}$, respectively, and did not differ between dietary treatments (Table 4.2). Hay nets used in the present study were comparable to the medium hay nets used by Glunk et al. (2014), who reported a mean TTC of 5.1 h and a DMIR of 0.99 kg $h^{-1}$. The slightly longer TTC and lower DMIR observed in the present study could be due to a number of reasons, including changes in forage type (i.e. alfalfa vs. grass hay), differences in feeding time (i.e. PM meal fed at 1600 vs. 2000 h), or variation among individual horses. Regardless, the lack of differences in DMI, TTC, and DMIR between reduced lignin and reference alfalfa hays indicate that both hay types were equally accepted by the horses in this study, and that rate of consumption was similar between alfalfa hay types.

### *Apparent Digestibility*

Apparent nutrient digestibility values for reduced lignin and reference alfalfa hays are shown in Table 4.3. Horses exhibited a 4% improvement in DMD when consuming reduced lignin alfalfa hay compared to reference alfalfa hay, averaging 64.4 and 61.7% for reduced lignin and reference alfalfa hays, respectively. These DMD values are comparable to results from previous studies, which have reported apparent DMD ranging from 57 to 73% for alfalfa hay (Cuddeford et al., 1995; Crozier et al., 1997; LaCasha et al., 1999; Sturgeon et al., 2000; Pearson et al., 2001, 2006; Edouard et al., 2008; Potts et al., 2010; Earing et al., 2010). To the authors' knowledge, this is the first study comparing DMD between reduced lignin and reference alfalfa hays fed to horses. However, this improvement in DMD for reduced lignin alfalfa hay is consistent with

results from Mertens and McCaslin (2008), who reported a 3 to 5% increase in DMD when reduced lignin alfalfa hay was fed to lambs. These improvements in DMD for reduced lignin alfalfa hay could be due to a number of reasons, the most likely of which include a reduction in forage ADL concentrations or a greater L:S for the reduced lignin alfalfa cultivar. It has been well established that the deposition of lignin in plant cell walls negatively affects forage digestibility (Albrecht et al., 1987; Jung et al., 1997; Reddy et al., 2005; Getachew et al., 2011). Therefore, a reduction in herbage lignin content will likely result in improvements in forage digestibility. At the same time, alfalfa leaves are known to be low in cell wall concentration and highly digestible, while alfalfa stems exhibit a lower digestibility as a result of high concentrations of cell wall polysaccharides (Albrecht et al., 1987; Julier and Huyghe, 1997; Milić et al., 2011; Marković et al., 2012; Yari et al., 2012; Lamb et al., 2012). As a result, changes in the proportion of leaf and stem material (i.e. L:S) within the plant could also alter forage digestibility. Further research is needed to pinpoint the cause behind the observed DMD improvement for reduced lignin alfalfa.

Crude protein digestibility did not differ between reduced lignin and reference alfalfa hays, averaging 78.4% across treatments. Past studies evaluating CPD for alfalfa hay has found similar results, reporting CPD ranging from 66 to 90% (Cuddeford et al., 1992, 1995; Crozier et al., 1997; LaCasha et al., 1999; Sturgeon et al., 2000; Pearson et al., 2001, 2006; Potts et al., 2010; Woodward et al., 2011). To the authors' knowledge, previous research has not compared CPD between reduced lignin and reference alfalfa hays. The similarity in CPD between alfalfa hays in the present study is likely due to a combination of factors, including the similar CP content between forages, the high

availability of readily-digested CP in alfalfa forage, and the ability of the microbial populations in the hindgut to utilize any remaining CP following pre-cecal digestion.

Alfalfa hay treatments were similar in ADF digestibility (ADFD) and NDFD, but there was a trend for increased ADL digestibility (ADLD) for reduced lignin alfalfa hay (30.8%) compared to reference alfalfa hay (23.3%). All fiber digestibility values observed in the present study are consistent with reports from previous research, which has documented alfalfa hay ADFD ranging from 21 to 55%, NDFD ranging from 24 to 57%, and ADLD ranging from 18 to 32% (Cuddeford et al., 1992, 1995; Crozier et al., 1997; LaCasha et al., 1999; Sturgeon et al., 2000; Pearson et al., 2001, 2006; Potts et al., 2010; Earing et al., 2010). The present study is the first report comparing apparent ADFD, NDFD, and ADLD between reduced lignin and reference alfalfa hays when fed to horses. Previous work in other species has reported significant improvements in NDFD when reduced lignin alfalfa hay was either fed to lambs (Mertens and McCaslin, 2008) or included in the ration for lactating dairy cows (Weakley et al., 2008). Similarly, studies have documented improvements in *in vitro* stem and total herbage NDFD for reduced lignin alfalfa (Guo et al., 2001; Grev et al., 2017, 2019; Getachew et al., 2018). The lack of differences in fiber digestibility between alfalfa hays in the present study could be related to several factors, including but not limited to a larger amount of variability in fiber digestion among the horses in the study (as indicated by greater SE for ADF, NDF, and ADL). Although ADFD and NDFD were not significantly greater for reduced lignin alfalfa hay in the present study, numerically there was an 8% improvement in both ADFD and NDFD for the reduced lignin alfalfa hay. This, combined with the trend for greater ADLD, the significant improvements in NDFD reported for other

species, and the increased NDFD results from several field studies, warrants further investigation into fiber digestibility for reduced lignin alfalfa forage.

### *Fecal Particle Size*

Fecal particle size distribution and MFPS did not differ between reduced lignin and reference alfalfa hay treatments (Table 4.4). For both hay treatments, MFPS was 0.89 mm, with the greatest proportion of fecal particles retained in the 1.0 to 2.0 mm sieve (31%), followed by the 0.5 to 1.0 mm sieve (29%). Mean fecal particle size results from the present study are comparable to those reported by Clauss et al. (2014), who reported MFPS ranging from 0.73 to 1.55 mm for ponies consuming grass hay at different intake levels. Similarly, Carmalt et al. (2005) and Lapinskas et al. (2017) reported MFPS ranging from 0.90 to 1.53 mm for horses consuming diets containing various combinations of hay and concentrates. Average MFPS in the present study is slightly lower than results reported by Miyaji et al. (2011), who found MFPS ranging from 1.17 to 2.97 mm for horses fed chopped or ground timothy hay. Differences in MFPS among studies are likely due to a number of aspects, including differences in feed type (i.e. hay vs. concentrate), diet nutrient composition, feeding level (i.e. *ad libitum* vs. maintenance), number and size of sieves used, sieving method (i.e. wet vs. dry), and variability between individual horses. Regardless, the lack of differences in fecal particle size distribution and MFPS between dietary treatments in the present study indicates that mastication and breakdown of feed was similar for each alfalfa hay type.

### *Mean Retention Time*

Marker concentrations for both liquid (Co) and particulate (Yb) phase matter were not above detectable concentrations at 72 h post-dose and in some instances, prior to that

time point; therefore, marker concentrations were not determined past the 72 h mark. Mean marker excretion for Co and Yb is shown in Figures 4.1 and 4.2, respectively. There was no effect of dietary treatment on marker concentration (average, AUC, peak, and TTP) or on MRT for both Co and Yb (Table 4.5). Mean retention times were similar when calculated using both Blaxter (1956) and Thielemans (1978) algebraic methods and ranged from 21.8 to 26.3 h for Co and from 25.4 to 31.8 h for Yb. There is a wide range in reported MRT in the literature for horses and ponies consuming forage or mostly forage diets, with liquid MRT ranging from 17.4 to 59.4 h and particulate MRT ranging from 21.3 to 63.7 h (Pearson and Merritt, 1991; Cuddeford et al., 1995; Drogoul et al., 2000, 2001; Pearson et al., 2001, 2006; Moore-Colyer et al., 2003; Goachet et al., 2009; Miyaji et al., 2011, 2014; Rodrigues et al., 2012; Clauss et al., 2014). This large variation in MRT is likely due to a number of different factors, including forage source, inclusion of concentrates in the diet, diet nutrient composition, feeding level, intake rate, marker and method used for MRT analysis, management strategies (i.e. time of feeding, use of hay nets, etc.), and variability between individual horses. However, the majority of reported MRT are between 19 and 34 h for liquid phase digesta and between 23 and 40 h for particulate phase digesta, both of which are ranges that encompass the values reported in the present study.

The longer MRT for Yb compared to Co indicates selective retention of particulate phase digesta within the equine gastrointestinal tract. Previous research using both liquid and particulate phase markers has also reported a longer MRT for particulate phase matter compared to liquid phase matter (Cuddeford et al., 1995; Drogoul et al., 2000; Goachet et al., 2009; Miyaji et al., 2011; Clauss et al., 2014), highlighting the

importance of utilizing markers specific to the dietary component of interest. In the present study, the lack of differences between dietary treatments for both liquid and particulate phase matter indicates that both hays traveled through the gastrointestinal tract at similar rates, regardless of alfalfa forage type. As such, any differences in apparent digestibility between forage treatments in the present study were likely not a result of differences in retention time within the gastrointestinal tract.

## SUMMARY AND CONCLUSIONS

Reduced lignin and reference alfalfa hays were similar in DM, CP, ADF, NDF, and equine DE content, while ADL concentrations were reduced by 9% for reduced lignin alfalfa hay compared to reference alfalfa hay. The nutrient profile for both hays was sufficient to meet the daily DE and CP requirements for adult horses at maintenance. Average stem length did not differ between alfalfa hay types, but L:S was greater for reduced lignin alfalfa hay compared to reference alfalfa hay.

Hay and water intakes, TTC, and DMIR were similar for horses consuming reduced lignin and reference alfalfa hays, indicating that both hay types were equally accepted by horses. Horses exhibited a 4% improvement in apparent DMD when consuming reduced lignin alfalfa hay, and there was a trend for increased ADLD for reduced lignin alfalfa hay compared to reference alfalfa hay. Apparent CPD, ADFD, and NDFD did not differ between alfalfa hay types. Similarly, fecal particle size distribution, MFPS, marker concentration, and MRT did not differ between reduced lignin and reference alfalfa hay treatments.

Altogether, these results indicate an improvement in DMD for reduced lignin alfalfa hay when fed to adult horses, with no change in forage consumption, fecal particle size, or retention time within the gastrointestinal tract. Future research should investigate changes in L:S and leaf retention for reduced lignin alfalfa hay, and should further explore changes in fiber digestibility between reduced lignin and reference alfalfa hays.

Table 4.1. Nutrient composition (DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; ADL, acid detergent lignin; DE, digestible energy) and forage characteristics of reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay fed to adult horses at maintenance.

| Nutrient | Reduced lignin (54HVX41) | Reference (WL355.RR) | *SE* |
|---|---|---|---|
| | | Nutrient Composition | |
| DM, g $kg^{-1}$ | 892 | 892 | *1.68* |
| CP, g $kg^{-1}$ | 196 | 202 | *3.53* |
| ADF, g $kg^{-1}$ | 343 | 348 | *11.70* |
| NDF, g $kg^{-1}$ | 430 | 435 | *8.06* |
| ADL, g $kg^{-1}$ | 74[b] | 81[a] | *4.73* |
| Equine DE, Mcal $kg^{-1}$ | 24 | 2.4 | *0.02* |
| | | Forage Characteristics | |
| Stem weight, % | 43.2[b] | 54.2[a] | *1.93* |
| Leaf weight, % | 56.8[a] | 45.8[b] | *1.93* |
| Leaf to stem ratio | 1.4 | 0.9 | *0.11* |
| Stem length, cm | 25.3 | 24.6 | *0.63* |

[ab]Within row, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

Table 4.2. Forage dry matter intake (DMI), water intake, total time to consumption (TTC), and DMI rate (DMIR) for adult horses fed reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay.

| Item | Reduced lignin (54HVX41) | Reference (WL355.RR) | *SE* |
|---|---|---|---|
| DMI, kg $d^{-1}$ | 8.9 | 8.8 | *0.54* |
| DMI, % of BW | 1.6 | 1.6 | *0.06* |
| DMI, g $kg^{-1}$ of $BW^{0.75}$ | 78.3 | 77.8 | *3.34* |
| Water, kg $d^{-1}$ | 34.1 | 34.5 | *2.45* |
| Water, % of BW | 6.2 | 6.3 | *0.34* |
| Water, g $kg^{-1}$ of $BW^{0.75}$ | 301.2 | 306.1 | *17.53* |
| TTC, h | 7.3 | 7.9 | *0.93* |
| DMIR, kg $h^{-1}$ | 0.72 | 0.67 | *0.11* |

[ab]Within row, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

Table 4.3. Apparent nutrient digestibility values (DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; ADL, acid detergent lignin) for reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay fed to adult horses at maintenance.

| Digestibility | Reduced lignin (54HVX41) | Reference (WL355.RR) | *SE* |
|---|---|---|---|
| DM, % | 64.4[a] | 61.7[b] | *1.00* |
| CP, % | 78.4 | 78.3 | *1.20* |
| ADF, % | 48.1 | 44.5 | *2.39* |
| NDF, % | 46.8 | 43.5 | *1.70* |
| ADL, % | 30.8 | 23.3 | *5.64* |

[ab]Within row, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

Table 4.4. Fecal particle size distribution (% of DM) and mean fecal particle size (MFPS) for adult horses fed reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay.

| Particle size distribution | Reduced lignin (54HVX41) | Reference (WL355.RR) | *SE* |
|---|---|---|---|
| >4.0 mm, % | 0.4 | 0.3 | *0.06* |
| 2.0-4.0 mm, % | 18.6 | 18.3 | *2.62* |
| 1.0-2.0 mm, % | 31.4 | 31.0 | *2.85* |
| 0.5-1.0 mm, % | 29.1 | 29.0 | *2.38* |
| 0.25-0.5 mm, % | 16.5 | 16.4 | *2.72* |
| <0.25 mm, % | 3.8 | 4.3 | *1.50* |
| MFPS, mm | 0.89 | 0.89 | *0.07* |

[ab]Within row, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

Table 4.5. Marker concentration values (average, avg; area under the curve, AUC; peak; and time to peak, TTP) and mean retention time (MRT) for liquid (Co) and particulate (Yb) phase matter in the digestive tract of adult horses fed reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay.

| Item | Reduced lignin (54HVX41) | Reference (WL355.RR) | *SE* |
|---|---|---|---|
| Liquid phase (Co) | | | |
| Avg, mg $kg^{-1}$ | 69.2 | 67.1 | *5.09* |
| AUC | 3577.4 | 4085.8 | *307.98* |
| Peak, mg $kg^{-1}$ | 254.0 | 267.2 | *24.06* |
| TTP, h | 17.2 | 20.8 | *1.34* |
| MRT[1], h | 21.8 | 25.8 | *1.66* |
| MRT[2], h | 22.2 | 26.3 | *1.62* |
| Particulate phase (Yb) | | | |
| Avg, mg $kg^{-1}$ | 65.6 | 82.0 | *113.03* |
| AUC | 2897.1 | 4143.8 | *328.75* |
| Peak, mg $kg^{-1}$ | 235.7 | 259.7 | *25.94* |
| TTP, h | 22.4 | 30.4 | *2.28* |
| MRT[1], h | 25.4 | 31.3 | *2.12* |
| MRT[2], h | 25.4 | 31.8 | *2.11* |

[ab]Within row, means without a common letter differ based on a Tukey's HSD test ($P \leq 0.05$)

[1]MRT calculated from Blaxter et al. (1956)

[2]MRT calculated from Thielemans et al. (1978)

Figure 4.1. Mean cobalt (Co) excretion for adult horses consuming reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay.

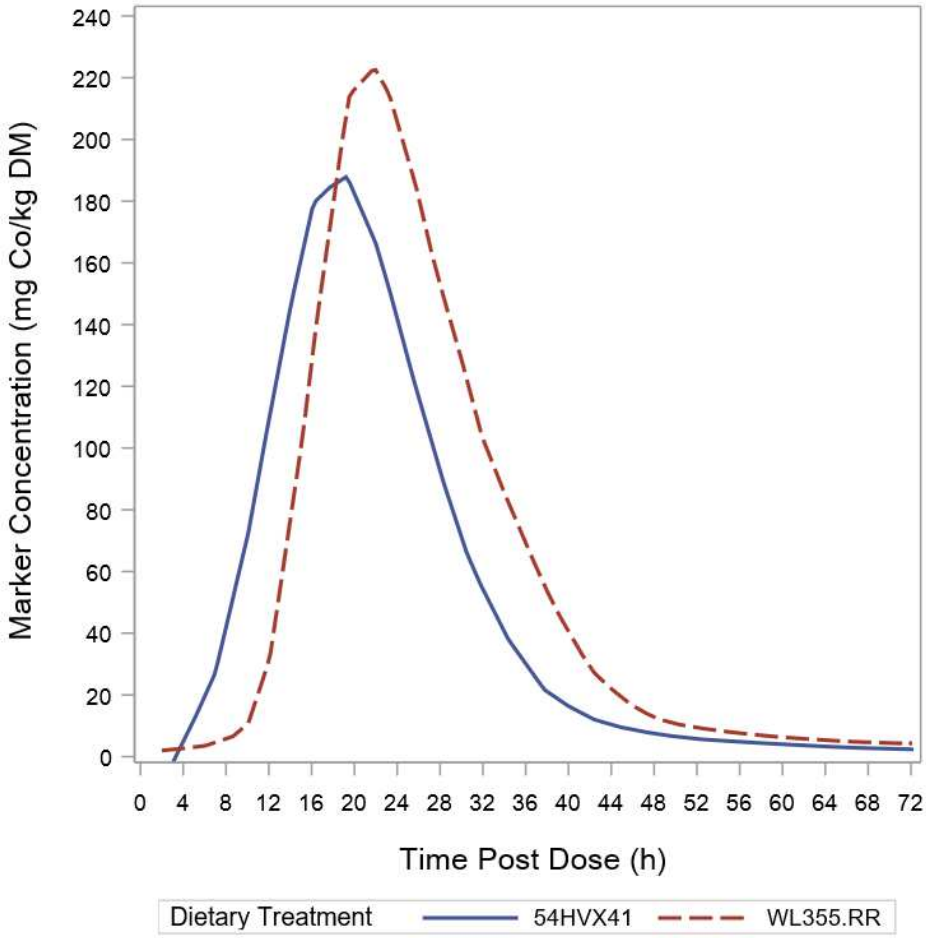
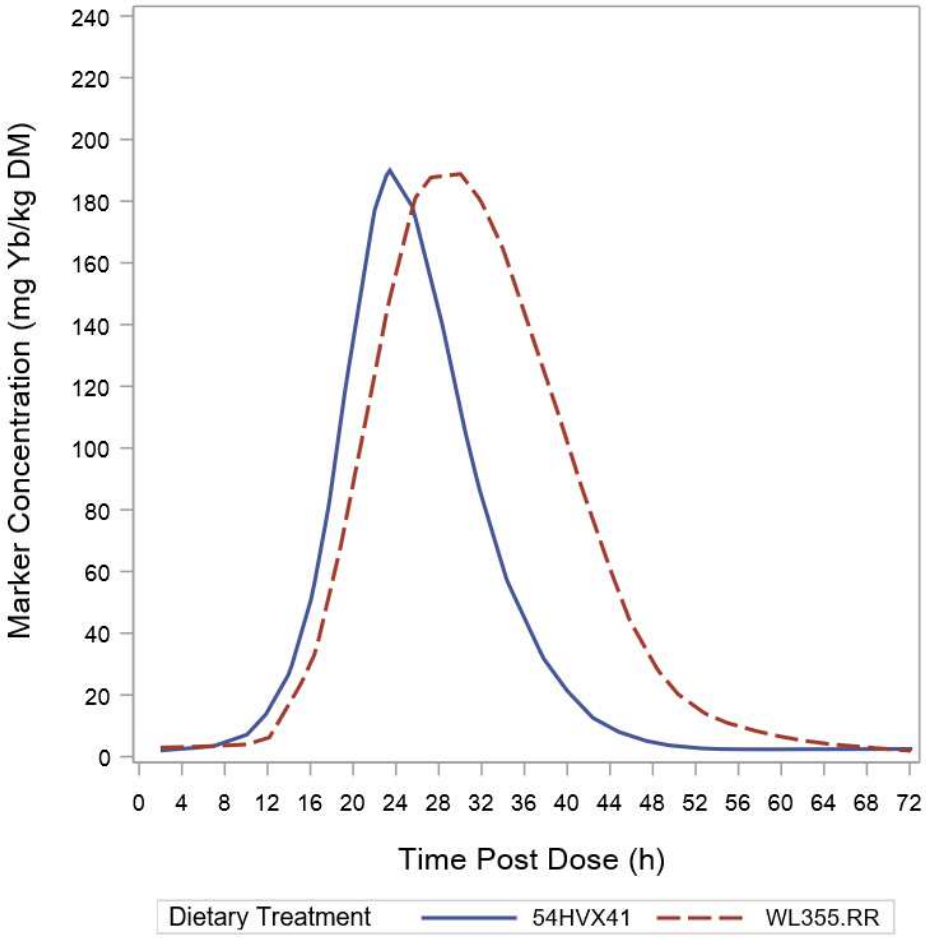


Figure 4.2. Mean ytterbium (Yb) excretion for adult horses consuming reduced lignin (54HVX41) and reference (WL355.RR) alfalfa hay.



## LITERATURE CITED


Albrecht, K.A., W.F. Wedin, and D.R. Buxton. 1987. Cell-wall composition and digestibility of alfalfa stems and leaves. Crop Science 27(4): 735–741. doi: 10.2135/cropsci1987.0011183X002700040027x.

Ankom Technology. 2013a. Acid detergent fiber in feeds filter bag technique. http://www.ankom.com/media/documents/Method_5_ADF_Method_A200_RevE_11_15_13.pdf (accessed 12 February 2014).

Ankom Technology. 2013b. Neutral detergent fiber in feeds filter bag technique. http://www.ankom.com/media/documents/Method_6_NDF_4013011_A200,A2001.pdf (accessed 12 February 2014).

AOAC. 2010. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD.

Blaxter, K.L., N.M. Graam, and F.W. Wainman. 1956. Some observations on the digestibility of food by sheep and on related problems. Brit. J. Nutrition 10: 69–91.

Buxton, D.R., and J.S. Hornstein. 1986. Cell-wall concentration and components in stratified canopies of alfalfa, birdsfoot trefoil, and red clover. Crop Science 26(1): 180–184. doi: 10.2135/cropsci1986.0011183X002600010043x.

Buxton, D.R., and J.R. Russell. 1988. Lignin Constituents and Cell-Wall Digestibility of Grass and Legume Stems. Crop Science 28(3): 553. doi: 10.2135/cropsci1988.0011183X002800030026x.

Carmalt, J.L., N.F. Cymbaluk, and H.G.G. Townsend. 2005. Effect of premolar and molar occlusal angle on feed digestibility, water balance, and fecal particle size in horses. Journal of the American Veterinary Medical Association 227(1): 110–113. doi: 10.2460/javma.2005.227.110.

Casler, M.D. 1986. Causal effects among forage yield and quality measures of smooth bromegrass. Can. J. Plant Sci. 66(3): 591–600. doi: 10.4141/cjps86-079.

Casler, M.D. 1987. In vitro digestibility of dry matter and cell wall constituents of smooth bromegrass forage. Crop Science 27(5): 931–934. doi: 10.2135/cropsci1987.0011183X002700050021x.

Casler, M.D., D.R. Buxton, and K.P. Vogel. 2002. Genetic modification of lignin concentration affects fitness of perennial herbaceous plants. Theor Appl Genet 104(1): 127–131. doi: 10.1007/s001220200015.

Clauss, M., K. Schiele, S. Ortmann, J. Fritz, D. Codron, et al. 2014. The effect of very low food intake on digestive physiology and forage digestibility in horses. J Anim Physiol Anim Nutr 98(1): 107–118. doi: 10.1111/jpn.12053.

Crozier, J.A., V.G. Allen, N.E. Jack, J.P. Fontenot, and M.A. Cochran. 1997. Digestibility, apparent mineral absorption, and voluntary intake by horses fed alfalfa, tall fescue, and caucasian bluestem. Journal of animal science 75(6): 1651–1658. doi: 10.2527/1997.7561651x.

Cuddeford, D., R.A. Pearson, R.F. Archibald, and R.H. Muirhead. 1995. Digestibility and gastro-intestinal transit time of diets containing different proportions of alfalfa and oat straw given to Thoroughbreds, Shetland ponies, Highland ponies and donkeys. Animal Science 61(2): 407–417. doi: 10.1017/S1357729800013953.

Cuddeford, D., A. Woodhead, and R. Muirhead. 1992. A comparison between the nutritive value of short-cutting cycle, high temperature-dried alfalfa and timothy hay for horses. Equine Veterinary Journal 24(2): 84–89. doi: 10.1111/j.2042-3306.1992.tb02788.x.

Digman, M., D. Undersander, K. Shinners, and C. Saxe. 2011. Best practices to hasten field drying of grasses and alfalfa. University of Wisconsin-Extension, Madison, WI.

Drogoul, C., A. de Fombelle, and V. Julliand. 2001. Feeding and microbial disorders in horses: 2: Effect of three hay:grain ratios on digesta passage rate and digestibility in ponies. Journal of Equine Veterinary Science 21(10): 487–491. doi: 10.1016/S0737-0806(01)70211-0.

Drogoul, C., C. Poncet, and J.L. Tisserand. 2000. Feeding ground and pelleted hay rather than chopped hay to ponies: 1. Consequences for in vivo digestibility and rate of passage of digesta. Animal Feed Science and Technology 87(1): 117–130. doi: 10.1016/S0377-8401(00)00187-5.

Earing, J. 2011. Comparison of Digestive Function in Young and Mature Horses. http://uknowledge.uky.edu/gradschool_diss/142.

Earing, J.E., B.D. Cassill, S.H. Hayes, E.S. Vanzant, and L.M. Lawrence. 2010. Comparison of in vitro digestibility estimates using the DaisyII incubator with in vivo digestibility estimates in horses. Journal of Animal Science 88(12): 3954–3963. doi: 10.2527/jas.2010-2989.

Edouard, N., G. Fleurance, W. Martin-Rosset, P. Duncan, J.P. Dulphy, et al. 2008. Voluntary intake and digestibility in horses: effect of forage quality with emphasis on individual variability. Animal 2(10): 1526–1533. doi: 10.1017/S1751731108002760.

Getachew, G., A.M. Ibáñez, W. Pittroff, A.M. Dandekar, M. McCaslin, et al. 2011. A comparative study between lignin down regulated alfalfa lines and their respective unmodified controls on the nutritional characteristics of hay. Animal Feed Science and Technology 170(3–4): 192–200. doi: 10.1016/j.anifeedsci.2011.09.009.

Getachew, G., E.A. Laca, D.H. Putnam, D. Witte, M. McCaslin, et al. 2018. The impact of lignin downregulation on alfalfa yield, chemical composition, and in vitro gas production. Journal of the Science of Food and Agriculture 98(11): 4205–4215. doi: 10.1002/jsfa.8942.

Gibbs, P.G., G.D. Potter, G.T. Schelling, J.L. Kreider, and C.L. Boyd. 1988. Digestion of Hay Protein in Different Segments of the Equine Digestive Tract. Journal of Animal Science 66(2): 400–406. doi: 10.2134/jas1988.662400x.

Glunk, E.C., M.R. Hathaway, W.J. Weber, C.C. Sheaffer, and K.L. Martinson. 2014. The Effect of Hay Net Design on Rate of Forage Consumption When Feeding Adult Horses. Journal of Equine Veterinary Science 34(8): 986–991. doi: 10.1016/j.jevs.2014.05.006.

Goachet, A.G., C. Philippeau, M. Varloud, and V. Julliand. 2009. Adaptations to standard approaches for measuring total tract apparent digestibility and gastro-intestinal retention time in horses in training. Animal feed science and technology 152(1): 141–151.

Grev, A.M., M.S. Wells, D.N. Catalano, K.L. Martinson, J.M. Jungers, et al. 2019. Morphology and Stem and Leaf Forage Nutritive Value for Reduced Lignin Alfalfa. Unpublished Manuscript.

Grev, A.M., M.S. Wells, D.A. Samac, K.L. Martinson, and C.C. Sheaffer. 2017. Forage Accumulation and Nutritive Value of Reduced Lignin and Reference Alfalfa Cultivars. Agronomy Journal 109(6): 2749–2761. doi: 10.2134/agronj2017.04.0237.

Guo, D., F. Chen, J. Wheeler, J. Winder, S. Selman, et al. 2001. Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. Transgenic Res 10(5): 457–464. doi: 10.1023/A:1012278106147.

Hall, M.B., W.B. Hoover, J.P. Jennings, and T.K. Miller. 1999. A method for partitioning neutral detergent soluble carbohydrates. J Sci Food Agric 79: 2079–2086.

Henneke, D.R., G.D. Potter, J.L. Kreider, and B.F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Veterinary Journal 15(4): 371–372. doi: 10.1111/j.2042-3306.1983.tb01826.x.

Julier, B., and C. Huyghe. 1997. Effect of growth and cultivar on alfalfa digestibility in a multi-site trial. Agronomie 17(9–10): 481–489.

Jung, H.G., D.R. Mertens, and A.J. Payne. 1997. Correlation of acid detergent lignin and klason lignin with digestibility of forage dry matter and neutral detergent fiber. Journal of Dairy Science 80(8): 1622–1628. doi: 10.3168/jds.S0022-0302(97)76093-4.

Jung, H.-J.G., D.A. Samac, and G. Sarath. 2012. Modifying crops to increase cell wall digestibility. Plant Science 185–186: 65–77. doi: 10.1016/j.plantsci.2011.10.014.

Kaiser, D.E., J.A. Lamb, and R. Eliason. 2011. Fertilizer Recommendations for Agronomic Crops in Minnesota: Alfalfa. https://www.extension.umn.edu/agriculture/nutrient-management/nutrient-lime-guidelines/fertilizer-recommendations-for-agronomic-crops-in-minnesota/alfalfa/ (accessed 13 March 2017).

Kalu, B.A., and G.W. Fick. 1981. Quantifying morphological development of alfalfa for studies of herbage quality. Crop Science 21(2): 267–271. doi: 10.2135/cropsci1981.0011183X002100020016x.

LaCasha, P.A., H.A. Brady, V.G. Allen, C.R. Richardson, and K.R. Pond. 1999. Voluntary intake, digestibility, and subsequent selection of Matua bromegrass, coastal bermudagrass, and alfalfa hays by yearling horses. Journal of animal science 77(10): 2766–2773. doi: 10.2527/1999.77102766x.

Lamb, J.F.S., G.J. Hans-Joachim, and H. Riday. 2012. Harvest Impacts on Alfalfa Stem Neutral Detergent Fiber Concentration and Digestibility and Cell Wall Concentration and Composition. Crop Science 52(5): 2402–2412. doi: 10.2135/cropsci2012.01.0027.

Lapinskas, S.L., K.A. Swartz, P. Ochonski, and W.B. Staniar. 2017. Quantification of dietary and fecal particle size. Journal of Equine Veterinary Science 52(Supplement C): 93–94. doi: 10.1016/j.jevs.2017.03.137.

Li, X., Y. Zhang, A. Hannoufa, and P. Yu. 2015. Transformation with TT8 and HB12 RNAi constructs in model forage (Medicago sativa, alfalfa) affects carbohydrate structure and metabolic characteristics in ruminant livestock systems. J. Agric. Food Chem. 63(43): 9590–9600. doi: 10.1021/acs.jafc.5b03717.

Littell, R.C., P.R. Henry, and C.B. Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedures. Journal of Animal Science; Champaign 76(4): 1216–31. doi: http://dx.doi.org/10.2527/1998.7641216x.

Liu, N., and P. Yu. 2011. Molecular clustering, interrelationships and carbohydrate conformation in hull and seeds among barley cultivars. Journal of Cereal Science 53(3): 379–383. doi: 10.1016/j.jcs.2011.02.011.

Marita, J.M., J. Ralph, R.D. Hatfield, D. Guo, F. Chen, et al. 2003. Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase. Phytochemistry 62(1): 53–65. doi: 10.1016/S0031-9422(02)00434-X.

Marković, J.P., R.T. Scaron, trbanović, D.V. Terzić, D.J. Djokić, et al. 2012. Changes in lignin structure with maturation of alfalfa leaf and stem in relation to ruminants nutrition. AJAR 7(2): 257–264. doi: 10.5897/AJAR11.1485.

Martin-Rosset, W., M. Doreau, S. Boulot, and N. Miraglia. 1990. Influence of level of feeding and physiological state on diet digestibility in light and heavy breed horses. Livestock Production Science 25(3): 257–264. doi: 10.1016/0301-6226(90)90021-W.

Mertens, D.R., and M. McCaslin. 2008. Evaluation of alfalfa hays with down-regulated lignin biosynthesis. Journal of Dairy Science. p. 170

Milić, D., D. Karagić, S. Vasiljević, A. Mikić, B. Mijić, et al. 2011. Leaf and stem chemical composition of divergent alfalfa cultivars. Biotechnology in Animal Husbandry 27(4): 1505–1511.

Miyaji, M., K. Ueda, H. Hata, and S. Kondo. 2011. Effects of quality and physical form of hay on mean retention time of digesta and total tract digestibility in horses. Animal Feed Science and Technology 165(1): 61–67. doi: 10.1016/j.anifeedsci.2011.02.003.

Miyaji, M., K. Ueda, H. Hata, and S. Kondo. 2014. Effect of grass hay intake on fiber digestion and digesta retention time in the hindgut of horses. Journal of Animal Science 92(4): 1574–1581. doi: 10.2527/jas.2013-6676.

Miyaji, M., K. Ueda, Y. Kobayashi, H. Hata, and S. Kondo. 2008. Fiber digestion in various segments of the hindgut of horses fed grass hay or silage. Animal Science Journal 79(3): 339–346. doi: 10.1111/j.1740-0929.2008.00535.x.

Moore-Colyer, M.J.S., H.J. Morrow, and A.C. Longland. 2003. Mathematical modelling of digesta passage rate, mean retention time and apparent digestibility of two different lengths of hay and big-bale grass silage in ponies. British Journal of Nutrition 90(1): 109–118. doi: 10.1079/BJN2003869.

NRC. 2007. Nutrient Requirements of Horses. 6th ed. The National Academies Press, Washington, D.C.

Ordakowski, A.L., D.S. Kronfeld, J.L. Holland, B.J. Hargreaves, L.S. Gay, et al. 2001. Alkanes as internal markers to estimate digestibility of hay or hay plus concentrate diets in horses. Journal of animal science 79(6): 1516–1522. doi: 10.2527/2001.7961516x.

Pagan, J.D. 1998. Measuring the digestible energy content of horse feeds. Advanced Equine Nutrition. Nottingham University Press, United Kingdom. p. 7–76

Palmgren Karlsson, C., J.E. Lindberg, and M. Rundgren. 2000. Associative effects on total tract digestibility in horses fed different ratios of grass hay and whole oats.

Livestock Production Science 65(1): 143–153. doi: 10.1016/S0301-6226(99)00178-5.

Pearson, R.A., R.F. Archibald, and R.H. Muirhead. 2001. The effect of forage quality and level of feeding on digestibility and gastrointestinal transit time of oat straw and alfalfa given to ponies and donkeys. British Journal of Nutrition 85(5): 599–606. doi: 10.1079/BJN2001321.

Pearson, R.A., R.F. Archibald, and R.H. Muirhead. 2006. A comparison of the effect of forage type and level of feeding on the digestibility and gastrointestinal mean retention time of dry forages given to cattle, sheep, ponies and donkeys. British Journal of Nutrition 95(1): 88–98. doi: 10.1079/BJN20051617.

Pearson, R.A., and J.B. Merritt. 1991. Intake, digestion and gastrointestinal transit time in resting donkeys and ponies and exercised donkeys given ad libitum hay and straw diets. Equine Veterinary Journal 23(5): 339–343. doi: 10.1111/j.2042-3306.1991.tb03734.x.

Potts, L., J. Hinkson, B. Graham, C. Löest, and J. Turner. 2010. Nitrogen Retention and Nutrient Digestibility in Geldings Fed Grass Hay, Alfalfa Hay, or Alfalfa Cubes. Journal of Equine Veterinary Science 30(6): 330–333. doi: 10.1016/j.jevs.2010.04.007.

Reddy, M.S.S., F. Chen, G. Shadle, L. Jackson, H. Aljoe, et al. 2005. Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (Medicago sativa L.). PNAS 102(46): 16573–16578. doi: 10.1073/pnas.0505749102.

Rodrigues, L.M., F.Q. de Almeida, M.B. Pereira, A.C.T. Miranda, A. Guimarães, et al. 2012. Roughage digestion evaluation in horses with total feces collection and mobile nylon bags. Revista Brasileira de Zootecnia 41(2): 341–346. doi: 10.1590/S1516-35982012000200016.

Sanderson, M.A., J.S. Hornstein, and W.F. Wedin. 1989. Alfalfa Morphological Stage and its Relation to in Situ Digestibility of Detergent Fiber Fractions of Stems. Crop Science 29(5): 1315. doi: 10.2135/cropsci1989.0011183X002900050045x.

Sewalt, V.J.H., W. Ni, H.G. Jung, and R.A. Dixon. 1997. Lignin impact on fiber degradation: Increased enzymatic digestibility of genetically engineered tobacco (Nicotiana tabacum) stems reduced in lignin content. J. Agric. Food Chem. 45(5): 1977–1983. doi: 10.1021/jf9609690.

Sheaffer, C.C., N.P. Martin, J.G. Jewett, J. Halgerson, R.D. Moon, et al. 2000. Sampling Requirements for Forage Quality Characterization of Rectangular Hay Bales. Agronomy Journal 92(1): 64. doi: 10.2134/agronj2000.92164x.

Staniar, W.B., J.R. Bussard, N.M. Repard, M.H. Hall, and A.O. Burk. 2010. Voluntary intake and digestibility of teff hay fed to horses. Journal of Animal Science 88(10): 3296–3303. doi: 10.2527/jas.2009-2668.

Sturgeon, L.S., L.A. Baker, J.L. Pipkin, J.C. Haliburton, and N.K. Chirase. 2000. The digestibility and mineral availability of Matua, Bermuda grass, and alfalfa hay in mature horses. Journal of Equine Veterinary Science 20(1): 45–48. doi: 10.1016/S0737-0806(00)80320-2.

Thielemans, M.-F., E. Francois, C. Bodart, and A. Thewis. 1978. Gastrointestinal transit in the pig: measurement using radioactive lanthanides and comparison with sheep. Ann. Biol. Anim. Biochim. Biophys 18(2A): 237–247.

Udén, P., P.E. Colucci, and P.J. Van Soest. 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. J. Sci. Food Agric. 31(7): 625–632. doi: 10.1002/jsfa.2740310702.

Weakley, D., D.R. Mertens, and M. McCaslin. 2008. Lactating cow responses to alfalfa hays with down-regulated lignin biosynthesis. Journal of Dairy Science. p. 170

Wilman, D., and P.R. Moghaddam. 1998. In vitro digestibility and neutral detergent fibre and lignin contents of plant parts of nine forage species. The Journal of Agricultural Science 131(1): 51–58.

Woodward, A.D., B.D. Nielsen, J. Liesman, T. Lavin, and N.L. Trottier. 2011. Protein quality and utilization of timothy, oat-supplemented timothy, and alfalfa at differing harvest maturities in exercised Arabian horses. Journal of Animal Science 89(12): 4081–4092. doi: 10.2527/jas.2010-3825.

Yari, M., R. Valizadeh, A.A. Naserian, G.R. Ghorbani, P. Rezvani Moghaddam, et al. 2012. Botanical traits, protein and carbohydrate fractions, ruminal degradability and energy contents of alfalfa hay harvested at three stages of maturity and in the afternoon and morning. Animal Feed Science and Technology 172(3–4): 162–170. doi: 10.1016/j.anifeedsci.2012.01.004.

## Bibliography


Abdouli, H., and S.B. Attia. 2007. Evaluation of a two-stage in vitro technique for estimating digestibility of equine feeds using horse faeces as the source of microbial inoculum. Animal Feed Science and Technology 132(1): 155–162. doi: 10.1016/j.anifeedsci.2006.03.005.

Albrecht, K.A., W.F. Wedin, and D.R. Buxton. 1987. Cell-wall composition and digestibility of alfalfa stems and leaves. Crop Science 27(4): 735–741. doi: 10.2135/cropsci1987.0011183X002700040027x.

Almeida, M.I., J. Lopez Donzeles, M.I. Leao, and P.R. Cecon. 2001. Internal and external markers to estimate the apparent digestibility of nutrients in horse diets. Proceedings of the 17th Equine Nutrition and Physiology Symposium. Equine Science Society, Lexington, KY. p. 477–481

Åman, P., and H. Graham. 1990. Chemical evaluation of polysaccharides in animal feeds. In: Wiseman, J. and Cole, D.J.A., editors, Feedstuff Evaluation. Butterworth-Heinemann. p. 161–177

Ankom Technology. 2013a. Acid detergent fiber in feeds filter bag technique. http://www.ankom.com/media/documents/Method_5_ADF_Method_A200_RevE_11_15_13.pdf (accessed 12 February 2014).

Ankom Technology. 2013b. Neutral detergent fiber in feeds filter bag technique. http://www.ankom.com/media/documents/Method_6_NDF_4013011_A200,A2001.pdf (accessed 12 February 2014).

AOAC. 2010. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD.

AOAC 990.03. 2010. Protein (Crude) in Animal Feed: Combustion Method. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD.

Applegate, C.S., and T.V. Hershberger. 1969. Evaluation of and Cecal Fermentation Techniques for Estimating the Nutritive Value of Forages for Equine. Journal of Animal Science 28(1): 18–22. doi: 10.2527/jas1969.28118x.

Austbø, D., and H. Volden. 2006. Influence of passage model and caecal cannulation on estimated passage kinetics of roughage and concentrate in the gastrointestinal tract of horses. Livestock Science 100(1): 33–43. doi: 10.1016/j.livprodsci.2005.11.002.

Baucher, M., M.A. Bernard-vailhé, B. Chabbert, J.-M. Besle, C. Opsomer, et al. 1999. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (Medicago sativa L.) and the effect on lignin composition and digestibility. Plant Mol Biol 39(3): 437–447. doi: 10.1023/A:1006182925584.

Beauchemin, K.A., and J.G. Buchanan-Smith. 1989. Evaluation of markers, sampling sites and models for estimating rates of passage of silage or hay in dairy cows. Animal Feed Science and Technology 27(1): 59–75. doi: 10.1016/0377-8401(89)90129-6.

Bernard, L., and M. Doreau. 2000. Use of rare earth elements as external markers for mean retention time measurements in ruminants. Reprod. Nutr. Dev. 40(2): 89–101. doi: 10.1051/rnd:2000122.

Blaxter, K.L., N.M. Graam, and F.W. Wainman. 1956. Some observations on the digestibility of food by sheep and on related problems. Brit. J. Nutrition 10: 69–91.

Bott, R.C., E.A. Greene, K. Koch, K.L. Martinson, P.D. Siciliano, et al. 2013. Production and Environmental Implications of Equine Grazing. Journal of Equine Veterinary Science 33(12): 1031–1043. doi: 10.1016/j.jevs.2013.05.004.

Brink, G., M. Hall, G. Shewmaker, D. Undersander, N. Martin, et al. 2010. Changes in alfalfa yield and nutritive value within individual harvest periods. Agronomy Journal 102(4): 1274–1282. doi: 10.2134/agronj2010.0080.

Brink, G.E., and G.C. Marten. 1989. Harvest management of alfalfa—Nutrient yield vs. forage quality, and relationship to persistence. Journal of Production Agriculture 2(1): 32–36. doi: 10.2134/jpa1989.0032.

Burnham, K.P., and D.R. Anderson. 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. Springer Science & Business Media, New York, NY.

Buxton, D.R., and J.S. Hornstein. 1986. Cell-wall concentration and components in stratified canopies of alfalfa, birdsfoot trefoil, and red clover. Crop Science 26(1): 180–184. doi: 10.2135/cropsci1986.0011183X002600010043x.

Buxton, D.R., J.S. Hornstein, W.F. Wedin, and G.C. Marten. 1985. Forage Quality in Stratified Canopies of Alfalfa, Birdsfoot Trefoil, and Red Clover. Crop Science 25(2): 273–279. doi: 10.2135/cropsci1985.0011183X002500020016x.

Buxton, D.R., and J.R. Russell. 1988. Lignin Constituents and Cell-Wall Digestibility of Grass and Legume Stems. Crop Science 28(3): 553. doi: 10.2135/cropsci1988.0011183X002800030026x.

Cancelado, R.E., and E.B. Radcliffe. 1979. Action Thresholds for Potato Leafhopper on Potatoes in Minnesota. J Econ Entomol 72(4): 566–569. doi: 10.1093/jee/72.4.566.

Carmalt, J.L., N.F. Cymbaluk, and H.G.G. Townsend. 2005. Effect of premolar and molar occlusal angle on feed digestibility, water balance, and fecal particle size in

horses. Journal of the American Veterinary Medical Association 227(1): 110–113. doi: 10.2460/javma.2005.227.110.

Casler, M.D. 1986. Causal effects among forage yield and quality measures of smooth bromegrass. Can. J. Plant Sci. 66(3): 591–600. doi: 10.4141/cjps86-079.

Casler, M.D. 1987. In vitro digestibility of dry matter and cell wall constituents of smooth bromegrass forage. Crop Science 27(5): 931–934. doi: 10.2135/cropsci1987.0011183X002700050021x.

Casler, M.D., D.R. Buxton, and K.P. Vogel. 2002. Genetic modification of lignin concentration affects fitness of perennial herbaceous plants. Theor Appl Genet 104(1): 127–131. doi: 10.1007/s001220200015.

Casler, M.D., and K.P. Vogel. 1999. Accomplishments and impact from breeding for increased forage nutritional value. Crop Science 39(1): 12–20. doi: 10.2135/cropsci1999.0011183X003900010003x.

Chasen, E.M., D.J. Undersander, and E.M. Cullen. 2015. Revisiting the economic injury level and economic threshold model for potato leafhopper (Hemiptera: Cicadellidae) in alfalfa. J Econ Entomol 108(4): 1748–1756. doi: 10.1093/jee/tov120.

Chavez, S.J., P.D. Siciliano, and G.B. Huntington. 2014. Intake estimation of horses grazing tall fescue or fed tall fescue hay. Journal of Animal Science 92(5): 2304–2308. doi: 10.2527/jas.2013-7119.

Clauss, M., K. Schiele, S. Ortmann, J. Fritz, D. Codron, et al. 2014. The effect of very low food intake on digestive physiology and forage digestibility in horses. J Anim Physiol Anim Nutr 98(1): 107–118. doi: 10.1111/jpn.12053.

Cochran, R.C., and M.L. Galyean. 1994. Measurement of in vivo forage digestion by ruminants. Forage Quality, Evaluation, and Utilization. ASA, CSSA, and SSSA, Madison, WI. p. 613–643

Crooker, B.A., J.H. Clark, and R.D. Shanks. 1982. Rare earth elements as markers for rate of passage measurements of individual feedstuffs through the digestive tract of ruminants. J. Nutr 112: 1353–1361.

Crozier, J.A., V.G. Allen, N.E. Jack, J.P. Fontenot, and M.A. Cochran. 1997. Digestibility, apparent mineral absorption, and voluntary intake by horses fed alfalfa, tall fescue, and caucasian bluestem. Journal of animal science 75(6): 1651–1658. doi: 10.2527/1997.7561651x.

Cuddeford, D., and D. Hughes. 1990. A comparison between chromium-mordanted hay and acid-insoluble ash to determine apparent digestibility of a chaffed, molassed hay/straw mixture. Equine Veterinary Journal 22(2): 122–125. doi: 10.1111/j.2042-3306.1990.tb04223.x.

Cuddeford, D., R.A. Pearson, R.F. Archibald, and R.H. Muirhead. 1995. Digestibility and gastro-intestinal transit time of diets containing different proportions of alfalfa and oat straw given to Thoroughbreds, Shetland ponies, Highland ponies and donkeys. Animal Science 61(2): 407–417. doi: 10.1017/S1357729800013953.

Cuddeford, D., A. Woodhead, and R. Muirhead. 1992. A comparison between the nutritive value of short-cutting cycle, high temperature-dried alfalfa and timothy hay for horses. Equine Veterinary Journal 24(2): 84–89. doi: 10.1111/j.2042-3306.1992.tb02788.x.

Digman, M., D. Undersander, K. Shinners, and C. Saxe. 2011. Best practices to hasten field drying of grasses and alfalfa. University of Wisconsin-Extension, Madison, WI.

Dove, H., and R.W. Mayes. 1991. The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. Aust. J. Agric. Res. 42(6): 913–952. doi: 10.1071/ar9910913.

Drogoul, C., A. de Fombelle, and V. Julliand. 2001. Feeding and microbial disorders in horses: 2: Effect of three hay:grain ratios on digesta passage rate and digestibility in ponies. Journal of Equine Veterinary Science 21(10): 487–491. doi: 10.1016/S0737-0806(01)70211-0.

Drogoul, C., C. Poncet, and J.L. Tisserand. 2000. Feeding ground and pelleted hay rather than chopped hay to ponies: 1. Consequences for in vivo digestibility and rate of passage of digesta. Animal Feed Science and Technology 87(1): 117–130. doi: 10.1016/S0377-8401(00)00187-5.

Earing, J. 2011. Comparison of Digestive Function in Young and Mature Horses. http://uknowledge.uky.edu/gradschool_diss/142.

Earing, J.E., B.D. Cassill, S.H. Hayes, E.S. Vanzant, and L.M. Lawrence. 2010. Comparison of in vitro digestibility estimates using the DaisyII incubator with in vivo digestibility estimates in horses. Journal of Animal Science 88(12): 3954–3963. doi: 10.2527/jas.2010-2989.

Edouard, N., G. Fleurance, W. Martin-Rosset, P. Duncan, J.P. Dulphy, et al. 2008. Voluntary intake and digestibility in horses: effect of forage quality with emphasis on individual variability. Animal 2(10): 1526–1533. doi: 10.1017/S1751731108002760.

Ehle, F.R. 1984. Influence of Feed Particle Density on Paniculate Passage from Rumen of Holstein Cow. Journal of Dairy Science 67(3): 693–697. doi: 10.3168/jds.S0022-0302(84)81354-5.

Fick, G.W., and R.S. Holthausen. 1975. Significance of Parts Other Than Blades and Stems in Leaf-stem Separations of Alfalfa Herbage 1. Crop Science 15(2): 259–262. doi: 10.2135/cropsci1975.0011183X001500020035x.

Fonnesbeck, P.V., R.K. Lydman, V. Noot, G. W, and L.D. Symons. 1967. Digestibility of the Proximate Nutrients of Forage by Horses. Journal of Animal Science 26(5): 1039–1045. doi: 10.2527/jas1967.2651039x.

Garner, H.E., D.P. Hutcheson, J.R. Coffman, A.W. Hahn, and C. Salem. 1977. Lactic Acidosis: a Factor Associated with Equine Laminitis. Journal of Animal Science 45(5): 1037–1041. doi: 10.2527/jas1977.4551037x.

Getachew, G., A.M. Ibáñez, W. Pittroff, A.M. Dandekar, M. McCaslin, et al. 2011. A comparative study between lignin down regulated alfalfa lines and their respective unmodified controls on the nutritional characteristics of hay. Animal Feed Science and Technology 170(3–4): 192–200. doi: 10.1016/j.anifeedsci.2011.09.009.

Getachew, G., E.A. Laca, D.H. Putnam, D. Witte, M. McCaslin, et al. 2018. The impact of lignin downregulation on alfalfa yield, chemical composition, and in vitro gas production. Journal of the Science of Food and Agriculture 98(11): 4205–4215. doi: 10.1002/jsfa.8942.

Gibbs, P.G., G.D. Potter, G.T. Schelling, J.L. Kreider, and C.L. Boyd. 1988. Digestion of Hay Protein in Different Segments of the Equine Digestive Tract. Journal of Animal Science 66(2): 400–406. doi: 10.2134/jas1988.662400x.

Glinsky, M.J., R.M. Smith, H.R. Spires, and C.L. Davis. 1976. Measurement of volatile fatty acid production rates in the cecum of the pony. Journal of animal science 42(6): 1465–1470.

Glunk, E.C., M.R. Hathaway, W.J. Weber, C.C. Sheaffer, and K.L. Martinson. 2014. The Effect of Hay Net Design on Rate of Forage Consumption When Feeding Adult Horses. Journal of Equine Veterinary Science 34(8): 986–991. doi: 10.1016/j.jevs.2014.05.006.

Goachet, A.G., C. Philippeau, M. Varloud, and V. Julliand. 2009. Adaptations to standard approaches for measuring total tract apparent digestibility and gastro-intestinal retention time in horses in training. Animal feed science and technology 152(1): 141–151.

Goachet, A.-G., M. Varloud, C. Philippeau, and V. Julliand. 2010. Long-term effects of endurance training on total tract apparent digestibility, total mean retention time and faecal microbial ecosystem in competing Arabian horses. Equine Veterinary Journal 42: 387–392. doi: 10.1111/j.2042-3306.2010.00188.x.

Goering, H.K., and P.J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, prcedures, and some applications). USDA Agr Handb. http://agris.fao.org/agris-search/search.do?recordID=US201301156229 (accessed 26 April 2017).

Goodson, J., W.J. Tyznik, J.H. Cline, and B.A. Dehority. 1988. Effects of an abrupt diet change from hay to concentrate on microbial numbers and physical environment in the cecum of the pony. Appl. Environ. Microbiol. 54(8): 1946–1950.

Grev, A.M., M.S. Wells, D.N. Catalano, K.L. Martinson, J.M. Jungers, et al. 2019. Morphology and Stem and Leaf Forage Nutritive Value for Reduced Lignin Alfalfa. Unpublished Manuscript.

Grev, A.M., M.S. Wells, D.A. Samac, K.L. Martinson, and C.C. Sheaffer. 2017. Forage Accumulation and Nutritive Value of Reduced Lignin and Reference Alfalfa Cultivars. Agronomy Journal 109(6): 2749–2761. doi: 10.2134/agronj2017.04.0237.

Griffin, T.S., K.A. Cassida, O.B. Hesterman, and S.R. Rust. 1994. Alfalfa Maturity and Cultivar Effects on Chemical and In Situ Estimates of Protein Degradability. Crop Science 34(6): 1654–1661. doi: 10.2135/cropsci1994.0011183X003400060043x.

Guay, K.A., H.A. Brady, V.G. Allen, K.R. Pond, D.B. Wester, et al. 2002. Matua bromegrass hay for mares in gestation and lactation. Journal of Animal Science 80(11): 2960–2966. doi: 10.2527/2002.80112960x.

Guo, D., F. Chen, K. Inoue, J.W. Blount, and R.A. Dixon. 2001a. Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: Impacts on lignin structure and implications for the biosynthesis of G and S lignin. The Plant Cell 13(1): 73–88. doi: 10.2307/3871154.

Guo, D., F. Chen, J. Wheeler, J. Winder, S. Selman, et al. 2001b. Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. Transgenic Res 10(5): 457–464. doi: 10.1023/A:1012278106147.

Hall, M.B., W.B. Hoover, J.P. Jennings, and T.K. Miller. 1999. A method for partitioning neutral detergent soluble carbohydrates. J Sci Food Agric 79: 2079–2086.

Hall, M.H., W.S. Smiles, and R.A. Dickerson. 2000. Morphological development of alfalfa cultivars selected for higher quality. Agronomy Journal 92(6): 1077–1080. doi: 10.2134/agronj2000.9261077x.

Hansen, T. 2014. Modeling Digestibility and Rate of Passage in Horses. http://uknowledge.uky.edu/animalsci_etds/37.

Hartnell, G.F., and L.D. Satter. 1979. Extent of Particulate Marker (Samarium, Lanthanum and Cerium) Movement from One Digesta Particle to Another. Journal of Animal Science 48(2): 375–380. doi: 10.2527/jas1979.482375x.

Henneke, D.R., G.D. Potter, J.L. Kreider, and B.F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Veterinary Journal 15(4): 371–372. doi: 10.1111/j.2042-3306.1983.tb01826.x.

Hintz, H.F., D.E. Hogue, E.F. Walker, J.E. Lowe, and H.F. Schryver. 1971. Apparent digestion in various segments of the digestive tract of ponies fed diets with varying roughage-grain ratios. Journal of Animal Science 32(2): 245–248.

Hintz, H.F., and R.G. Loy. 1966. Effects of Pelleting on the Nutritive Value of Horse Rations. Journal of Animal Science 25(4): 1059–1062. doi: 10.2527/jas1966.2541059x.

Hoffman, P.C., S.J. Sievert, R.D. Shaver, D.A. Welch, and D.K. Combs. 1993. In Situ Dry Matter, Protein, and Fiber Degradation of Perennial Forages. Journal of Dairy Science 76(9): 2632–2643. doi: 10.3168/jds.S0022-0302(93)77599-2.

Holland, J.L., D.S. Kronfeld, D. Sklan, and P.A. Harris. 1998. Calculation of fecal kinetics in horses fed hay or hay and concentrate. Journal of animal science 76(7): 1937–1944. doi: 10.2527/1998.7671937x.

Hussein, H.S., L.A. Vogedes, G.C.J. Fernandez, and R.L. Frankeny. 2004. Effects of cereal grain supplementation on apparent digestibility of nutrients and concentrations of fermentation end-products in the feces and serum of horses consuming alfalfa cubes. Journal of Animal Science 82(7): 1986–1996. doi: 10.2527/2004.8271986x.

Inoue, K., V.J.H. Sewalt, G.M. Ballance, W. Ni, C. Stürzer, et al. 1998. Developmental expression and substrate specificities of alfalfa caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase in relation to lignification. Plant Physiol. 117(3): 761–770. doi: 10.1104/pp.117.3.761.

Janis, C. 1976. The Evolutionary Strategy of the Equidae and the Origins of Rumen and Cecal Digestion. Evolution 30(4): 757–774. doi: 10.2307/2407816.

Jouany, J.-P., J. Gobert, B. Medina, G. Bertin, and V. Julliand. 2008. Effect of live yeast culture supplementation on apparent digestibility and rate of passage in horses fed a high-fiber or high-starch diet. Journal of Animal Science 86(2): 339–347. doi: 10.2527/jas.2006-796.

Juan, N.A., C.C. Sheaffer, D.K. Barnes, D.R. Swanson, and J.H. Halgerson. 1993. Leaf and Stem Traits and Herbage Quality of Multifoliolate Alfalfa. Agronomy Journal 85(6): 1121–1127. doi: 10.2134/agronj1993.00021962008500060005x.

Julier, B., and C. Huyghe. 1997. Effect of growth and cultivar on alfalfa digestibility in a multi-site trial. Agronomie 17(9–10): 481–489.

Julliand, V., A. de Fombelle, C. Drogoul, and E. Jacotot. 2001. Feeding and microbial disorders in horses: Part 3—Effects of three hay:grain ratios on microbial profile

and activities. Journal of Equine Veterinary Science 21(11): 543–546. doi: 10.1016/S0737-0806(01)70159-1.

Jung, H.-J.G., and J.F.S. Lamb. 2003. Identification of lucerne stem cell wall traits related to in vitro neutral detergent fibre digestibility. Animal Feed Science and Technology 110(1): 17–29. doi: 10.1016/j.anifeedsci.2003.08.003.

Jung, H.G., and J.F.S. Lamb. 2006. Stem Morphological and Cell Wall Traits Associated with Divergent In Vitro Neutral Detergent Fiber Digestibility in Alfalfa Clones. Crop Science 46(5): 2054–2061. doi: 10.2135/cropsci2005.12.0470.

Jung, H.G., D.R. Mertens, and A.J. Payne. 1997a. Correlation of acid detergent lignin and klason lignin with digestibility of forage dry matter and neutral detergent fiber. Journal of Dairy Science 80(8): 1622–1628. doi: 10.3168/jds.S0022-0302(97)76093-4.

Jung, H.-J.G., D.A. Samac, and G. Sarath. 2012. Modifying crops to increase cell wall digestibility. Plant Science 185–186: 65–77. doi: 10.1016/j.plantsci.2011.10.014.

Jung, H.G., C.C. Sheaffer, D.K. Barnes, and J.L. Halgerson. 1997b. Forage quality variation in the U.S. alfalfa core collection. Crop Science 37(4): 1361–1366. doi: 10.2135/cropsci1997.0011183X003700040054x.

Kaiser, D.E., J.A. Lamb, and R. Eliason. 2011. Fertilizer Recommendations for Agronomic Crops in Minnesota: Alfalfa. https://www.extension.umn.edu/agriculture/nutrient-management/nutrient-lime-guidelines/fertilizer-recommendations-for-agronomic-crops-in-minnesota/alfalfa/ (accessed 13 March 2017).

Kallenbach, R.L., C.J. Nelson, and J.H. Coutts. 2002. Yield, quality, and persistence of grazing- and hay-type alfalfa under three harvest frequencies. Agronomy Journal 94(5): 1094–1103. doi: 10.2134/agronj2002.1094.

Kalu, B.A., and G.W. Fick. 1981. Quantifying morphological development of alfalfa for studies of herbage quality. Crop Science 21(2): 267–271. doi: 10.2135/cropsci1981.0011183X002100020016x.

Kalu, B.A., and G.W. Fick. 1983. Morphological stage of development as a predictor of alfalfa herbage quality. Crop Science 23(6): 1167–1172. doi: 10.2135/cropsci1983.0011183X002300060033x.

Kilcher, M.R., and D.H. Heinrichs. 1974. Contribution of Stems and Leaves to the Yield and Nutrient Level of Irrigated Alfalfa at Different Stages of Development. Can. J. Plant Sci. 54(4): 739–742. doi: 10.4141/cjps74-125.

Koller, B.L., H.F. Hintz, J.B. Robertson, and P.J. Van Soest. 1978. Comparative Cell Wall and Dry Matter Digestion in the Cecum of the Pony and the Rumen of the

Cow Using and Nylon Bag Techniques. Journal of Animal Science 47(1): 209–215. doi: 10.2527/jas1978.471209x.

LaCasha, P.A., H.A. Brady, V.G. Allen, C.R. Richardson, and K.R. Pond. 1999. Voluntary intake, digestibility, and subsequent selection of Matua bromegrass, coastal bermudagrass, and alfalfa hays by yearling horses. Journal of animal science 77(10): 2766–2773. doi: 10.2527/1999.77102766x.

Lamb, J.F.S., G.J. Hans-Joachim, and H. Riday. 2012. Harvest Impacts on Alfalfa Stem Neutral Detergent Fiber Concentration and Digestibility and Cell Wall Concentration and Composition. Crop Science 52(5): 2402–2412. doi: 10.2135/cropsci2012.01.0027.

Lamb, J.F.S., H.-J.G. Jung, and D.A. Samac. 2014. Environmental Variability and/or Stability of Stem Fiber Content and Digestibility in Alfalfa. Crop Science 54(6): 2854–2863. doi: 10.2135/cropsci2014.04.0323.

Lamb, J.F.S., H.-J.G. Jung, C.C. Sheaffer, and D.A. Samac. 2007. Alfalfa Leaf Protein and Stem Cell Wall Polysaccharide Yields under Hay and Biomass Management Systems. Crop Science 47(4): 1407–1415. doi: 10.2135/cropsci2006.10.0665.

Lamb, J.F.S., C.C. Sheaffer, and D.A. Samac. 2003. Population Density and Harvest Maturity Effects on Leaf and Stem Yield in Alfalfa. Agronomy Journal 95(3): 635–641. doi: 10.2134/agronj2003.6350.

Lapinskas, S.L., K.A. Swartz, P. Ochonski, and W.B. Staniar. 2017. Quantification of dietary and fecal particle size. Journal of Equine Veterinary Science 52(Supplement C): 93–94. doi: 10.1016/j.jevs.2017.03.137.

Lattimer, J.M., S.R. Cooper, D.W. Freeman, and D.L. Lalman. 2007. Effect of yeast culture on in vitro fermentation of a high-concentrate or high-fiber diet using equine fecal inoculum in a Daisy II incubator. Journal of Animal Science 85(10): 2484–2491. doi: 10.2527/jas.2006-655.

Ledoux, D.R., J.E. Williams, T.E. Stroud, and M. Ellersieck. 1985. Ytterbium as a particulate phase marker for measurement of digesta passage rate in ruminants. Nutrition reports international 32(1): 111–115.

Lewis, L.D. 1995. Equine clinical nutrition: feeding and care. Williams & Wilkins, Baltimore, MD.

Li, Z., Z. Li, D. Combs, and D. Undersander. 2015a. Effect of reduced lignin alfalfa on forage quality at three harvest intervals. Journal of Animal Science/Journal of Dairy Science, Orlando, FL. p. 675

Li, X., Y. Zhang, A. Hannoufa, and P. Yu. 2015b. Transformation with TT8 and HB12 RNAi constructs in model forage (Medicago sativa, alfalfa) affects carbohydrate

structure and metabolic characteristics in ruminant livestock systems. J. Agric. Food Chem. 63(43): 9590–9600. doi: 10.1021/acs.jafc.5b03717.

Lindberg, J.E., B. Essen-Gustavsson, K. Dahlborn, M. Gottlieb-Vedi, and A. Jansson. 2006. Exercise response, metabolism at rest and digestibility in athletic horses fed high-fat oats. Equine Veterinary Journal 38(S36): 626–630.

Littell, R.C., P.R. Henry, and C.B. Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedures. Journal of Animal Science; Champaign 76(4): 1216–31. doi: http://dx.doi.org/10.2527/1998.7641216x.

Liu, N., and P. Yu. 2011. Molecular clustering, interrelationships and carbohydrate conformation in hull and seeds among barley cultivars. Journal of Cereal Science 53(3): 379–383. doi: 10.1016/j.jcs.2011.02.011.

Lowman, R.S., M.K. Theodorou, J.J. Hyslop, M.S. Dhanoa, and D. Cuddeford. 1999. Evulation of an in vitro batch culture technique for estimating the in vivo digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum. Animal Feed Science and Technology 80(1): 11–27. doi: 10.1016/S0377-8401(99)00039-5.

Luckett, C.R., and T.J. Klopfenstein. 1970. Leaf-To-Stem Ratio and Composition of Alfalfa from Five Harvesting Systems. Journal of Animal Science 31(1): 126–129. doi: 10.2527/jas1970.311126x.

Mader, T.L., R.G. Teeter, and G.W. Horn. 1984. Comparison of Forage Labeling Techniques for Conducting Passage Rate Studies. Journal of Animal Science 58(1): 208–212. doi: 10.2527/jas1984.581208x.

Marita, J.M., J. Ralph, R.D. Hatfield, D. Guo, F. Chen, et al. 2003. Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase. Phytochemistry 62(1): 53–65. doi: 10.1016/S0031-9422(02)00434-X.

Marković, J.P., R.T. Scaron, trbanović, D.V. Terzić, D.J. Djokić, et al. 2012. Changes in lignin structure with maturation of alfalfa leaf and stem in relation to ruminants nutrition. AJAR 7(2): 257–264. doi: 10.5897/AJAR11.1485.

Martin-Rosset, W., M. Doreau, S. Boulot, and N. Miraglia. 1990. Influence of level of feeding and physiological state on diet digestibility in light and heavy breed horses. Livestock Production Science 25(3): 257–264. doi: 10.1016/0301-6226(90)90021-W.

Martin-Rosset, W., and J.P. Dulphy. 1987. Digestibility interactions between forages and concentrates in horses: Influence of feeding level - Comparison with sheep. Livestock Production Science 17: 263–276. doi: 10.1016/0301-6226(87)90071-6.

Mayes, R.W., and H. Dove. 2000. Measurement of dietary nutrient intake in free-ranging mammalian herbivores. Nutrition Research Reviews 13(1): 107–138. doi: 10.1079/095442200108729025.

Medina, B., I.D. Girard, E. Jacotot, and V. Julliand. 2002. Effect of a preparation of on microbial profiles and fermentation patterns in the large intestine of horses fed a high fiber or a high starch diet. Journal of Animal Science 80(10): 2600–2609. doi: 10.2527/2002.80102600x.

Mertens, D.R., and L.O. Ely. 1982. Relationship of Rate and Extent of Digestion to Forage Utilization-A Dynamic Model Evaluation. Journal of Animal Science 54(4): 895–905. doi: 10.2527/jas1982.544895x.

Mertens, D.R., and M. McCaslin. 2008. Evaluation of alfalfa hays with down-regulated lignin biosynthesis. Journal of Dairy Science. p. 170

Milić, D., D. Karagić, S. Vasiljević, A. Mikić, B. Mijić, et al. 2011. Leaf and stem chemical composition of divergent alfalfa cultivars. Biotechnology in Animal Husbandry 27(4): 1505–1511.

Min, D. 2016. Effects of cutting interval between harvests on dry matter yield and nutritive value in alfalfa. American Journal of Plant Sciences 07(08): 1226–1231. doi: 10.4236/ajps.2016.78118.

Miraglia, N., D. Bergero, B. Bassano, M. Tarantola, and G. Ladetto. 1999. Studies of apparent digestibility in horses and the use of internal markers. Livestock Production Science 60(1): 21–25. doi: 10.1016/S0301-6226(99)00043-3.

Miyaji, M., K. Ueda, H. Hata, and S. Kondo. 2011. Effects of quality and physical form of hay on mean retention time of digesta and total tract digestibility in horses. Animal Feed Science and Technology 165(1): 61–67. doi: 10.1016/j.anifeedsci.2011.02.003.

Miyaji, M., K. Ueda, H. Hata, and S. Kondo. 2014. Effect of grass hay intake on fiber digestion and digesta retention time in the hindgut of horses. Journal of Animal Science 92(4): 1574–1581. doi: 10.2527/jas.2013-6676.

Miyaji, M., K. Ueda, Y. Kobayashi, H. Hata, and S. Kondo. 2008a. Fiber digestion in various segments of the hindgut of horses fed grass hay or silage. Animal Science Journal 79(3): 339–346. doi: 10.1111/j.1740-0929.2008.00535.x.

Miyaji, M., K. Ueda, H. Nakatsuji, T. Tomioka, Y. Kobayashi, et al. 2008b. Mean retention time of digesta in the different segments of the equine hindgut. Animal Science Journal 79(1): 89–96. doi: 10.1111/j.1740-0929.2007.00502.x.

Moore, J.E., and D.J. Undersander. 2002. Relative forage quality: An alternative to relative feed value and quality index. Proceedings of the 13th Annual Florida

Ruminant Nutrition Symposium. University of Florida Extension, Gainesville, Florida. p. 16–32

Moore-Colyer, M.J.S., H.J. Morrow, and A.C. Longland. 2003. Mathematical modelling of digesta passage rate, mean retention time and apparent digestibility of two different lengths of hay and big-bale grass silage in ponies. British Journal of Nutrition 90(1): 109–118. doi: 10.1079/BJN2003869.

Morrison, I.M. 1979. Carbohydrate chemistry and rumen digestion. Proceedings of the Nutrition Society 38(03): 269–274. doi: 10.1079/PNS19790048.

Mowat, D.N., R.S. Fulkerson, W.E. Tossell, and J.E. Winch. 1965. The in vitro digestibility and protein content of leaf and stem portions of forages. Can. J. Plant Sci. 45(4): 321–331. doi: 10.4141/cjps65-065.

Nordkvist, E., and P. Åman. 1986. Changes during growth in anatomical and chemical composition and in-vitro degradability of lucerne. J. Sci. Food Agric. 37(1): 1–7. doi: 10.1002/jsfa.2740370102.

NRC. 2007. Nutrient Requirements of Horses. 6th ed. The National Academies Press, Washington, D.C.

Nyberg, M.A., G.D. Potter, P.G. Gibbs, J. Schumacher, M. Murray-Gerzik, et al. 1995. Flow rate through the equine small intestine determined with soluble and insoluble indicators. 14th Equine Nutrition ans Physiology Symposium (ENPS Ed.). p. 36–41

Oba, M., and M.S. Allen. 1999. Evaluation of the importance of the digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. Journal of Dairy Science 82(3): 589–596. doi: 10.3168/jds.S0022-0302(99)75271-9.

Ordakowski, A.L., D.S. Kronfeld, J.L. Holland, B.J. Hargreaves, L.S. Gay, et al. 2001. Alkanes as internal markers to estimate digestibility of hay or hay plus concentrate diets in horses. Journal of animal science 79(6): 1516–1522. doi: 10.2527/2001.7961516x.

Orton, R.K., I.D. Hume, and R.A. Leng. 1985. Effects of exercise and level of dietary protein on digestive function in horses. Equine Veterinary Journal 17(5): 386–390. doi: 10.1111/j.2042-3306.1985.tb02530.x.

Owens, F.N., and C.F. Hanson. 1992. External and Internal Markers for Appraising Site and Extent of Digestion in Ruminants1. Journal of Dairy Science 75(9): 2605–2617. doi: 10.3168/jds.S0022-0302(92)78023-0.

Pagan, J.D. 1998. Measuring the digestible energy content of horse feeds. Advanced Equine Nutrition. Nottingham University Press, United Kingdom. p. 7–76

Pagan, J.D., P. Harris, T. Brewster-Barnes, S.E. Duren, and S.G. Jackson. 1998. Exercise Affects Digestibility and Rate of Passage of All-Forage and Mixed Diets in Thoroughbred Horses. J. Nutr. 128(12): 2704S-2707S.

Palmgren Karlsson, C., J.E. Lindberg, and M. Rundgren. 2000. Associative effects on total tract digestibility in horses fed different ratios of grass hay and whole oats. Livestock Production Science 65(1): 143–153. doi: 10.1016/S0301-6226(99)00178-5.

Palmonari, A., M. Fustini, G. Canestrari, E. Grilli, and A. Formigoni. 2014. Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content. Journal of Dairy Science 97(12): 7729–7734. doi: 10.3168/jds.2014-8123.

Parkins, J.J., D.H. Snow, and S. Adams. 1982. The apparent digestibility of "complete diet" cubes given to Thoroughbred horses and the use of chromic oxide as an inert faecal marker. Br. Vet. J. 138(4): 350–355.

Pearson, R.A., R.F. Archibald, and R.H. Muirhead. 2001. The effect of forage quality and level of feeding on digestibility and gastrointestinal transit time of oat straw and alfalfa given to ponies and donkeys. British Journal of Nutrition 85(5): 599–606. doi: 10.1079/BJN2001321.

Pearson, R.A., R.F. Archibald, and R.H. Muirhead. 2006. A comparison of the effect of forage type and level of feeding on the digestibility and gastrointestinal mean retention time of dry forages given to cattle, sheep, ponies and donkeys. British Journal of Nutrition 95(1): 88–98. doi: 10.1079/BJN20051617.

Pearson, R.A., and J.B. Merritt. 1991. Intake, digestion and gastrointestinal transit time in resting donkeys and ponies and exercised donkeys given ad libitum hay and straw diets. Equine Veterinary Journal 23(5): 339–343. doi: 10.1111/j.2042-3306.1991.tb03734.x.

Penning, P.D., and R.H. Johnson. 1983a. The use of internal markers to estimate herbage digestibility and intake: 1. Potentially indigestible cellulose and acid insoluble ash. The Journal of Agricultural Science 100(1): 127–131. doi: 10.1017/S0021859600032512.

Penning, P.D., and R.H. Johnson. 1983b. The use of internal markers to estimate herbage digestibility and intake: 2. Indigestible acid detergent fibre. The Journal of Agricultural Science 100(1): 133–138. doi: 10.1017/S0021859600032524.

Poore, M.H., J.A. Moore, and R.S. Swingle. 1990. Differential passage rates and digestion of neutral detergent fiber from grain and forages in 30, 60 and 90% concentrate diets fed to steers. Journal of animal science 68(9): 2965–2973. doi: 10.2527/1990.6892965x.

Potter, G.D., F.F. Arnold, D.D. Householder, D.H. Hansen, and K.M. Brown. 1992. Digestion of starch in the small or large intestine of the equine. Pferdeheilkunde 1(4): 107–111.

Potts, L., J. Hinkson, B. Graham, C. Löest, and J. Turner. 2010. Nitrogen Retention and Nutrient Digestibility in Geldings Fed Grass Hay, Alfalfa Hay, or Alfalfa Cubes. Journal of Equine Veterinary Science 30(6): 330–333. doi: 10.1016/j.jevs.2010.04.007.

Probst, A., and R. Smith. 2011. Harvest frequency effects on yield, persistence, and regrowth rate among new alfalfa cultivars. Forage and Grazinglands 9(1): 1–8. doi: 10.1094/FG-2011-0926-01-RS.

Putnam, D.H., S. Orloff, and L.R. Teuber. 2005. Strategies for balancing quality and yield using cutting schedules and varieties. Proceedings of the 35th California Alfalfa & Forage Symposium. University of California Cooperative Extension, Visalia, California. p. 237–252

Reddy, M.S.S., F. Chen, G. Shadle, L. Jackson, H. Aljoe, et al. 2005. Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (Medicago sativa L.). PNAS 102(46): 16573–16578. doi: 10.1073/pnas.0505749102.

Rodrigues, L.M., F.Q. de Almeida, M.B. Pereira, A.C.T. Miranda, A. Guimarães, et al. 2012. Roughage digestion evaluation in horses with total feces collection and mobile nylon bags. Revista Brasileira de Zootecnia 41(2): 341–346. doi: 10.1590/S1516-35982012000200016.

Rosenfeld, I., and D. Austbø. 2009. Effect of type of grain and feed processing on gastrointestinal retention times in horses. Journal of Animal Science 87(12): 3991–3996. doi: 10.2527/jas.2008-1150.

Rosenfeld, I., D. Austbø, and H. Volden. 2006. Models for estimating digesta passage kinetics in the gastrointestinal tract of the horse. Journal of Animal Science 84(12): 3321–3328. doi: 10.2527/jas.2006-039.

Sales, J. 2012. A review on the use of indigestible dietary markers to determine total tract apparent digestibility of nutrients in horses. Animal Feed Science and Technology 174(3): 119–130. doi: 10.1016/j.anifeedsci.2012.03.005.

Sanderson, M.A., J.S. Hornstein, and W.F. Wedin. 1989. Alfalfa Morphological Stage and its Relation to in Situ Digestibility of Detergent Fiber Fractions of Stems. Crop Science 29(5): 1315. doi: 10.2135/cropsci1989.0011183X002900050045x.

Sanderson, M.A., and W.F. Wedin. 1988. Cell wall composition of alfalfa stems at similar morphological stages and chronological age during spring growth and summer regrowth. Crop Science 28(2): 342–347. doi: 10.2135/cropsci1988.0011183X002800020034x.

Schaafstra, F.J.W.C., D.A. van Doorn, J.T. Schonewille, F.C. Wartena, M.V. Zoon, et al. 2015. Evaluation of methodological aspects of digestibility measurements in ponies fed different grass hays. Journal of Animal Science 93(10): 4742. doi: 10.2527/jas.2015-9240.

Schneider, B.H., and W.P. Flatt. 1975. The evaluation of feeds through digestibility experiments. University of Georgia Press, Athens, GA.

Schnurr, J.A., H.-J.G. Jung, and D.A. Samac. 2007. A Comparative Study of Alfalfa and Stem Traits: Morphology, Chemical Composition, and Ruminal Digestibility. Crop Science 47(4): 1672–1680. doi: 10.2135/cropsci2006.12.0762.

Sellers, A.F., and J.E. Lowe. 1986. Review of large intestinal motility and mechanisms of impaction in the horse. Equine Veterinary Journal 18(4): 261–263. doi: 10.1111/j.2042-3306.1986.tb03621.x.

Sewalt, V.J.H., W. Ni, H.G. Jung, and R.A. Dixon. 1997. Lignin impact on fiber degradation: Increased enzymatic digestibility of genetically engineered tobacco (Nicotiana tabacum) stems reduced in lignin content. J. Agric. Food Chem. 45(5): 1977–1983. doi: 10.1021/jf9609690.

Sheaffer, C.C., D. Cash, N.J. Ehlke, J.C. Henning, J.G. Jewett, et al. 1998. Entry × Environment Interactions for Alfalfa Forage Quality. Agronomy Journal 90(6): 774–780. doi: 10.2134/agronj1998.00021962009000060010x.

Sheaffer, C.C., G.D. Lacefield, and V.L. Marble. 1988. Cutting schedules and stands. Alfalfa and Alfalfa Improvement. ASA, CSSA, and SSSA, Madison, WI. p. 411–437

Sheaffer, C.C., and G.C. Marten. 1990. Alfalfa cutting frequency and date of fall cutting. Journal of Production Agriculture 3(4): 486–491. doi: 10.2134/jpa1990.0486.

Sheaffer, C.C., N.P. Martin, J.G. Jewett, J. Halgerson, R.D. Moon, et al. 2000a. Sampling Requirements for Forage Quality Characterization of Rectangular Hay Bales. Agronomy Journal 92(1): 64. doi: 10.2134/agronj2000.92164x.

Sheaffer, C.C., N.P. Martin, J.F.S. Lamb, G.R. Cuomo, J.G. Jewett, et al. 2000b. Leaf and stem properties of alfalfa entries. Agronomy Journal 92(4): 733–739. doi: 10.2134/agronj2000.924733x.

Sheaffer, C.C., M.S. Wells, and J. Larson. 2016. Alfalfa Field Crop Trials. Minnesota Agricultural Experiment Station. https://www.maes.umn.edu/publications/field-crop-trials/alfalfa (accessed 3 March 2017).

Smith, S.R., J.H. Bouton, and C.S. Hoveland. 1989. Alfalfa Persistence and Regrowth Potential under Continuous Grazing. Agronomy Journal 81(6): 960–965. doi: 10.2134/agronj1989.00021962008100060023x.

Smolders, E.A.A., A. Steg, and V.A. Hindle. 1990. Organic matter digestibility in horses and its prediction. Netherlands Journal of Agricultural Science 38(3B): 435–447.

Staniar, W.B., J.R. Bussard, N.M. Repard, M.H. Hall, and A.O. Burk. 2010. Voluntary intake and digestibility of teff hay fed to horses. Journal of Animal Science 88(10): 3296–3303. doi: 10.2527/jas.2009-2668.

Stevens, D.M., J.V. Ryssen, and J.P. Marais. 2002. The use of n-alkane markers to estimate the intake and apparent digestibility of ryegrass and Kikuyu by horses. South African Journal of Animal Science 32(1): 50–56. doi: 10.4314/sajas.v32i1.3791.

Sturgeon, L.S., L.A. Baker, J.L. Pipkin, J.C. Haliburton, and N.K. Chirase. 2000. The digestibility and mineral availability of Matua, Bermuda grass, and alfalfa hay in mature horses. Journal of Equine Veterinary Science 20(1): 45–48. doi: 10.1016/S0737-0806(00)80320-2.

Teeter, R.G., F.N. Owens, and T.L. Mader. 1984. Ytterbium Chloride as a Marker for Particulate Matter in the Rumen. Journal of Animal Science 58(2): 465–473. doi: 10.2527/jas1984.582465x.

Thielemans, M.-F., E. Francois, C. Bodart, and A. Thewis. 1978. Gastrointestinal transit in the pig: measurement using radioactive lanthanides and comparison with sheep. Ann. Biol. Anim. Biochim. Biophys 18(2A): 237–247.

Tilley, J.M.A., and R.A. Terry. 1963. A Two-Stage Technique for the in Vitro Digestion of Forage Crops. Grass and Forage Science 18(2): 104–111. doi: 10.1111/j.1365-2494.1963.tb00335.x.

Todd, L.K., W.C. Sauer, R.J. Christopherson, R.J. Coleman, and W.R. Caine. 1995. The effect of level of feed intake on nutrient and energy digestibilities and rate of feed passage in horses. Journal of Animal Physiology and Animal Nutrition 73(1–5): 140–148. doi: 10.1111/j.1439-0396.1995.tb00413.x.

Tremblay, G.F., G. Bélanger, K.B. McRae, and R. Michaud. 2002. Leaf and stem dry matter digestibility and ruminal undegradable proteins of alfalfa cultivars. Can. J. Plant Sci. 82(2): 383–393. doi: 10.4141/P01-122.

Udén, P., P.E. Colucci, and P.J. Van Soest. 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. J. Sci. Food Agric. 31(7): 625–632. doi: 10.1002/jsfa.2740310702.

Undersander, D. 2016. 2016 Alfalfa Trial Results. 2016 Alfalfa Trial Results. http://fyi.uwex.edu/forage/alfalfa-trial-results-2016/ (accessed 5 April 2017).

Undersander, D., D. Cosgrove, E. Cullen, C. Grau, M. Rice, et al. 2011. Alfalfa Management Guide. https://www.agronomy.org/files/publications/alfalfa-management-guide.pdf (accessed 23 February 2017).

Undersander, D., M. McCaslin, C. Sheaffer, D. Whalen, D. Miller, et al. 2009. Low lignin alfalfa: Redefining the yield/quality tradeoff. UC Cooperative Extension, Reno, Nevada. p. 1–4

Van Soest, P.J., J.B. Robertson, and B.A. Lewis. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. Journal of Dairy Science 74(10): 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2.

Van Soest, P.J., R.H. Wine, and L.A. Moore. 1966. Estimation of the true digestibility of forages by the in vitro digestion of cell walls. Estimation of the true digestibility of forages by the in vitro digestion of cell walls. https://www.cabdirect.org/cabdirect/abstract/19670700081 (accessed 4 September 2017).

Van Weyenberg, S., J. Sales, and G.P.J. Janssens. 2006. Passage rate of digesta through the equine gastrointestinal tract: A review. Livestock Science 99(1): 3–12. doi: 10.1016/j.livprodsci.2005.04.008.

Vogel, K.P., and R.A. Masters. 2001. Frequency grid: A simple tool for measuring grassland establishment. Journal of Range Management 54(6): 653–655. doi: 10.2307/4003666.

Weakley, D., D.R. Mertens, and M. McCaslin. 2008. Lactating cow responses to alfalfa hays with down-regulated lignin biosynthesis. Journal of Dairy Science. p. 170

Weir, W.C., L.G. Jones, and J.H. Meyer. 1960. Effect of cutting interval and stage of maturity on the digestibility and yield of alfalfa. Journal of Animal Science 19(1): 5–19. doi: 10.2527/jas1960.1915.

Wells, M.S., K.L. Martinson, D.J. Undersander, and C.C. Sheaffer. 2014. A survey investigating alfalfa winter injury in Minnesota and Wisconsin from the winter of 2012-2013. Forage and Grazinglands 12(1): 1–7. doi: 10.2134/FG-2013-0051-RS.

Wilman, D., and P.R. Moghaddam. 1998. In vitro digestibility and neutral detergent fibre and lignin contents of plant parts of nine forage species. The Journal of Agricultural Science 131(1): 51–58.

Woodward, A.D., B.D. Nielsen, J. Liesman, T. Lavin, and N.L. Trottier. 2011. Protein quality and utilization of timothy, oat-supplemented timothy, and alfalfa at differing harvest maturities in exercised Arabian horses. Journal of Animal Science 89(12): 4081–4092. doi: 10.2527/jas.2010-3825.

Yari, M., R. Valizadeh, A.A. Naserian, G.R. Ghorbani, P. Rezvani Moghaddam, et al. 2012. Botanical traits, protein and carbohydrate fractions, ruminal degradability and energy contents of alfalfa hay harvested at three stages of maturity and in the afternoon and morning. Animal Feed Science and Technology 172(3–4): 162–170. doi: 10.1016/j.anifeedsci.2012.01.004.

Yari, M., R. Valizadeh, A.A. Naserian, A. Jonker, A. Azarfar, et al. 2014. Effects of including alfalfa hay cut in the afternoon or morning at three stages of maturity in high concentrate rations on dairy cow performance, diet digestibility and feeding behavior. Animal Feed Science and Technology 192: 62–72. doi: 10.1016/j.anifeedsci.2014.04.001.

Yu, P., D.A. Christensen, J.J. McKinnon, and J.D. Markert. 2003a. Effect of variety and maturity stage on chemical composition, carbohydrate and protein subfractions, in vitro rumen degradability and energy values of timothy and alfalfa. Can. J. Anim. Sci. 83(2): 279–290. doi: 10.4141/A02-053.

Yu, P., D.A. Christensen, J.J. McKinnon, and J.D. Markert. 2003b. Effect of variety and maturity stage on chemical composition, carbohydrate and protein subfractions, in vitro rumen degradability and energy values of timothy and alfalfa. Can. J. Anim. Sci. 83(2): 279–290. doi: 10.4141/A02-053.