Solubilization of nonstructural carbohydrates as a function of soaking interval and water temperature in southern forages commonly fed to equids

by

Brooklyne Michele Wassel

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 7, 2017

Keywords: alfalfa, bermudagrass, hay soaking, perennial peanut, southern forages, total nonstructural carbohydrate

Approved by

Elizabeth L. Wagner, Chair, Associate Professor of Animal Sciences
Russell B. Muntifering, Professor of Animal Sciences
Bernhard Kaltenboeck, Professor of Veterinary Microbiology
Courteney M. Holland, Equine Extension Specialist

Abstract

Nonstructural carbohydrates (NSC) play a major role in the diet of equids prone to laminitis or afflicted with metabolic conditions such as insulin resistance. Although the threshold of <10% dietary NSC has been set as a guideline for these horses, the execution of feeding below this limit is not fully understood due to the complexities by which water leaches NSC from plants. A study was conducted to determine kinetic characteristics of NSC and DM solubilization in forages commonly fed in the southeastern USA to ascertain appropriate feeding management practices. Samples (180 g) of 4 hays: alfalfa (*Medicago sativa*), perennial peanut (*Arachis glabrata*), and Coastal and Tifton-85 varieties of bermudagrass (*Cynodon dactylon*) were evaluated in 50°C and 28°C soaking liquor at 0-, 15-, 30-, 60-, 120-, 360-, and 720-min soaking intervals. Bale was defined as a replicate with a minimum of 5 replicates per treatment. Samples were dried, ground, and analyzed for total nonstructural carbohydrates (TNC) using wet chemistry. Nonlinear regression models were constructed utilizing JMP Pro 12 (SAS Inc.) to measure the percentage of TNC and DM remaining after each soaking interval. Significance was set at $P < 0.05$ to determine the effect of treatment on the regression model. Soaking interval was significant with respect to TNC loss for all hay types except alfalfa which trended toward significance ($P = 0.07$). Water temperature had no effect on loss of TNC. Solubilization prediction equations were created for each hay. Percentage of remaining TNC was defined by [a + b * Exp (c * t)] where a is the point at which TNC solubilization is complete, b is the potentially soluble TNC fraction, c is rate of solubilization as a function of time, and t is the

soaking interval. The grass hays reached maximum solubilization of TNC within approximately 2 h whereas the legumes took approximately 4 h. Percentage of DM remaining was defined by [a * Exp (- b * t) + c * Exp (- d * t)] where a is the point at which DM solubilization rate is reduced, c is the remaining DM that can be solubilized, and b and d are rates of solubilization as a function of time. Regardless of forage type, quality, or maturity, these formulas can be used to design effective soaking treatments to obtain desired TNC concentrations below recommended thresholds.

Acknowledgments

I would like to first and foremost thank my mentor, Dr. Wagner, for allowing me to embark on this journey which has solidified my love for academia. Thank you for always answering my numerous questions in and out of the classroom. Thank you to my committee members for their continued support throughout my project and its many twists and turns; without my committee, this project would not have been the success that it was. I am grateful for Dr. Lisa Sheehy and Dr. Edzard van Santen for their assistance with statistics.

Thank you to my forage fairy godmother, Susan Sladden, who took me under her wing, welcomed me into her world, and showed me what it truly meant to work in a lab. I can honestly say I have the utmost respect for not only what she does in the lab daily but also for who she is as a person. It was an honor to be welcomed into her lab and work beside her.

Thank you to my fellow graduate students for braving the heat to trim hay, getting soaking wet in the lab, and rallying around me and this project. I would like to extend a special thank you to the graduate student who demonstrated what it is to truly be selfless and put others above themselves. I could not possibly have made this journey without her. Stephanie Turner, it was an absolute honor to be your teammate through this, and I will forever call you my friend.

Last but certainly not least, I would like to thank my friends and family for giving me the support I needed even if that meant making sure I was not bombarded by phone calls during some of the stressful weeks. Your love and support from afar was appreciated. Thank you to my other half, Kyle Hall, for giving me the daily support and encouragement throughout this

program. It seemed like a bumpy road at times, but you were there every step of the way. Thank you for everything!

Table of Contents

Abstract ii

Acknowledgments iv

List of Tables viii

List of Figures ix

List of Abbreviations xi

I. Review of Literature 1

Nonstructural Carbohydrates 1

Conditions Affected by Nonstructural Carbohydrates 3

Insulin Resistance 3

Equine Metabolic Syndrome 4

Laminitis 5

Polysaccharide Storage Myopathy 6

Hay Soaking 7

Introduction 7

Factors Affecting Solubilization 9

Future Research Implications 11

II. Solubilization of nonstructural carbohydrates as a function of soaking interval and water temperature in southern forages commonly fed to equids 13

Introduction 13

Materials and Methods 14
Results 19
Discussion 36
Conclusion 40
Literature Cited 42
Appendix 1. Non-pure samples identified pre-treatment in 2 hay types 47
Appendix 2. Mold contaminated hay samples identified post soaking treatment and 60°C drying 48
Appendix 3. Calculating total nonstructural carbohydrate on %DM basis given titer amount and standard curve for the batch of Shaffer-Somogyi reagent used 50
Appendix 4. Alfalfa hay composition means post-soaking for up to 720 min 51
Appendix 5. Perennial peanut hay composition means post-soaking for up to 720 min 52
Appendix 6. Coastal bermudagrass hay composition means post-soaking for up to 720 min 53
Appendix 7. Tifton-85 bermudagrass hay composition means post-soaking for up to 720 min 54
Appendix 8. Mean water temperatures for cold and hot water during hay soaking treatments through 720 min 55
Appendix 9. Measures of ambient temperature (°C) and relative humidity (%) during the soaking of 4 hay types in hot and cold water for up to 720 min. 56

List of Tables

Table 1. Composition of 4 hay types prior to soaking 20

Table 2. Effect of mold contamination on the solubilization of total nonstructural carbohydrate (TNC) and dry matter (DM) in 2 hay types soaked in hot (50°C) and cold (28°C) water for up to 720 min 20

Table 3. Effect of time and water temperature on the solubilization of total nonstructural carbohydrates in 4 hay types soaked in hot (50°C) and cold (28°C) water for up to 720 min 21

Table 4. Solubilization equation characteristics of 4 forages where percentage of remaining total nonstructural carbohydrate (y) = a + b * Exp (c * t) 26

Table 5. Effect of time and water temperature on the solubilization of dry matter in 4 hay types soaked in hot (50°C) and cold (28°C) water for up to 720 min 26

Table 6. Solubilization equation characteristics of 4 forages where percentage of remaining DM (y) = a * Exp (-b * t) + c * Exp (-d * t) 35

List of Figures

Figure 1. Loss of total nonstructural carbohydrates from alfalfa hay soaked in hot (50°C) and cold (28°C) water for up to 720 min 22

Figure 2. Loss of total nonstructural carbohydrates from perennial peanut hay soaked in hot (50°C) and cold (28°C) water for up to 720 min 23

Figure 3. Loss of total nonstructural carbohydrates from Coastal bermudagrass hay soaked in hot (50°C) and cold (28°C) water for up to 720 min 24

Figure 4. Loss of total nonstructural carbohydrates from Tifton-85 bermudagrass hay soaked in hot (50°C) and cold (28°C) water for up to 720 min 25

Figure 5. Dry matter loss in alfalfa hay soaked in cold (28°C) water for up to 720 min 28

Figure 6. Dry matter loss in alfalfa hay soaked in hot (50°C) water for up to 720 min 29

Figure 7. Dry matter loss in perennial peanut hay soaked in cold (28°C) and hot (50°C) water for up to 720 min..... 30

Figure 8. Dry matter loss in Coastal bermudagrass hay soaked in cold (28°C) water for up to 720 min 31

Figure 9. Dry matter loss in Coastal bermudagrass hay soaked in hot (50°C) water for up to 720 min 32

Figure 10. Dry matter loss in Tifton-85 bermudagrass hay soaked in cold (28°C) water for up to 720 min 33

Figure 11. Dry matter loss in Tifton-85 bermudagrass hay soaked in hot (50°C) water for up

to 720 min 34

List of Abbreviations

ADF	Acid detergent fiber
BCS	Body condition score
C3	Cool-season
C4	Warm-season
CFU	Colony forming unit
DDFT	Deep digital flexor tendon
DM	Dry matter
EMS	Equine Metabolic Syndrome
GLUT-4	Glucose transporter 4
IR	Insulin Resistance
IVDMD	In vitro dry matter digestibility
LPS	Lipopolysaccharide
NDF	Neutral detergent fiber
NIRS	Near-infrared spectroscopy
NSC	Nonstructural carbohydrate
OST	Oral sugar test
P3	Phalanx 3
PSSM	Polysaccharide Storage Myopathy
RAO	Recurrent Airway Obstruction

TNC	Total nonstructural carbohydrate
WSC	Water soluble carbohydrate

I. Review of Literature

Nonstructural Carbohydrates

Water soluble carbohydrates (WSC) comprise of mono-, di-, and oligosaccharides, fructans, and a small portion of ß-glucans. Nonstructural carbohydrates (NSC) contain all WSC constituents as well as the starch fraction (NRC, 2007) and can be determined through near-infrared spectroscopy (Martinson et al., 2012a,b) or enzymatic wet chemistry (Mullenix et al., 2012). This NSC fraction of the plant cell plays a vital role in the equine diet in that it provides energy in the form of glucose which can be readily utilized by the horse or stored as glycogen in muscle for rapid use during exercise (NRC, 2007). Due to horses' heavy reliance on blood glucose as an energy source and its availability to the equine muscles in the form of glycogen during work, NSC is one dietary component that is heavily considered when formulating feed or calculating feeding management protocols (Hoffman et al., 2001).

The effect of NSC on the glycemic response of horses is important when considering equids that might not respond normally to glucose or may require careful attention due to metabolic concerns. Metabolically challenged horses, specifically NSC-sensitive horses, are those that do not respond normally to NSC intake with appropriate postprandial insulin concentrations, or have decreased tissue responses to insulin; these horses have a recommended intake of <10% dietary NSC due to their metabolic sensitivities (Borgia et al., 2009).

Nonstructural carbohydrate load in meals and its effect on glycemic response has been studied. The glycemic response following NSC consumption can be studied utilizing oral sugar tests (OST) that stimulate in part the release of incretin hormones from the small intestine, which

in turn leads to a rise in insulin concentration that down-regulates glucagon following the ingestion of sugar as a part of a meal (Schuver et al., 2014). A study by Gordon et al. (2007) compared two isocaloric diets. The diet consisting of 50% more NSC, also above the 10% NSC threshold recommended for metabolically challenged horses (Borgia et al., 2009), resulted in greater postprandial average glucose concentrations and peak glucose concentrations. However, consumption rate of the two isocaloric experimental feeds, as well as time to reach peak glucose, remained similar (Gordon et al., 2007). Postprandial insulin followed similar patterns with respect to high- and low-NSC diets. In two similar studies that evaluated the effect of high- and low-NSC diets on postprandial glycemic response, average and peak insulin concentrations were significantly greater in diets with greater NSC content (Gordon et al., 2007; Pratt-Phillips et al., 2014). If concentrates high in NSC are the only feed source available, horses may eat smaller, more frequent meals (difference between two and three meals a day) or may utilize feeding obstacles to slow the rate of ingestion. Both techniques will result in decreased insulin response to the NSC-rich meal, which is necessary to maintain the health of certain metabolically sensitive horses (Pratt-Phillips et al., 2014).

Numerous carbohydrates, including oligofructose, rapidly ferment in the cecum. If the concentration of consumed sugars, starches, and oligosaccharides (NSC) overwhelms the capacity of the small intestine, the result is an increase of lactic acid within the cecum which lowers the pH within the tract (Suagee et al., 2015). This decrease in pH within the cecum may damage the lining of the epithelium, which could lead to further complications such as allowing lipopolysaccharides circulating in blood which promotes an overall inflammatory response (Suagee et al., 2015). Although NSC are a vital energy source for equids, the associated

metabolic pathways must be carefully considered when managing metabolically challenged horses due to the possibility of exacerbating a chronic condition and possibly eliciting a new one.

Conditions Affected by Nonstructural Carbohydrates

Insulin Resistance

Feeding meals rich in NSC, either in volume or concentration, produce greater postprandial insulin concentrations that can result in decreased insulin sensitivity in numerous tissues. The result is insulin resistance (IR) with a higher baseline insulin concentration if practices continue over time (Pratt-Phillips et al., 2014). High-starch and sugar diets can also lead to IR by perpetuating increased proinflammatory cytokines in plasma (Suagee et al., 2015; Vick et al., 2007). Thus, these feeding management practices along with other factors can lead to IR, even in previously healthy horses.

Insulin resistance can be defined as a decrease in insulin sensitivity, and it affects numerous pathways within the body. Normally, insulin stimulates a cell-signaling cascade that leads to the down-regulation of gluconeogenesis, inhibition of lipolysis, and increase triglyceride and fatty acid synthesis. Within the skeletal muscle cells, glucose uptake is facilitated by glucose transporter 4 (GLUT-4) transport proteins, which are recruited once an increase of insulin is released (Geor, 2008). This skeletal muscle glucose uptake by GLUT-4 is important due to the glycogen storage capacity in equine muscle, which is comparable to the glycogen stores found in the human liver. In horses afflicted with IR, GLUT-4 synthesis, movement, and function are decreased, which ultimately limits the glycogen stores in skeletal muscle, although the exact mechanism of inhibition is not fully understood (Frank, 2011).

Because ingesting soluble carbohydrates leads to a release of insulin and the resulting insulin signaling cascade, large amounts of nonstructural carbohydrates are to be avoided when

maintaining a horse afflicted with IR. Concentrates that are high in NSC should be removed from the diet due to the high glycemic response. Low-NSC forage should replace fresh forage typically available in pasture situations. Hay harvested at a later maturity should be utilized because it is less likely to have a large amount of NSC; further, the hay should be soaked to remove additional amounts of WSC (Longland et al., 2010; Gordon et al., 2007). Reducing dietary NSC leads to lower blood glucose and insulin concentrations both in the postprandial peak and during fasting. This reduction in dietary NSC results in more consistent glycemic levels compared with high glycemic meals (Gordon et al., 2007; Pratt-Phillips et al., 2014).

Equine Metabolic Syndrome

Insulin resistance is often seen in conjunction with other metabolic conditions and together makes up the disorder Equine Metabolic Syndrome (EMS). Equine Metabolic Syndrome describes horses typically characterized as obese with a body condition score (BCS) greater than 7 (Henneke et al., 1983; Geor, 2008) and are afflicted with IR, hyperinsulinemia (Elzinga et al., 2016b), dyslipidemia (Elzinga et al., 2016a), systemic inflammation, lower fecal microbial diversity (Elzinga et al., 2016b), and suffer from laminitic events (Frank, 2011). These horses are most commonly diagnosed following veterinary calls or hospitalization due to severe symptoms (Frank, 2011).

In order to maintain any quality of life for afflicted equids, management of EMS is critical. Management strategies should include dietary restrictions both in forage volume and low NSC concentrations to reduce physiological symptoms of IR and EMS (Frank, 2011; Divers, 2008; Collins et al., 2015; Secombe and Lester, 2012) and should also include an exercise regimen to decrease BCS and its effects on systemic inflammation (Secombe and Lester, 2012). Hay that is high in NSC is shown to cause a higher glycemic response, so hay considered low

(4%) or moderate (10%) in NSC should be preferentially fed to EMS horses (Collins et al., 2015; McGowan et al., 2013). Weight loss of approximately 1% per week has been seen in horses fed forage with <10% NSC at 1.25% of body weight on a dry matter basis (Argo et al., 2015; Borgia et al., 2009). Furthermore, because horses will eat between 2% and 5% of their body weight in forage daily, this reduction in total intake could cause problems if not managed properly with protocols such as utilizing dry paddocks and slow feeder hay nets to extend forage consumption throughout the day (McGowan et al., 2013).

Laminitis

The previously discussed metabolic conditions are predominantly problematic for horse owners because horses affected by these conditions are at an increased risk of laminitis. Although the true etiology of laminitis is not fully understood, the connections between IR and EMS with laminitis have been well established (Geor, 2008; Frank, 2011). Laminitis, also known as founder, occurs when the blood flow to the laminar "bridge" between the horse's hoof wall and pedal bone is restricted and causes death of the laminar tissue. As the laminar tissue is compromised, the ability to maintain the placement of the pedal bone is also compromised. The deep digital flexor tendon (DDFT) has a dorsal attachment to the pedal bone, and thus when the caudal structural fixture, the laminae, is weakened, the DDFT pulls the bone and causes rotation towards the ground surface.

While acute laminitis is typically connected with sudden diet changes such as an ingestion of a large carbohydrate meal from a horse gaining access to a feed room, chronic laminitis is most commonly correlated with pasture associated laminitis. Pasture associated laminitis is most common when pasture forage has a high concentration of rapidly fermentable carbohydrate storage or the horse has continuous or excessive access to forage that is moderately

high in NSC (Secombe and Lester, 2012). Laminitis has been clinically induced in otherwise healthy horses in multiple studies using oligofructose (Tadros et al., 2013) and other sugar compounds (Martinson et al., 2012a; Weiss et al., 1998). This carbohydrate component to laminitis is not fully understood in its mechanism but has made management practices clear; management entails restricting NSC feeding to below 10% and increasing exercise when possible (Divers, 2008; Secombe and Lester, 2012; Taylor et al., 2014; Geor, 2008).

Polysaccharide Storage Myopathy

Polysaccharide storage myopathy (PSSM) is yet another metabolic condition horses can exhibit. Unlike IR in which horses have decreased insulin sensitivity, PSSM horses have an increase in insulin sensitivity and blood glucose uptake (Secombe and Lester, 2012). Polysaccharide Storage Myopathy is a hereditary glycogen storage disorder found most commonly in American Quarter Horses, the most popular breed in the United States comprising over 41% of the total equine population (USDA, 2017), so the effect on the equine industry is substantial (Secombe and Lester, 2012). In horses afflicted with PSSM, a mutation in the gene coding for muscle glycogen synthetase results in a linear synthesis of glycogen but an inability to branch the storage molecule leading to an increased concentration of amylase-resistant polysaccharide within type II skeletal muscle. This increase enhances blood glucose uptake and insulin sensitivity. These inflicted horses experience energy deficits during submaximal exercise and often exhibit muscular symptoms including: muscle pain, stiffening, atrophy, and fasciculations (Secombe and Lester, 2012).

Management of PSSM horses should include low-NSC feedstuffs as NSC has been shown to increase pain associated with muscle fasciculation. Caloric needs can be addressed by adding fat to the diet. This feeding practice will reduce the uptake of glucose by affected type II

muscle cells and encourage cells to utilize fats as an energy source during submaximal exercise, reducing the tissue's need for glycogen. Forage with <10% NSC is once again the recommendation for feeding these metabolically challenges horses (Borgia et al., 2009; Secombe and Lester, 2012).

Hay Soaking

Introduction

The prevalence of metabolism disorders in equids is increasing both in the U.S. and the UK (Suagee et al., 2015). Such disorders require dietary restrictions in the form of decreased NSC in feedstuffs, including forages (Geor, 2008). Because so many metabolic conditions prescribe low-NSC feeds for management, research with how to achieve low-NSC feedstuffs is paramount. Low-NSC concentrate is now becoming more readily available to consumers, but horses require long-stem forage as a primary feed source to maintain digestive health. Therefore, offering low-NSC concentrate alone is not enough. Forage must meet requirements of these metabolically challenged animals as well.

Owners of these horses must be aware of the nutritional values before feeding in order to select forages that meet the nutritional needs of these specific horses. Forage in the form of hay is typically harvested to achieve high yields as well as high quality (Ball et al., 2010). Quality hay is typically desirable, but not necessarily in the case of IR, EMS, laminitis prone, and PSSM horses as quality largely correlates to digestible energy in the form of NSC and protein concentrations. Though more mature forage (Secombe and Lester, 2012), forage silage (Muller et al., 2016), or different forage types may be valid options for achieving appropriate forage quality and NSC concentrations for metabolically afflicted horses, horse owners may be restricted to one forage type due to reasons including geographic location or financial

constraints. When forage options are limited, forage must be adapted to meet the metabolic needs of the animal. Two methods of forage adaptation to reduce NSC exist: hay soaking (Martinson et al., 2012a,b; Martinson et al., 2011a; Longland et al., 2013) and hay steaming (Earing et al., 2013; Moore-Colyer et al., 2016). The most common practice, hay soaking, involves soaking forage in water to reduce WSC concentrations of the hay so they fall below the recommended threshold of <10%NSC (Borgia et al., 2009; Earing et al., 2013). Soaking provides owners an avenue to adapt forage in a cost-effective manner and, if conducted properly, results in a safe forage source for the horse (Martinson et al., 2012a).

The threshold set at <10% NSC has never been scientifically validated as an official cutoff for any of the conditions it aims to treat (Watts, 2004). Although percentages of NSC on either side of the threshold have been studied (Gordon et al., 2007), the thresholds for alleviating clinical symptoms of IR, EMS, and PSSM has never been individually established (McGowan et al., 2013). Due to the lack of experimental evidence, the recommendation to reduce NSC to less than 10% is generally a ceiling value, as the true threshold is unknown thus there are notable problems with this management strategy.

There is no visual way to evaluate the concentration of NSC in forage; thus, it must be sent for testing in order to verify the true percentage present (Moore-Colyer et al., 2016). Additionally, NSC concentrations vary from bale-to-bale within the same cutting of a hay species, so even if one bale was tested pre- and post-soak, it would still serve only as an estimate for the remaining bales (Longland et al., 2010). Nonstructural carbohydrate testing is typically conducted using near-infrared spectroscopy (NIRS) (Martinson et al., 2012a; Earing et al., 2013). While convenient, NIRS is not a reliable form of evaluating NSC unless the machine is

calibrated for the specific type of forage or forage mixture, and its evaluation becomes less accurate as the NSC concentrations decreases (Sladden, personal communication, 2016).

Factors Affecting Solubilization

Soaking protocols are highly variable, both in studies and application. Reportedly, WSC is leached at different rates depending on the water to forage ratio (Longland et al., 2011), temperature of the soaking liquor (Martinson et al., 2012a,b), soaking interval (Martinson et al., 2012a,b), quality of the forage (Martinson et al., 2012a,b), forage components present (Collins, 1991), forage compaction (Longland et al., 2011), agitation (Longland et al., 2013; Earing et al., 2013), and forage species (Collins et al., 2015; Martinson et al., 2012a,b; Martinson et al., 2011a; Longland et al., 2013). Because of the vast number of components associated with solubility of WSC, research is still considered limited in this area, as only a few combinations of these variables have been evaluated.

Hay type is also noted to affect the solubilization of NSC. Hay type primarily refers to the cell makeup resulting from cool-season (C3) versus warm-season (C4) plants. The majority of forage and soaking research has focused on C3 plants which are the primary forages fed to horses in England and the northern United States. Plants classified as C3 store carbohydrate primarily as fructan in the plant stem, which has been long blamed for pasture-induced laminitis (Longland and Byrd, 2006). Fructan comprises of glucose and fructose molecules in various formations depending on the specific type of fructan and is allowed to accumulate without a regulatory process as long as carbohydrate is plentiful within the plant (Longland and Byrd, 2006; Verspreet et al., 2015).

Warm-season (C4) forages, more prominent in the diet of horses living in warmer climates such as the southern United States, store carbohydrate as starch in plant leaves, which is

more readily leached than the fructan in C3 plant stems (Longland and Byrd, 2006). Starch is a storage polysaccharide made up of both amylopectin and amylose. Warm-season plants have a regulatory cut off mechanism for maximal amounts of stored starch; thus, they will not accumulate the large concentration of carbohydrate stores compared with C3 plants which do not have a fructan accumulations shut-off point (Longland and Byrd, 2006). Because these C4 plants start with a lower maximum stored carbohydrate and store carbohydrate in leaves, it is easier to limit-feed NSC utilizing C4 plants (McIntosh et al., 2013). Within these classifications of cool- and warm- season forages, cultivars or varieties also affect solubilization of NSC. Soaking forage or utilizing a more mature forage with a higher stem-to-leaf ratio, C4 plants should be able to fall below the necessary threshold easier than C3 plants (Muller et al., 2016; Geor, 2013).

Several factors can affect NSC solubilization during the soaking process. Plant maturity affects the solubilization due to increased lignification with more mature forage because soluble carbohydrates are tied up and cannot leave the cell (Martinson et al., 2011a). The ability of water to make physical contact with plant cells affects solubility; thus, compact hay and loose hay have been researched (Longland et al., 2011). Soaking liquor temperature affects solubility; warmer liquor has been noted to increase the rate of solubilization across numerous forage types (Martinson et al., 2012b). The amount of time the forage remains in contact with the water, the greater the NSC leaching (Martinson et al., 2011a).

Whereas numerous factors affect solubilization rates, several forage components are affected as well. Hay soaking solubilizes NSC (Martinson et al., 2012a,b) along with DM (Martinson et al., 2012a; Longland et al., 2011) and some water-soluble vitamins and minerals (Martinson et al., 2012b; Moore-Colyer, 1996; McGowan et al., 2013). Water-soluble carbohydrate constituents (fructan, sucrose, glucose, and fructose) are solubilized at varying rates

with fructose being the least soluble simple sugar (Longland et al., 2011; Longland et al., 2013; Muller et al., 2016). Fructan is not as readily leached as other NSC fractions. Therefore, hays that store large amounts of fructan are not a preferred starting point for forage that needs to be adapted to feed NSC sensitive horses (Longland et al., 2011; Longland et al., 2013; Martinson et al., 2012a).

Future Research Implications

Metabolically challenged horses, or those requiring reduced NSC intakes, are often managed by feeding low-starch concentrate meals along with forage that has been treated to reduce NSC content. This reduction in overall fed NSC helps to manage insulinemic and glycemic responses in these horses to mitigate symptoms or future complications from metabolic conditions (Collins et al., 2015). Because NSC is water-soluble, the most common and cost-effective method of NSC reduction in forage is to soak hay for a given time interval in water (Martinson et al., 2012a). A threshold of tolerated NSC percentage in forage fed to this group of horses has been set as <10% (Borgia et al., 2009), but the point at which forage species and specific varieties reach this threshold reportedly varies greatly between studies due to the variability of solubilization of NSC within different forage types (Longland et al., 2011; Martinson et al., 2012a,b). Though research has been conducted with numerous hay types (Longland et al., 2011; Martinson et al., 2012a,b) and soaking intervals (Longland et al., 2011; Martinson et al., 2012a,b), unless the same maturity, quality, and type of hay is soaked, too many assumptions must be made by owners of metabolically challenged horses to construct an efficient and safe soaking protocol for a given forage.

Most research investigating hay soaking and NSC reduction focuses primarily on cool-season (C3) forage (Martinson et al., 2012a,b) or mixed meadow varieties (Longland et al., 2011;

Longland et al., 2013). Due to the physiology of C3 plants, parallels cannot easily be drawn between C3 forages that have been the focus of previous research and the warm-season (C4) forages commonly fed in the southeastern United States (Longland and Byrd, 2006). As a result, more research is needed to specifically characterize the solubilization of NSC in warm-season forages commonly fed to equids within this affected group. Understanding solubilization characteristics of NSC will allow owners of afflicted equids to construct a valid and effective management plan which includes soaking for the appropriate time interval to accurately falling below the <10% recommended threshold.

II. Solubilization of nonstructural carbohydrates as a function of soaking interval and water temperature in southern forages commonly fed to equids

Introduction

Several types of metabolically challenged horses, such as those with insulin resistance or polysaccharide storage myopathy, require low-nonstructural carbohydrate (NSC) diets as part of their treatment plans. Nonstructural carbohydrates are the primary dietary concern of horses within this category of afflicted equids because NSC exacerbate these particular metabolic conditions. Following a meal that is high in NSC, an influx of blood glucose results in an increase of insulin in the bloodstream that can cause severe damage to metabolically vulnerable horses over time. Thus, the reduction of NSC in the diet should be of the utmost importance in managing horses with these metabolic disorders. Because of the water-soluble nature of NSC, the soaking of a forage prior to feeding is a common procedure horse owners use to obtain these low levels of NSC in the diet. Unfortunately, research on the soaking of hay and the subsequent solubilization of NSC is limited.

If hay is soaked for too short of an interval, the resulting forage may still contain an unsafe proportion of NSC and can cause irreparable damage if consumed, but hay soaked for too long of an interval may be depleted of water-soluble vitamins and minerals, so the balance of the two is paramount. Previous studies have established soaking intervals for specific forage species, but those often include mixed-species, meadow hays or other cool-season forages that are not as commonly fed in the southern United States where warm-season forages are prominent. Investigation of these forages is justified, as warm- and cool-season forages store and accumulate

NSC differently, and this difference could cause differences in the rates of solubilization. Furthermore, some studies report exact percentages of NSC following soaking procedures, but the data can only be used to describe the specific hay sample evaluated in each study, and numerous assumptions must be made to extrapolate information on soaking intervals to other forage species, maturities, and cuttings. With a prediction equation, horse owners may use the known, starting nutritive value of the forage and use the equation to determine the appropriate soaking interval to fall below 10% NSC, as this is the recommended threshold for afflicted horses. Thus, this study aims to create a prediction model to eliminate many of the assumptions made when designing feeding programs for this special group of metabolically challenged horses.

Materials and Methods

Four hay types: alfalfa (*Medicago sativa*), perennial peanut (*Arachis glabrata*), and Coastal and Tifton-85 varieties of bermudagrass (*Cynodon dactylon*) were utilized in the experiment. Square bale served as experimental unit for each hay type, with a minimum of 5 replicates per treatment. The alfalfa cultivar used in this experiment was a second cutting of Alfagraze, and it was produced in Danville, Alabama (34.41° lat, -87.09° long). Both the perennial peanut and Tifton-85 bermudagrass hays were produced in Tifton, Georgia (31.45° lat, -83.51° long) and were second cuttings. Lastly, Coastal bermudagrass was a second cutting which was produced in Valdosta, Georgia (30.83° lat, -83.28° long). Hays were stored for 7 mo where they were under the protection of a shed and raised on pallets but were subjected to weathering both by rain and sunlight. Hay bales were moved to the Auburn University Wilson Beef Teaching Center metabolism stall room where they were protected from further weathering

damage. All hays were stacked on designated pallets apart from one another so that cross contamination was unlikely.

Sampling the hays was performed in the metabolism room at the Stanley P. Wilson Beef Teaching Center. Each bale was individually processed. During processing, the bale was placed on a sheet of cardboard to limit environmental contamination. The strings were cut and the bale ends removed and discarded so that only the middle 1/3 of the bale remained. Each flake of hay from the middle third was laid flat and individually trimmed to remove a perimeter of 4-5 cm to remove portions that were subject to weathering during storage. Trimming was performed using a rotary cutter (Fiskars Classic Stick Rotary Cutter) and shears (Fiskars Shear Ease Grass Shears). The remaining center portion of each flake was placed in a large container and mixed with other sections from the same bale.

Once the inner flake sections from the bale were thoroughly mixed to create an aggregate of the bale, samples of each bale were created from the contents of the mixing tub. Samples (180 g) of hay were placed in two layers of Grade 10 cheesecloth and bundled to help contain leaf material. During the weighing process, each sample was examined for the presence of foreign material or evidence of non-pure stands. Non-pure stands were noted as debris such as sticks or weeds but were not disturbed or removed during this process. The largest contamination of stand was noted in the perennial peanut samples in which *Panicum spp* were identified (Appendix 1). Bundles were then secured with a plastic cable tie and randomly assigned to one of 13 treatments. Treatments consisted of 0-, 15-, 30-, 60-, 120-, 360-, and 720-min soaking intervals in both 50ºC and 28ºC soaking liquor. Sample bundles were stored in plastic bags loosely tied and hung to avoid environmental contamination.

Treatments were applied in the Department of Animal Sciences' nutrition laboratories. The treatment room was under central control and stayed at an average of 25.5°C and 62% ambient humidity throughout the experiment.

Eight 18.93-liter buckets were used as soaking vessels. Vessels were thoroughly washed with soap and rinsed well before the start of each day and between treatments to eliminate possible environmental contamination. Four vessels were used for each soaking liquor temperature. In order to emulate the soaking liquor: sample weight ratio most commonly utilized in industry, each vessel held 14 L of soaking liquor to give a 1 L:12.857 g soaking liquor to sample weight ratio. Soaking liquor temperatures were selected to most closely resemble current industry practices, and thus were measured as either hot water using only the hot water tap or cold using only the cold water tap. The cold water was consistently 28°C. Hot water treatment vessels and water baths were initially filled with 55°C water and allowed to cool to 50°C before starting the hot water soaking treatments. Temperature of the soaking liquor and ambient room temperature were recorded prior to introducing the hay bundles to the vessels.

Large water baths (72.39 cm diam.) filled with 22 L of soaking liquor helped stabilize the soaking liquor temperature and rate of decline across the 4 soaking vessels within each water bath. The hot and cold water soaking liquor temperature treatments received a water bath of 50ºC and 28ºC respectively. The 4 soaking vessels, one per hay type, were arranged in the same orientation in each water bath and for each run to reduce any possible effect of the room or air flow.

Samples were submerged in the soaking vessel by dropping them into the soaking liquor and quickly thrusting them to the bottom of the vessel. Samples were then allowed to float without agitation or restriction in the soaking liquor for the duration of the soaking period.

Samples remained in the soaking liquor for the specified period of time and were promptly pulled from the water at the conclusion of the soaking interval. Samples were allowed to air dry while suspended for 30 minutes before each bundle was opened and contents were transferred to a resealable plastic bag. Air was pressed from each bag prior to sealing and the bags were placed in a <4°C freezer to await further analysis. Samples of the soaking liquor were also collected and stored at <4°C to be analyzed for use in a future project.

Samples were dried in 60°C drying ovens for 5 days. Upon inspection, 56 samples were visibly contaminated with mold (Appendix 2). These samples were noted as "Little" (12 samples) or "Heavy" (44 samples) mold. "Little mold" displayed mold contamination that was not easily detected but could be found if the sample was pulled apart and closely inspected. "Heavy mold" was noted as samples that had mold visibly protruding without disrupting the sample. When pulled apart, the "Heavy mold" was physically more difficult to separate than a non-contaminated or "Little mold" sample.

To combat further mold contamination, the drying procedure was adapted for the remaining samples to include thorough mixing of the samples on d 2 and d 4 of drying. Mixing the hay consisted of adding air to the sample bundle to aid the drying process by exposing the inner hay thus increasing air flow. To further aid the drying process and decrease the mold contamination risk, cloth forage bags were utilized to wick away moisture. This modified method resulted in 0 further identified mold contaminations.

Following the drying process, samples were weighed at their stable ambient temperature and humidity weights. The first group of samples was fully ground using a Model 4 Thomas-Wiley Laboratory Mill with a 1-mm screen. The remaining samples were first course ground through a hammer mill followed by fine grinding by the Wiley mill as described.

Once all the samples were finely ground, they were analyzed for dry matter and ash content. Fibers were analyzed sequentially through an Ankom Fiber Analyzer for NDF and ADF according to Van Soest et al. (1991). Total nonstructural carbohydrate (TNC) analysis was performed. Sample sizes were adjusted from 0.25 g to 1.00 g to account for the low TNC percentage. All TNC samples were run in duplicate. Samples were placed in 600 mL beakers. Fifty mL of 0.05 N H_2SO_4 were added down the side of each beaker so as not to cause loss of sample. Samples boiled for 1 h on a fiber rack. After the allotted time, beakers were immediately placed in an ice bath, given a 2-2.8 mL dose of 1 N NaOH as needed to raise pH substantially, and each given a stir bar.

The pH of the contents in each beaker was finely adjusted to 4.4-4.6 using 1 N NaOH, 0.1 N NaOH, 1 N H_2SO_4, and 0.1 N H_2SO_4 dropwise. Deionized water (4.5 pH) was used to rinse the sides of the beaker and pH probe. Once pH was within the optimum range, 1 mL of G-ZYME 480 (25% α amylase 75% glucoamylase, DuPont) was added and gently mixed with beaker contents. Beakers were covered and incubated at 60ºC for 1 hour. Post incubation, samples were filtered through glass wool into 250 mL volumetric flasks utilizing a vacuum pump. For samples with larger protein concentrations, the filtering process was amended to incorporate a stainless-steel filter apparatus (Millipore) and filtered under pressure through a 0.2 micron membrane filter or Watman #1 filter paper. Pressure was applied until all liquid was extracted. This adapted method was used to help combat the protein layer that prevented vacuum filtration. Filtering processes were determined to be not different from one another through evaluation of lab standards.

Following filtration, 2 mL of 1 N NaOH was added to each volumetric flask, and the flasks were brought to volume with deionized water. Each flask was inverted for several seconds

to ensure even mixing of the contents. Ten mL aliquots were pulled from each volumetric and dispensed into test tubes. Each test tube was given a vigorous 10 mL dose of Shaffer-Somogyi reagent (AOAC, 1995) and capped with glass condensers. Test tubes boiled for 15 min in a hot water bath. Samples went immediately into an ice bath following boiling; once samples were thoroughly cooled, 2 mL of potassium iodide potassium oxalate (2.5 g of each component in 100 mL of deionized water), a stir bar, and 5 mL of 1 N H_2SO_4 was added. The samples rested for a few minutes before dispensing a second 5 mL dose of 1 N H_2SO_4. Once the second dose was added, the sample was given one drop of Fast Break® (WinField United, St. Paul, MN) to reduce foaming during titration and 2 mL of 1% starch solution. Each sample was titrated using 0.02 N sodium thiosulfate until the color changed from a dark purple to a bright, translucent light blue. A back light was used to help distinguish color changes. Titer was recorded and entered into Excel with the standard curve equation previously established for each Shaffer-Somogyi batch (Appendix 3).

Statistical analysis was performed using JMP Pro 12 software (SAS Inc.). Fit Y by X function was utilized and a nonlinear regression model was fitted. Models 3P and 4P were determined as best fits for TNC solubilization and DM solubilization respectively following JMP Pro 12 guidelines.

Results

Diet composition of the 4 hay types utilized in the experiment were determined pre-soak (Table 1). Post-soaking compositions for DM, TNC, and ash were found for each of the 4 hay types (Appendix 4, 5, 6, and 7). Water temperatures were recorded at regular time intervals during each run to validate the consistency with which the soaking liquor temperatures cooled to

room temperature (Appendix 8). Similarly, relative humidity values were recorded during the 30-min drying interval post-soak as well as ambient temperature recorded throughout the treatment times (Appendix 9). The mean ambient temperature (25.58°C) and relative humidity (61.89%) remained relatively consistent throughout the experiment.

Table 1. Composition of 4 hay types prior to soaking

Hay type	DM	DM basis (%)					n
		NDF	ADF	CP	TNC	Ash	
Alfalfa	85.12	57.49	38.09	16.24	5.44	7.22	8
Perennial peanut	84.64	52.11	29.17	12.33	8.84	7.42	8
Coastal bermudagrass	87.07	73.78	34.93	11.26	9.08	6.27	8
Tifton-85 bermudagrass	86.73	77.07	41.10	11.45	6.18	4.42	7

Mold effects

Mold was found during the experiment at the conclusion of the 60°C drying phase. Mold occurred most often in the alfalfa and perennial peanut hays (25 and 28 samples, respectively) compared with the Coastal bermudagrass and Tifton-85 bermudagrass hays (1 and 2 samples, respectively). Mold was evaluated using least square means for its potential effects on TNC and DM (Table 2). Mold did not have an effect on the solubilization of DM or TNC solubilization; thus, the mold was not included in further analysis as a covariant.

Table 2. Effect of mold contamination on the solubilization of total nonstructural carbohydrate (TNC) and dry matter (DM) in 2 hay types soaked in hot (50°C) and cold (28°C) water for up to 720 min

Hay type	Parameter	
	TNC	DM
Alfalfa	$P = 0.2057$	$P = 0.1851$
Perennial peanut	$P = 0.9948$	$P = 0.6350$

Total nonstructural carbohydrate solubilization

Soaking liquor temperature and soaking interval were evaluated using least square means for their effect on TNC solubilization (Table 3). Soaking interval was significant on the solubilization of TNC in perennial peanut (P <0.001), Coastal bermudagrass (P <0.001), and Tifton-85 bermudagrass (P = 0.001). Alfalfa hay showed a trend toward significance (P = 0.079) with respect to soaking interval. Water temperature (28°C or 50°C) did not have an effect on solubilization for any of the four hay types (P = 0.241), and thus was not considered in further analysis of TNC solubilization.

Table 3. Effect of time and water temperature on the solubilization of total nonstructural carbohydrates in 4 hay types soaked in hot (50°C) and cold (28°C) water for up to 720 min

Hay type	Parameter	
	Time	Temperature
Alfalfa	P = 0.079	P = 0.783
Perennial peanut	P < 0.001	P = 0.590
Coastal bermudagrass	P < 0.001	P = 0.241
Tifton-85 bermudagrass	P = 0.001	P = 0.987

Percentage of TNC remaining was analyzed for all hay types utilizing a nonlinear decay model (Figures 1, 2, 3, and 4). Alfalfa, perennial peanut, Coastal bermudagrass and Tifton-85 bermudagrass all showed a rapid solubilization of TNC within the first hr of treatment in both cold and hot soaking liquor. All hays reached a TNC solubilization limit in the study in which the TNC within the hay was no longer solubilized in the soaking vessel. Alfalfa had 43.86% TNC remaining at the end of 720 min of soaking, whereas perennial peanut hay had 65.68% TNC remaining. For the bermudagrass varieties studied, Coastal had 69.48% TNC remaining and Tifton-85 had 54.22% TNC following soaking.

Figure 1. Loss of total nonstructural carbohydrates from alfalfa hay soaked in hot (50°C) and cold (28°C) water for up to 720 min

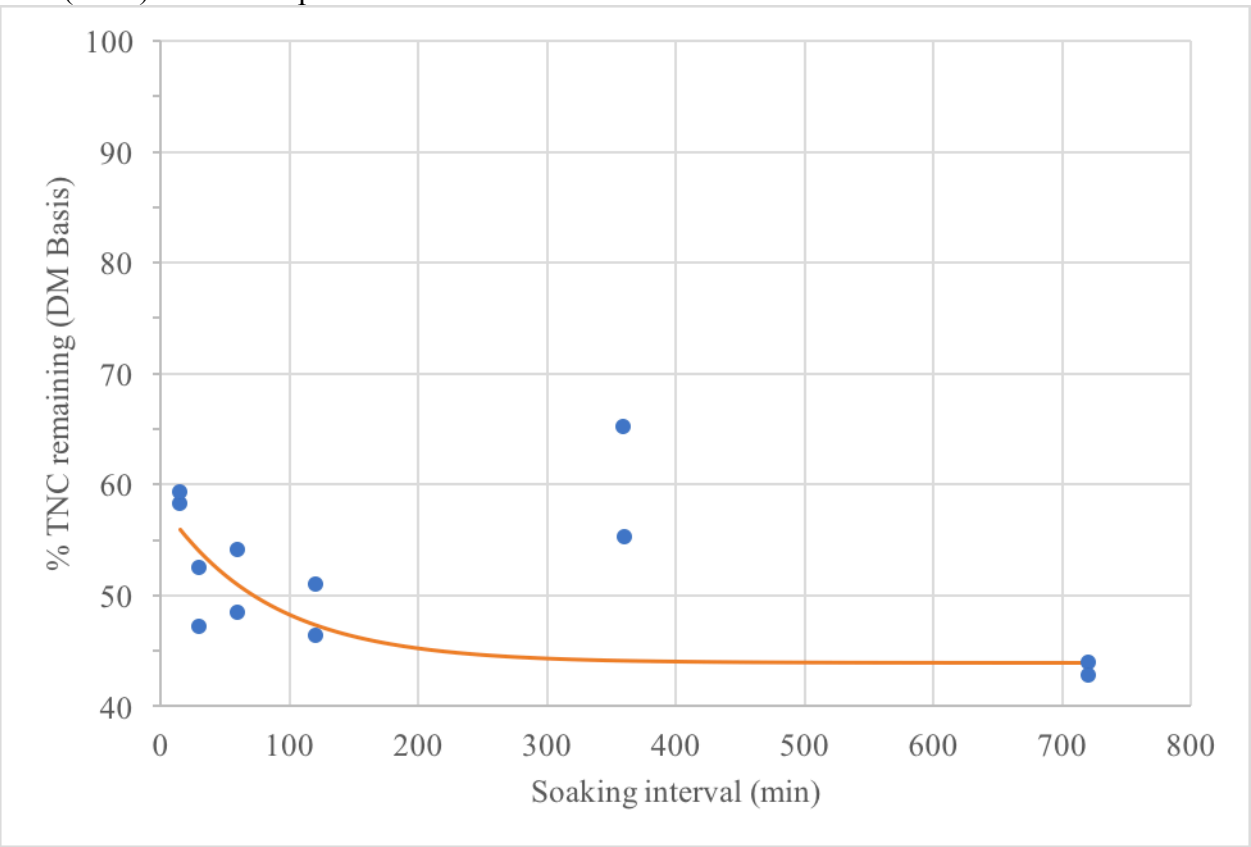


Figure 2. Loss of total nonstructural carbohydrates from perennial peanut hay soaked in hot (50°C) and cold (28°C) water for up to 720 min

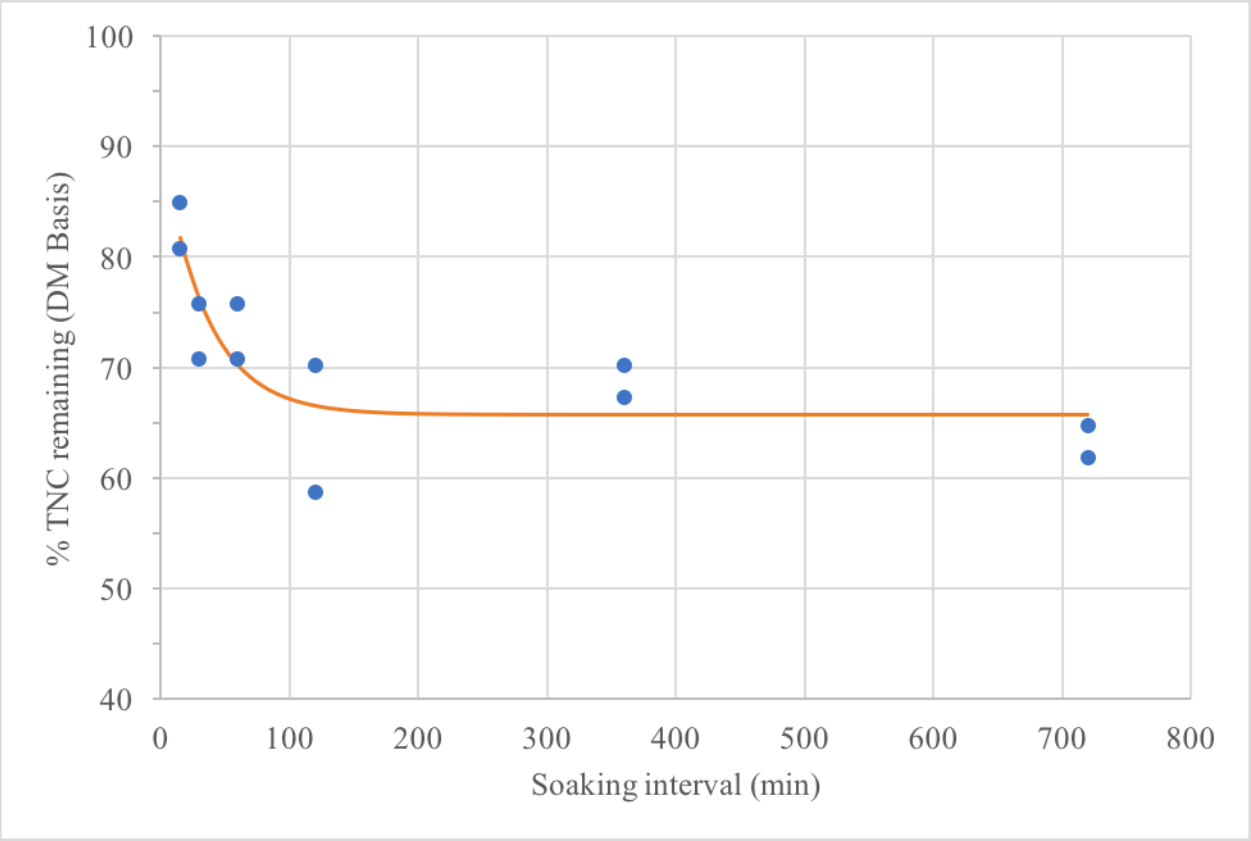


Figure 3. Loss of total nonstructural carbohydrates from Coastal bermudagrass hay soaked in hot (50°C) and cold (28°C) water for up to 720 min

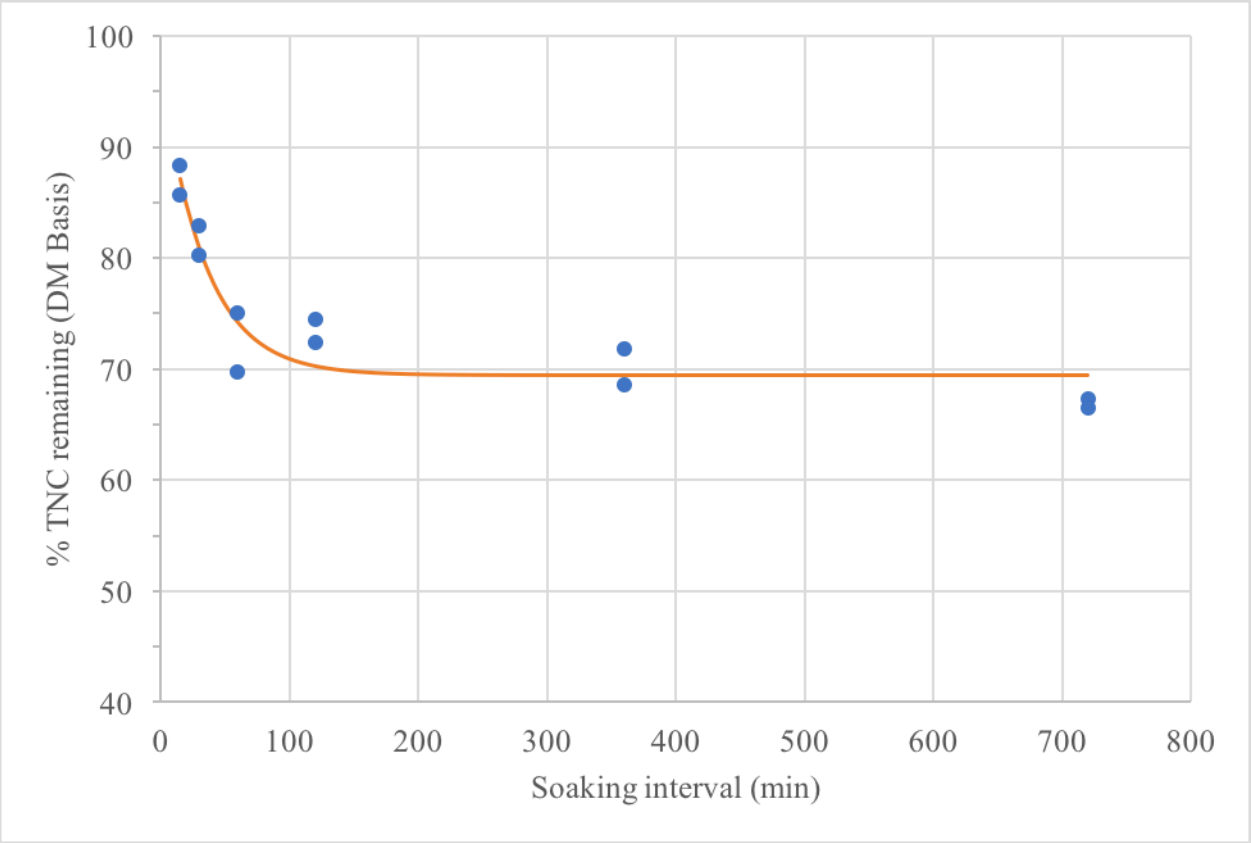
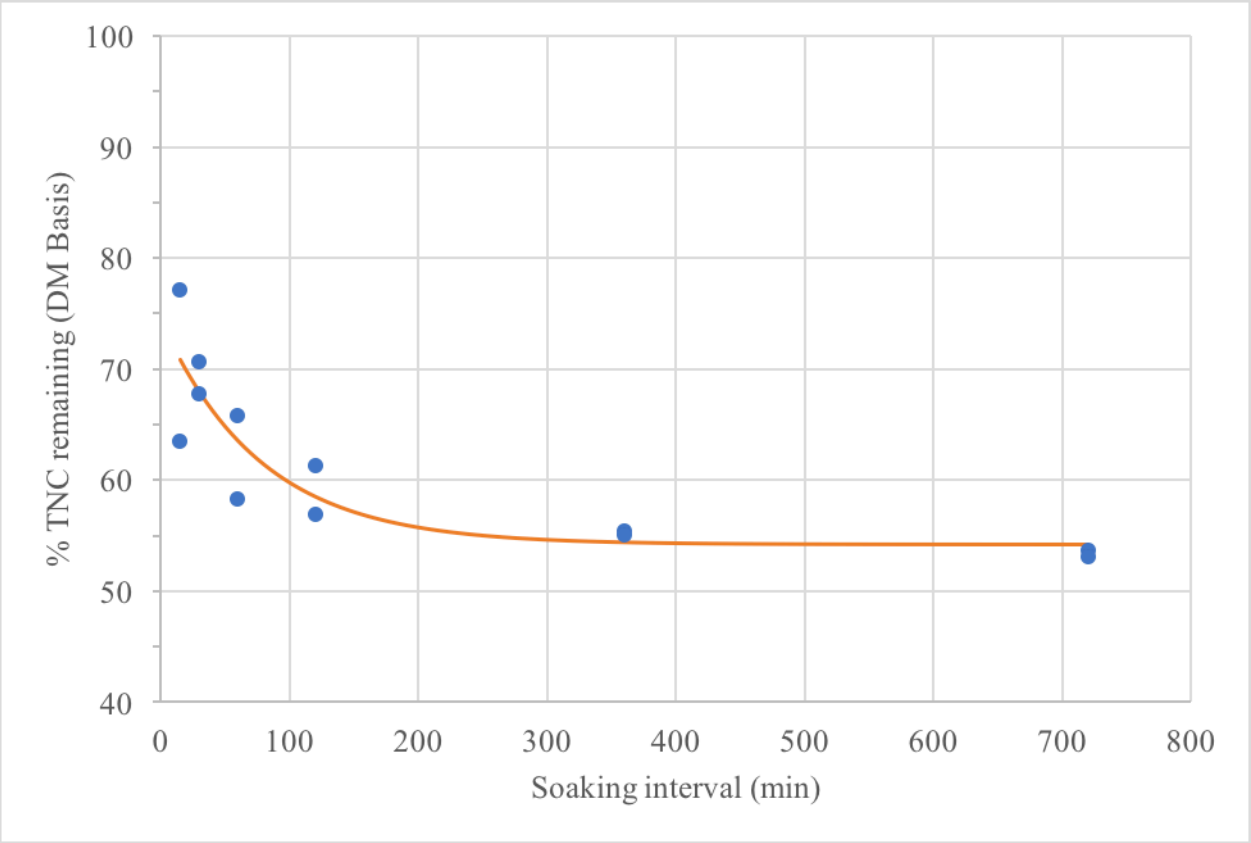


Figure 4. Loss of total nonstructural carbohydrates from Tifton-85 bermudagrass hay soaked in hot (50°C) and cold (28°C) water for up to 720 min



Each hay type had a different nonlinear prediction equation, though all were fitted with the same nonlinear decay model (Table 4). The prediction equation for determining the solubilization of forage given a certain soaking interval is determined as [y = a + b * Exp (c * t)] where y is equal to the percentage of remaining TNC, a is the point at which TNC solubilization is complete, b is the potentially soluble fraction, c is the rate of solubilization as a function of time, and t is the soaking interval in either 28°C or 50°C soaking liquor. For the alfalfa prediction equation, means for the 360 min soaking treatments were excluded from the analysis as they did not fit the line (Figure 1).

Table 4. Solubilization equation characteristics of 4 forages where percentage of remaining total nonstructural carbohydrate (y) = a + b * Exp (c * t)

Hay Type	Equation variable			
	a	b	c	R^2
Alfalfa[†]	43.864 ± 2.605	14.511 ± 4.064	-0.012 ± 0.008	0.666
Perennial peanut	65.684 ± 2.063	24.579 ± 8.391	-0.028 ± 0.016	0.702
Coastal bermudagrass	69.477 ± 1.268	27.417 ± 5.355	-0.029 ± 0.009	0.882
Tifton-85 bermudagrass	54.221 ± 2.125	20.109 ± 4.218	-0.013 ± 0.006	0.767

[†]Alfalfa equation does not include treatments at 360 min.
y = % of TNC remaining following a soaking treatment.
a = point at which solubilization is complete.
b = potentially soluble fraction.
c = rate of solubilization as a function of time.
t = soaking interval in min.

Dry matter solubilization

Effect of time and soaking liquor temperature on the solubilization of DM was analyzed using least square means (Table 5). Time had an effect on the solubilization of all hay types (P<0.001). Temperature had an effect on the solubilization of DM in alfalfa (P=0.038) and Tifton-85 bermudagrass (P=0.014). Temperature had a trend towards significance on affected DM in Coastal bermudagrass (P=0.077) but had no effect in perennial peanut hay (P=0.156). Due to temperature not having an effect on DM solubilization in perennial peanut hay, it was not included in further analysis for this hay type.

Table 5. Effect of time and water temperature on the solubilization of dry matter in 4 hay types soaked in hot (50°C) and cold (28°C) water for up to 720 min

Hay Type	Parameter	
	Time	Temperature
Alfalfa	$P < 0.001$	$P = 0.038$
Perennial peanut	$P < 0.001$	$P = 0.156$
Coastal bermudagrass	$P < 0.001$	$P = 0.077$
Tifton-85 bermudagrass	$P < 0.001$	$P = 0.014$

Dry matter was evaluated for the four hay types post-soaking treatment and fitted with biexponential nonlinear models (Figures 5, 6, 7, 8, 9, 10, and 11). Alfalfa, perennial peanut, Coastal bermudagrass, and Tifton-85 bermudagrass hays all displayed a rapid solubilization of DM within the first 30 to 60 min followed by a slower solubilization of DM for the remainder of the treatment times up to 720 min.

Figure 5. Dry matter loss in alfalfa hay soaked in cold (28°C) water for up to 720 min

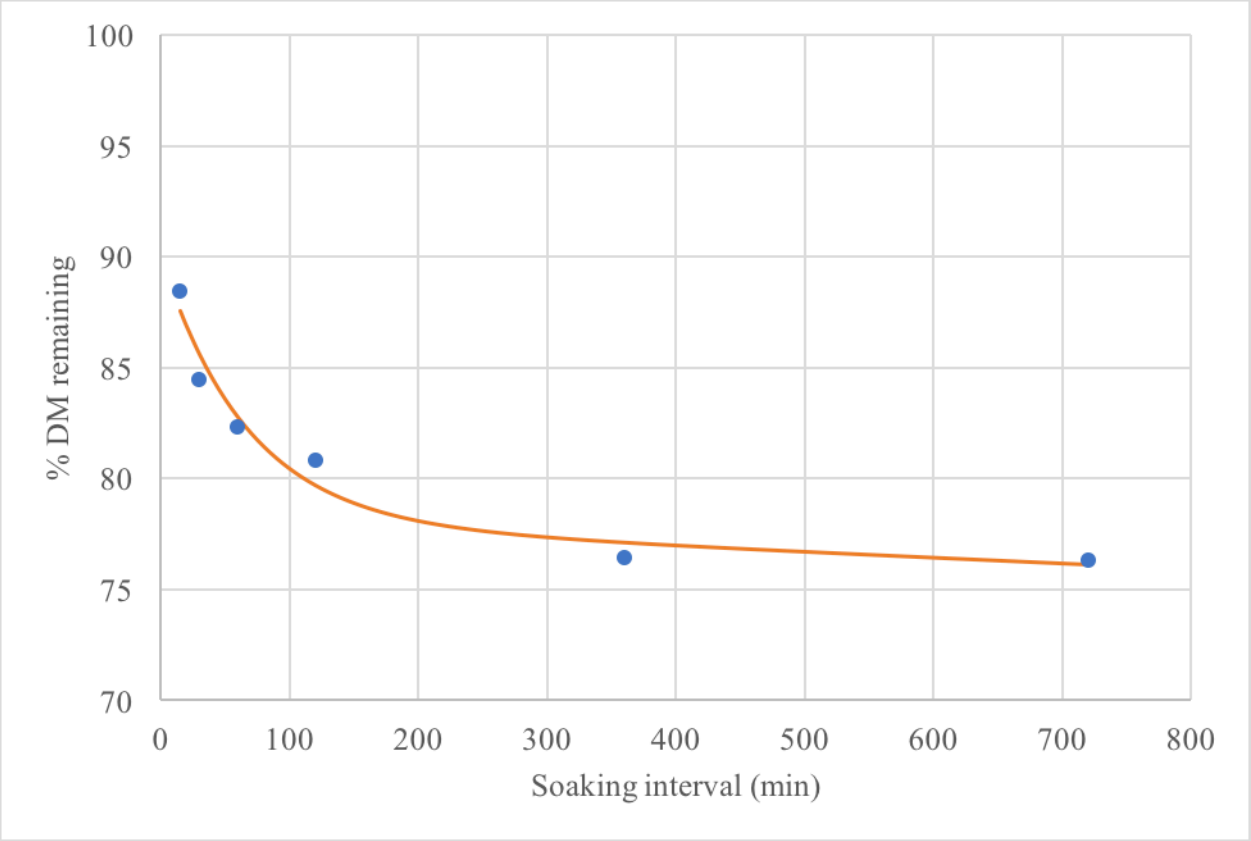


Figure 6. Dry matter loss in alfalfa hay soaked in hot (50°C) water for up to 720 min

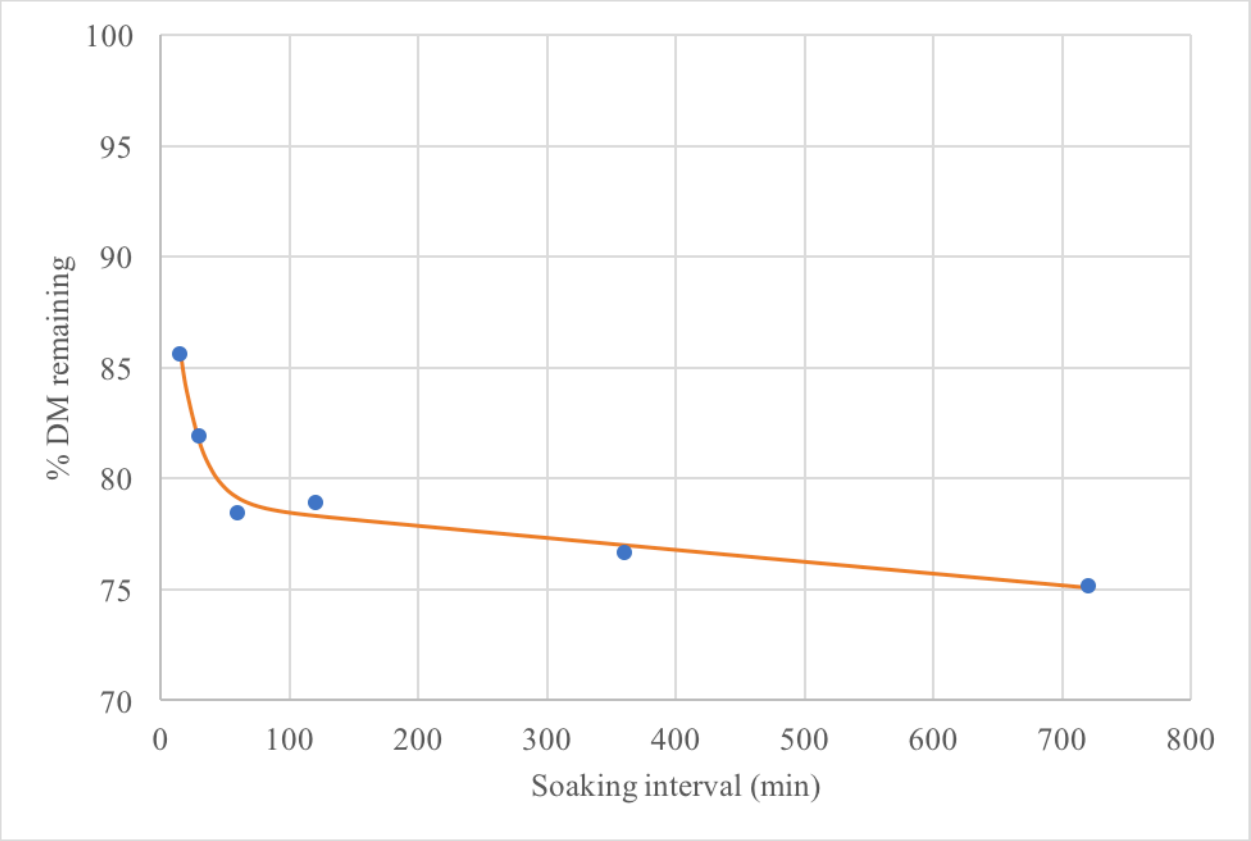


Figure 7. Dry matter loss in perennial peanut hay soaked in cold (28°C) and hot (50°C) water for up to 720 min

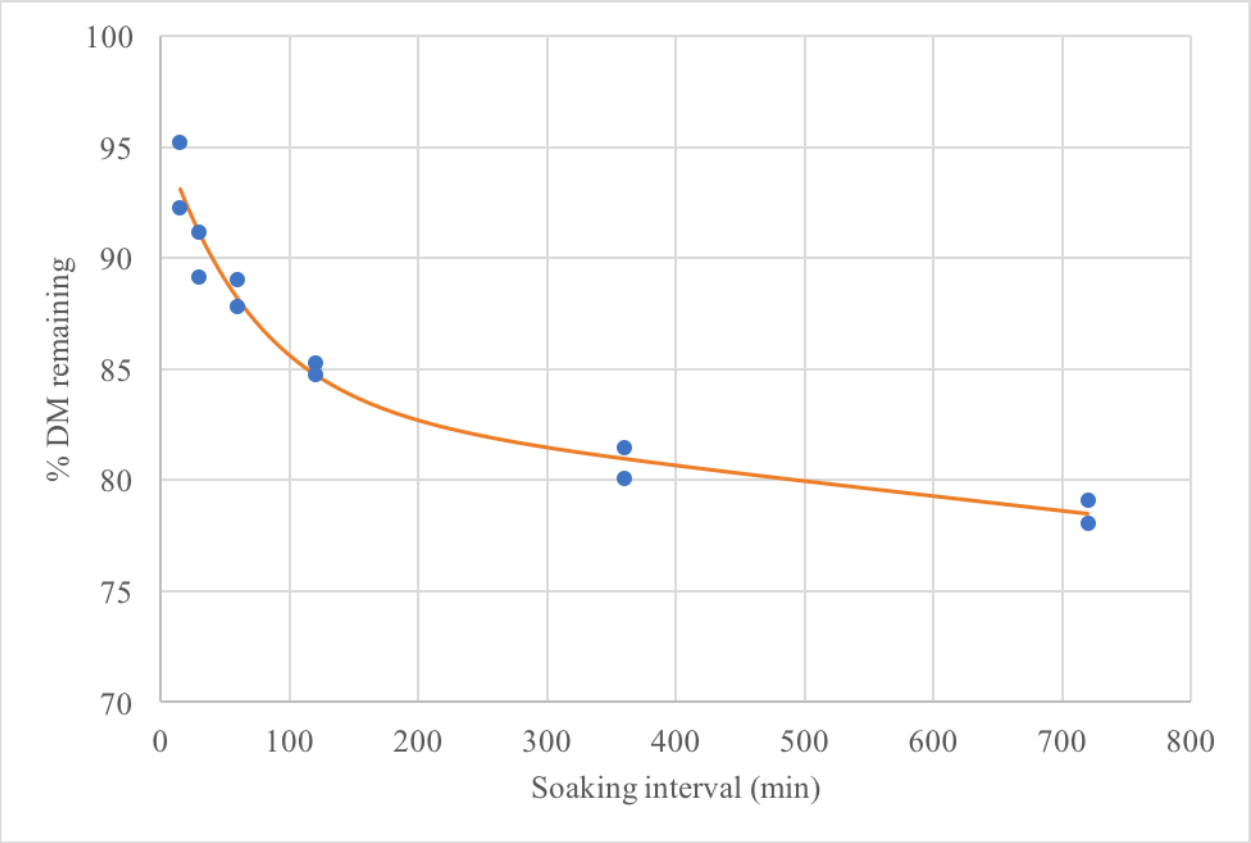


Figure 8. Dry matter loss in Coastal bermudagrass hay soaked in cold (28°C) water for up to 720 min

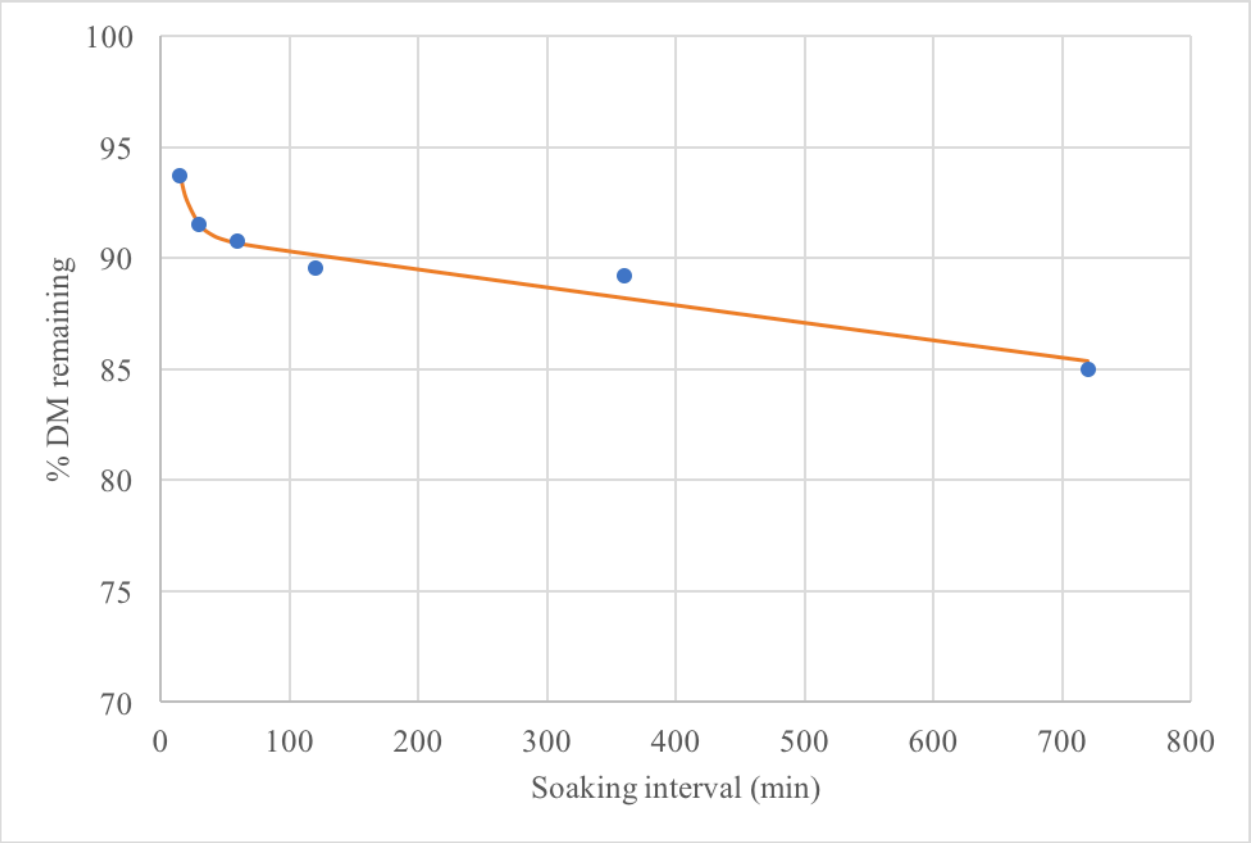


Figure 9. Dry matter loss in Coastal bermudagrass hay soaked in hot (50°C) water for up to 720 min

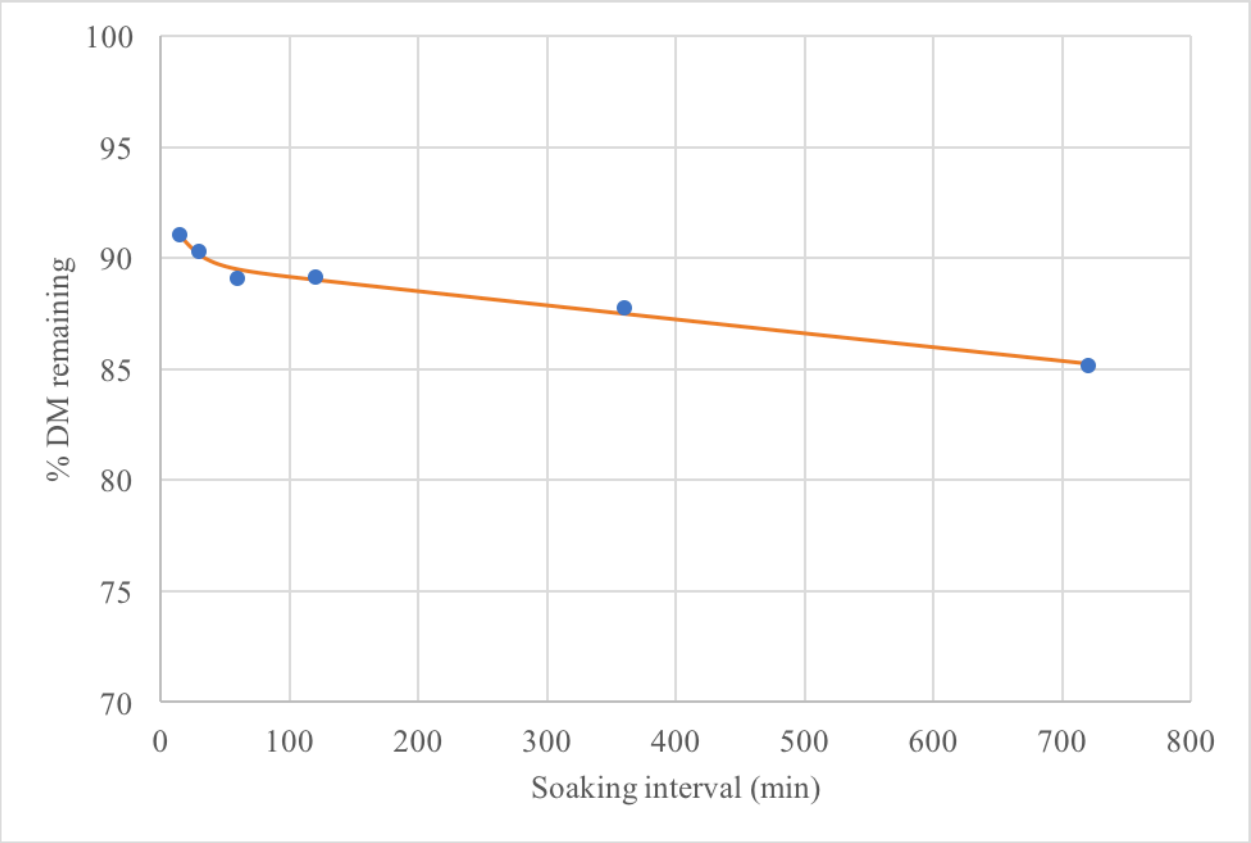


Figure 10. Dry matter loss in Tifton-85 bermudagrass hay soaked in cold (28°C) water for up to 720 min

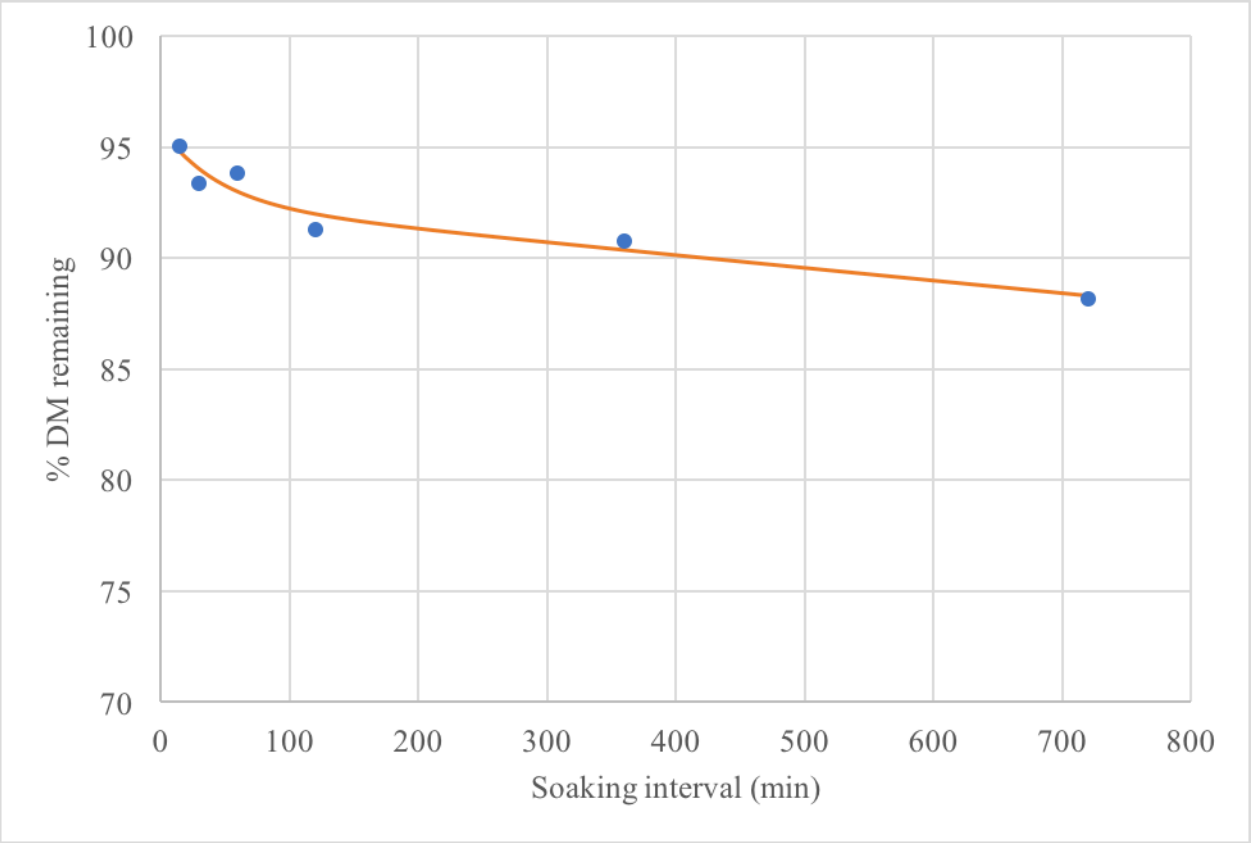
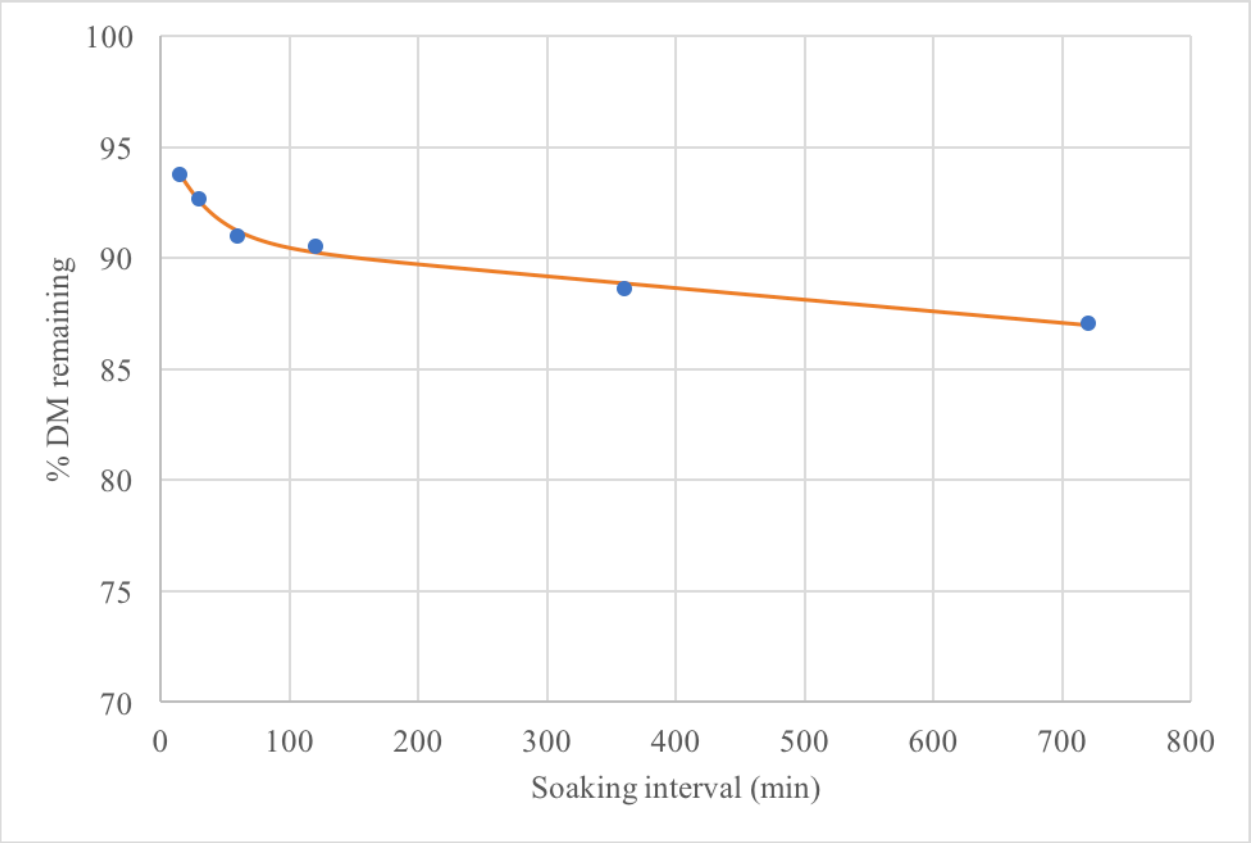


Figure 11. Dry matter loss in Tifton-85 bermudagrass hay soaked in hot (50°C) water for up to 720 min



Nonlinear prediction equations were determined for each hay type (Table 6). All four hays were fitted with a biexponential nonlinear model to determine a prediction equation where the percentage of DM remaining can be determined by [a * Exp (-b * t) + c * Exp (-d * t)]. In the prediction equation, a is equal to the point at which the rate of solubilization is reduced, b and d are rates of solubilization as a function of time, c is percentage of DM that is left to be solubilized by the second rate of solubilization, and t is the soaking interval in 28°C or 50°C.

Table 6. Solubilization equation characteristics of 4 forages where percentage of remaining DM (y) = a * Exp (-b * t) + c * Exp (-d * t)

Hay Type	Temperature	Equation variable				R^2
		a	b	c	d	
Alfalfa	28°C	77.989 ± 3.281	3.415 ± 7.31e-5	12.025 ± 3.143	0.015 ± 0.009	0.965
	50°C	78.961 ± 0.732	7.03e-5 ± 2.09e-5	16.499 ± 5.276	0.059 ± 0.021	0.985
Perennial peanut	28°C, 50°C	83.378 ± 2.128	8.38e-5 ± 4.42e-5	12.186 ± 2.008	0.014 ± 0.006	0.965
Coastal bermudagrass	28°C	91.142 ± 0.754	9.09e-5 ± 2.01e-5	11.49 ± 19.48	0.097 ± 0.116	0.964
	50°C	89.794 ± 0.365	7.19e-5 ± 9.24e-6	3.493 ± 2.966	0.062 ± 0.057	0.988
Tifton-85 bermudagrass	28°C	92.465 ± 1.730	6.42e-5 ± 3.48e-5	3.383 ± 2.107	0.023 ± 0.037	0.941
	50°C	90.787 ± 0.469	5.93e-5 ± 1.03e-5	4.943 ± 0.962	0.031 ± 0.013	0.993

y = percentage of remaining DM.
a = point at which the rate of solubilization is reduced.
b = rate of solubilization as a function of soaking interval.
c = percentage of DM that is left to be solubilized by the second rate of solubilization.
d = rate of solubilization as a function of soaking interval.

Discussion

Mold

Mold contamination of each sample was characterized by either "High" or "Low" in the current study. Roberts et al. (1987) found that using a relative mold index determined by visual determination is an accurate means of reporting concentrations of chitin and subsequently that of the mold contamination in a hay sample. The findings of Roberts et al. (1987) validates the characterization of visual mold determination in this study as valid means of describing mold contamination.

Mold was found most often in the legume hays compared with the grass hays in the present study. Per Gregory et al. (1963), thermophilic mold thrives at 60°C, the temperature of the drying oven used in the current study and also prefers the wettest portion of the hay. The legume hays were not conducive to air circulation throughout the sample during drying due to the compact nature of the hay samples, whereas the Coastal and Tifton-85 bermudagrass samples allowed more air flow through the sample during the drying process. It is hypothesized that this was a major contributing factor to the development of mold contamination; there was an absence of visible mold when subsequent samples were fluffed to facilitate air circulation throughout the drying period.

Furthermore, mold increases availability of WSC to leaching through the heating process in high-moisture forage (Earing et al., 2013). The mold contamination in this study was discovered while samples were contained in metal pans and were no longer in contact with water; thus, it is possible mold may have affected the WSC portion, but due to the lack of water surrounding the sample, WSC remained and was not leached, leading to a lack of an effect of mold when TNC was evaluated.

Mold in forage is to be expected and is reported by Martinson et al. (2011b) to appear at varying rates depending on the baling process utilized. These investigators found orchardgrass baled at high- and low-moistures experienced mold; low moisture produced 2.7 x 10^4 CFU/g, whereas the high-moisture hay experienced 24.8 x 10^6 CFU/g. The National Research Council (2015) also noted that the presence and percentage of mold contamination depend on the type of stored forage when evaluating the mold concentrations in hay, haylage, and silage; thus, it is not surprising that mold was present in the current study. Furthermore, Earing et al. (2013) noted decreases in mold contamination (>90% mold decrease) following steaming hay; however, researchers hypothesized the combination of DM loss and an increase in bale moisture and temperature may lead to an increase in mold formation in hay post-steaming if not fed immediately.

Mold concentrations above 500,000 CFU/g are detrimental to animal health and should be avoided (Adams et al., 1993). Although mold did not affect the solubilization of TNC or DM, mold did cause a numerical difference and should be considered when deciding on soaking protocol in terms of equine management practices, as horses are exceptionally sensitive to mold (Smith and Girish, 2008). Earing et al. (2013) found that steaming hay for 90 min decreased mold concentrations from 268,102 to 24,729 CFU/g. However, hay that has been subjected to moisture is at a greater risk for eliciting mold growth (Earing et al., 2013). If hay has been soaked or steamed, it should be fed as soon as possible to decrease the likelihood of mold contamination reaching levels that are considered unsafe for horses.

Total nonstructural carbohydrate

Soaking interval had an effect on solubilization of TNC in all hay types with the exception of alfalfa, and is in agreement with Martinson et al. (2012a) and Longland et al. (2013)

who found that soaked forage lost NSC over time. However, the finding that the TNC solubilization in alfalfa did not differ among soaking intervals is in conflict with the findings of Martinson et al. (2012a) who soaked 2 maturities of alfalfa hay in both 22°C and 39°C water temperatures and found significant NSC after 15-min in 22°C for alfalfa hay in bud as well as 60-min in 39°C for flowering alfalfa hay. Furthermore, time had a significant effect at each soaking interval (15-, 30-, 60-, 120-, 360-, and 720-min). These results agree with Muller et al. (2016) who found time impacted the leaching of NSC at all time intervals investigated, 0-, 12-, and 24-hr. However, previous studies have not investigated all of the soaking intervals included in the current study.

Studies by Martinson et al. (2012a), Longland et al. (2013), and Muller et al. (2016) all found an increased leaching of WSC and NSC occurred at the longest soaking interval investigated (12-, 16-, and 24-hr respectively); this was not the case in the current study. This study reached an apparent cessation of solubilization of TNC once hays reached an asymptote, which may be a result of the forage quality of the hays used in the current study compared with others in which a higher quality forage was utilized. If the present study had utilized forage that had not been subjected to weathering, these results may have agreed, but weathering is suspected of greatly impacting the quality and leaching TNC prior to the start of the current study. Anderson et al. (1981) discussed the effect of weathering on alfalfa round bales, and reported IVDMD decreased from 61.4 to 46.9 after being exposed to weathering.

Although differences between the total solubilization across the hay types may have differed from previous studies, some similarities remained. The rapid solubilization seen during the early treatment intervals agrees with previous studies. Martinson et al. (2012b) found that after 15-30 min, the decrease in NSC is not worth the potential loss of minerals following longer

soaking intervals. Hay type had a significant effect on the solubilization of TNC. Alfalfa hay appeared to lose the greatest percentage of NSC, 56%, compared with perennial peanut which only lost 34%. Alfalfa reached a point at which no more solubilization of NSC occurs at 43.9% of TNC whereas perennial peanut reached the point at which solubilization no longer occurs at 65.7% TNC. Coastal bermudagrass hay had NSC that was not as readily solubilized (69.4% remaining) as Tifton-85 bermudagrass hay (54.2% remaining). These results that suggest legumes may leach TNC more rapidly than grass hays, and is in conflict with Martinson et al. (2011a) who stated that relative to grasses, soaking alfalfa would have little effect on carbohydrate removal. However, Martinson et al. (2011a) compared legumes to C3 forages and not C4 forages which may account for the variability. Lastly, this is the first study that sought to characterize the solubilization of TNC opposed to the raw NSC concentration of each sample, which might account for some differences in previous studies compared to the current study.

Dry matter

Time had a significant effect on the solubilization of DM in all 4 hay types used in the current study. Martinson et al. (2012a) found that alfalfa and orchardgrass DM loss was greatest following the longest soaking interval, 12 hr in cold (22°C) water, in which alfalfa lost an average of 25.5% DM and orchardgrass lost an average of 18.5% DM. It is speculated that in the previous study by Martinson et al. (2012a) as well as the current study, the large DM losses seen post-soak in alfalfa hay, 76% DM remaining in cold and 75% DM remaining in hot in the current study, could be attributed to leaf shatter and subsequent loss as well as DM solubilization. Leaf shatter in the current study was characterized by leaf matter that was unable to be collected post-soak due to the lack of substance in which alfalfa was the most notable.

However, unlike TNC solubilization, the solubilization of DM was affected by soaking liquor temperature in alfalfa and Tifton-85 bermudagrass, but only showed a trend toward significance in Coastal bermudagrass. Interestingly, perennial peanut DM solubilization was unaffected by soaking liquor temperature. Martinson et al. (2012a) found temperature was significant when using 22°C and 39°C water to soak flowering and bud alfalfa hay as well as vegetative and flowering orchardgrass hays for 15-, 30-, 60-, 720-min. Researchers reported DM losses of 23, 28, 17, and 20% for the 4 forages, respectively, following the 720-min soak in 39°C liquor.

Unlike TNC, which appeared to reach the point at which no more solubilization occurs, DM solubilization appeared to reach a second rate of solubilization that was slower than the first rate. All hay types fit the biexponenial nonlinear model well, and agree with the findings of Argo et al. (2015) who found DM continued to decrease through soaking treatments of 0, 7, and 16 hr. Additionally, Longland et al. (2011) also analyzed DM loss and found DM to decrease over time throughout soaking hay for 0-, 20-, 40-, 180-, and 960-min, and did not report as asymptote as that found when the current study analyzed TNC.

Conclusions

Horses with metabolic disorders often require low-NSC diets. There is a need to reduce the NSC fraction of hays, and the most popular strategy is through soaking. Extent of solubilization of TNC in forage is dependent on soaking interval and hay type, and follows a nonlinear regression in which a point at which no further solubilization does occur. Similarly, DM solubilization was not only dependent on soaking interval and forage type but was also dependent on temperature of the soaking liquor in all hays tested apart from perennial peanut; it

also follows a nonlinear regression. However, DM solubilization appears to continue through extended soaking intervals and does not reach an asymptote within the time intervals tested in the current study. The prediction equations can be utilized to estimate the appropriate soaking intervals for the given forage to fall below recommended NSC concentrations. If soaking is considered the best method of reducing NSC intake, the reductions in DM and subsequent increases in feed costs may deter owners from utilizing this method.

Future research is needed to establish the limit of soaking C4 forages so as not to disrupt the other nutrients in the forage. Furthermore, mold and bacterial concentrations of soaked hay should be investigated further to check the quality of the forage before feeding to horses. Lastly, environmental effects should be considered further with regards to the disposal of the soaking liquor.

Literature Cited

Adams, R. S., K. B. Kephart, V. A. Ishler, L. J. Hutchinson, and G. W. Roth. 1993. Mold and mycotoxin problems in livestock feeding. Dep. of Dairy and Animal Science, Extension Publ. DAS 93-21. Pennsylvania State University, College of Agricultural Sciences and Cooperative Extension, University Park, PA.

AOAC. 1995. Official Methods of Analysis, 16th Ed. Association of Official Analytical Chemists, Washington, D.C.

Argo, C. McG., A. H. A. Dugdale, C. M. McGowan. 2015. Considerations for the use of restricted, soaked grass hay diets to promote weight loss in management of equine metabolic syndrome and obesity. Vet. J. 206:170-177.

Ball, D., D. Blade, G. Lacefield, N. Martin, and B. Pinkerton. 2010. Minimizing losses in hay storage and feeding. ANR-1356.

Borgia, L., S. J. Valberg, K. Watts, and J. Pagan. 2009. Glycemic/ insulemic response to feeding hay with different water soluble carbohydrate content in healthy and polysaccharide storage myopathy-affected horses. J. Equine Vet. Sci. 29:355-357.

Collins, M. 1991. Hay curing and water soaking: effects on composition and digestion of alfalfa leaf and stem components. Crop Sci. 31:219-223.

Collins, J. L., R. M. Hoffman, and H. S. Spooner. 2015. The effect of dry versus soaked hay on glycemic response in horses. J. Equine Vet. Sci. 35:425.

Divers, T. J. 2008. Preventing endocrine-related laminitis. J. Equine Vet. Sci. 28:317-319.

Earing, J. E., M. R. Hathaway, C. C. Sheaffer, B. P. Hetchler, L. D. Jacobson, J. C. Paulson, and K. L. Martinson. 2013. Effect of steaming on forage nutritive values and dry matter intake by horses. J. Anim. Sci. 91:5813-5820.

Elzinga, S., P. Wood, and A. A. Adams. 2016. Plasma lipidomic and inflammatory cytokine profiles of horses with equine metabolic syndrome. J. Equine Vet. Sci. 40:49-55.

Elzinga, S. P., J. S. Weese, and A. A. Adams. 2016. Comparison of the fecal microbiota in horses with equine metabolic syndrome and metabolically normal controls fed a similar all-forage diet. J. Equine Vet. Sci. 44:9-16.

Frank, N. 2011. Equine metabolic syndrome. Vet. Clin. Equine 27:73-92.

Geor, R. J. 2008. Metabolic predispositions to laminitis in horses and ponies: obesity, insulin resistance and metabolic syndromes. J. Equine Vet. Sci. 28:753-759.

Geor, R. J. 2013. Nutritional management of insulin resistance. J. Equine Vet. Sci. 33:838-859.

Gordon, M. E., M. L. Jerina, S. L. King, K. E. Davison, J. K. Young, R. H. Raub. 2007. The effects of nonstructural carbohydrate content and feeding rate on glucose and insulin response to meal feeding in equine. J. Equine Vet. Sci. 27:489-493.

Gregory, P. H., M. H. Lacey, G. N. Festenstein, and F. A. Skinner. 1963. Microbial and biochemical changes during the moulding of hay. J. Gen. Microbiol. 33:147-174.

Henneke, D. R., G. D. Potter, and J. L. Kreider. 1983. Relationship between condition score, physical measurement and body fat percentage in mares. Equine Vet. J. 15:371-372.

Hoffman, R. M., J. A. Wilson, D. S. Kronfeld, W. L. Cooper, L. A. Lawrence, D. Sklan, and P. A. Harris. 2001. Hydrolyzable carbohydrates in pasture, hay, and horse feeds: direct assay and season variation. J. Anim. Sci. 79:500-506.

Longland, A. C., and B. M. Byrd. 2006. Pasture nonstructural carbohydrates and equine laminitis. J. Nutr. 136:2099-2102.

Longland, A. C., C. Barfoot, and P. A. Harris. 2010. Insulin resistance – hay analysis and subsequent feeding management. J. Equine Vet. Sci. 30:110.

Longland, A. C., C. Barfoot, and P. A. Harris. 2011. Effects of soaking on the water-soluble carbohydrate and crude protein content of hay. Vet. Rec. doi:10.1136/vr.d157.

Longland, A. C., C. Barfoot, and P. A. Harris. 2013. Effect of period, water temperature and agitation loss of water-soluble carbohydrates and protein from grass hay: implications for equine feeding management. Vet. Rec. doi:10.1136/vr.101820.

Martinson, K., H. Jung, M. Hathaway, and C. Sheaffer. 2011a. The effect of soaking hay on dry matter loss and fructan removal. J. Equine Vet. Sci. 31:278-279.

Martinson, K., W. Coblentz, and C. Sheaffer. 2011b. The effect of harvest moisture and bale wrapping on forage quality, temperature, and mold in orchardgrass hay. J. Equine Vet. Sci. 31:711-716.

Martinson, K., H. Jung, M. Hathaway, and C. Sheaffer. 2012a. The effect of soaking on carbohydrate removal and dry matter loss in orchardgrass and alfalfa hays. J. Equine Vet. Sci. 32:332-338.

Martinson, K. L., M. Hathaway, H. Jung, and C. Sheaffer. 2012b. The effect of soaking on protein and mineral loss in orchardgrass and alfalfa hay. J. Equine Vet. Sci. 32:776-782.

McGowan, C. M., A. H. Dugdale, G. L. Pinchbeck, C. McG. Argo. 2013. Dietary restriction in combination with a nutraceutical supplement for the management of equine metabolic syndrome in horses. Vet. J. 196:153-159.

McIntosh, B., G. Bates, D. McIntosh, and J. Beeler. 2013. Effects of cutting height, nitrogen fertilization, and time of day on nonstructural carbohydrate content of bermudagrass (Cynodon dactylon). J. Equine Vet. Sci. 33:369.

Moore-Colyer, M. J. S. 1996. Effects of soaking hay fodder for horses on dust and mineral content. Anim. Sci. 63:337-342.

Moore-Colyer, M. J. S., J. L. E. Taylor, and R. James. 2016. The effect of steaming and soaking on the repirable particle, bacteria, mould, and nutrient content in hay for horses. J. Equine Vet. Sci. 39:62-68.

Mullenix, M .K., E. J. Bungenstab, J. C. Lin, B. E. Gamble, and R. B. Muntifering. 2012. Case study: productivitiy, quality characteristics, and beef cattle performance from cool-season annual forage mixtures. Prof. Anim. Sci. 28:379-386.

Muller, C. E., K. Nostell, and J. Brojer. 2015. Microbial counts in forages for horses- effect of storage time and of water soaking before feeding. J. Equine Vet. Sci. 35:622-627.

Muller, C. E., K. Nostell, and J. Brojer. 2016. Methods for reduction of water soluble carbohydrate content in grass forages for horses. Livestock Sci. 186:46-52.

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

Pratt-Phillips, S., J. Kutzner-Mulligan, R. Marvin, H. Brown, C. Sykes, and J. Federico. 2014. The effect of feeding two or three meals per day of either low or high nonstructural carbohydrate concentrates on postprandial glucose and insulin concentrations in horses. J. Equine Vet. Sci. 34:1251-1256.

Roberts, C. A., K. J. Moore, D. W. Graffis, H. W. Kirby, and R. P. Walgenbach. 1987. Chitin as an estimate of mold in hay. Crop Sci. 27:783-785.

Schuver, A., N. Frank, K. A. Chameroy, and S. B. Elliott. 2014. Assessment of insulin and glucose dynamics by using an oral sugar test in horses. J. Equine Vet. Sci. 34:465-470.

Secombe, C. J., and G. D. Lester. 2012. The role of diet in the prevention and management of several equine diseases. Anim. Feed Sci. Tech. 173:86-101.

Sladden, S. E. 2016. Near-infrared spectroscopy and forage analysis. Personal Communication.

Smith, T. K., and C. K. Girish. 2008. The effects of feed born mycotoxins on equine performance and metabolism. Transworld Research Network. 48-70.

Suagee, J. K., R. K. Splan, K. L. Swyers, R. J. Geor, and B. A. Corl. 2015. Effects of high-sugar and high-starch diets on postprandial inflammatory protein concentrations in horses. J. Equine Vet. Sci. 35:191-197.
Tadros, E. M., N. Frank, and D. W. Horohov. 2013. Inflammatory cytokine gene expression in blood during the development of oligofructose-induced laminitis in horses. J. Equine Vet. Sci. 33:802-808.

Taylor, D., A. Sperandeo, J. Schumacher, T. Passler, A. Wooldridge, R. Bell, A. Cooner, L. Guidry, H. Matz-Creel, I. Ramey, and P. Ramey. 2014. Clinical outcome of 14 obese, laminitic horses managed with the same rehabilitation protocol. J. Equine Vet. Sci. 34:556-564.

USDA. 2017. APHIS Info Sheet: Demographics of the U.S. equine population, 2015.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:583-3597.

Verspreet, J., E. Dornez, W. Van de Ende, J. A. Delcour, and C. M. Courtin. 2015. Cereal grain fructans: structure, variability and potential health effects. Trend in Food Sci. and Technology. 43:32-42.

Vick, M. M., A. A. Adams, B. A. Murphy, D. R. Sessions, D. W. Horohov, R. F. Cook, B. J. Shelton, and B. P. Fitzgerald. 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85:1144-1155.

Watts, K. 2004. Forage and pasture management for laminitic horses. Clinical techniques equine practice. 3:88-95.

Weiss, D. J., O. A. Evanson, D. McClenahan, J. J. Fagliari, C. T. Dunnwiddle, and R. E. Wells. 1998. Effect of a competitive inhibitor of platelet aggregation on experimentally unduced laminitis in ponies. Am. J. Vet. Res. 59:814-817.

Appendices

Appendix 1. Non-pure samples identified pre-treatment in 2 hay types

Sample characteristic		Treatment		Contaminant
Hay Type	Bale	Time	Temperature	
Tifton-85 bermudagrass	2			Common bermudagrass
Perennial Peanut	1	30	Hot	Stick
Perennial Peanut	2	60	Cold	Stick
Perennial Peanut	2	60	Hot	Stick
Perennial Peanut	3			*Panicum spp*
Perennial Peanut	3	15	Cold	Stick
Perennial Peanut	3	60	Cold	Stick
Perennial Peanut	3	120	Cold	Stick
Perennial Peanut	3	120	Hot	Stick
Perennial Peanut	3	720	Hot	Stick
Perennial Peanut	3	0		Stick
Perennial Peanut	4			*Panicum spp*
Perennial Peanut	6	30	Hot	Stick
Perennial Peanut	6	120	Cold	Stick

Appendix 2. Mold contaminated hay samples identified post soaking treatment and 60°C drying

Sample characteristic		Treatment		Level of Mold
Hay Type	Bale	Time	Temperature	
Coastal	6	15	Cold	High
Tifton-85	3	120	Cold	High
Tifton-85	3	120	Hot	High
Alfalfa	1	15	Cold	High
Alfalfa	1	15	Hot	Low
Alfalfa	1	30	Cold	Low
Alfalfa	1	30	Hot	High
Alfalfa	1	120	Cold	High
Alfalfa	1	720	Cold	Low
Alfalfa	2	15	Cold	Low
Alfalfa	2	30	Cold	High
Alfalfa	2	30	Hot	High
Alfalfa	2	360	Cold	High
Alfalfa	2	720	Cold	Low
Alfalfa	3	15	Cold	High
Alfalfa	3	30	Cold	High
Alfalfa	3	30	Hot	High
Alfalfa	3	120	Hot	High
Alfalfa	3	720	Hot	High
Alfalfa	4	15	Cold	High
Alfalfa	4	15	Hot	High
Alfalfa	5	15	Cold	High
Alfalfa	5	15	Hot	High
Alfalfa	5	720	Cold	Low
Alfalfa	6	15	Cold	High
Alfalfa	6	15	Hot	High
Alfalfa	6	360	Cold	High
Alfalfa	6	720	Cold	Low
Perennial Peanut	1	15	Hot	High
Perennial Peanut	1	30	Cold	High
Perennial Peanut	1	30	Hot	High
Perennial Peanut	1	120	Cold	High
Perennial Peanut	1	120	Hot	Low
Perennial Peanut	1	720	Cold	High
Perennial Peanut	1	720	Hot	High
Perennial Peanut	2	15	Cold	High
Perennial Peanut	2	15	Hot	High
Perennial Peanut	2	30	Cold	High
Perennial Peanut	2	30	Hot	High
Perennial Peanut	2	120	Cold	High
Perennial Peanut	2	720	Cold	High
Perennial Peanut	3	15	Hot	High

Appendix 2 continued. Mold contaminated hay samples identified post soaking treatment and 60°C drying

Perennial Peanut	3	30	Cold	High
Perennial Peanut	3	30	Hot	High
Perennial Peanut	3	120	Cold	High
Perennial Peanut	3	720	Cold	High
Perennial Peanut	4	15	Cold	High
Perennial Peanut	4	15	Hot	High
Perennial Peanut	4	720	Cold	Low
Perennial Peanut	5	15	Cold	Low
Perennial Peanut	5	15	Hot	High
Perennial Peanut	6	15	Cold	High
Perennial Peanut	6	15	Hot	High
Perennial Peanut	6	60	Cold	Low
Perennial Peanut	6	60	Hot	High
Perennial Peanut	6	720	Cold	Low

Appendix 3. Calculating total nonstructural carbohydrate on %DM basis given titer amount and standard curve for the batch of Shaffer-Somogyi reagent used

1.	[Weight of sample (g) * DM] * 1000 = DM (mg)
2.	Enzyme blank titer (mL) – Sample titer (mL) = Adjusted titer (mL)
3.	[(Slope of standard curve * 0.01) * Adjusted titer (mL)] + (Y-intercept of standard curve * 0.01) = Equation derived number
4.	Equation derived number * 25 = Dilution correction
5.	Dilution correction / DM (mg) = % TNC on DM basis

All %TNC figures should be calculated in duplicate and analyzed for coefficient of variation to lie below 5%

Appendix 4. Alfalfa hay composition means post-soaking for up to 720 min

Treatment		DM	DM basis (%)		n
Time (min)	Temperature (°C)		TNC	Ash	
15	28	75.26	3.17	5.06	8
15	50	72.88	3.23	4.94	8
30	28	71.90	2.58	5.15	8
30	50	69.74	2.87	4.61	8
60	28	69.50	2.93	4.73	5
60	50	66.22	2.63	4.55	5
120	28	68.40	2.55	4.46	6
120	50	67.23	2.80	4.14	8
360	28	65.06	3.57	4.36	7
360	50	64.89	2.99	4.20	6
720	28	64.94	2.41	3.96	8
720	50	63.97	2.35	4.05	8

Appendix 5. Perennial peanut hay composition means post-soaking for up to 720 min

Treatment		DM	DM basis (%)		n
Time (min)	Temperature (°C)		TNC	Ash	
15	28	80.55	7.08	6.76	8
15	50	78.05	7.45	6.66	8
30	28	77.13	7.03	6.64	8
30	50	75.40	6.34	6.30	8
60	28	74.55	6.24	6.33	5
60	50	74.07	6.74	6.20	5
120	28	72.10	6.31	6.11	8
120	50	71.75	5.20	5.83	8
360	28	68.68	5.87	5.70	7
360	50	67.83	6.17	5.44	7
720	28	66.02	5.46	5.62	8
720	50	66.90	5.72	5.36	8

Appendix 6. Coastal bermudagrass hay composition means post-soaking for up to 720 min

Treatment		DM	DM basis (%)		n
Time (min)	Temperature (°C)		TNC	Ash	
15	28	81.58	7.93	5.17	8
15	50	79.25	7.73	4.79	8
30	28	79.79	7.45	4.57	8
30	50	78.64	7.22	4.25	8
60	28	79.00	6.80	4.16	8
60	50	77.63	5.81	3.84	6
120	28	77.93	6.70	3.63	8
120	50	77.42	6.52	3.47	7
360	28	76.78	6.44	3.15	8
360	50	76.40	6.17	3.00	8
720	28	73.98	6.07	2.98	8
720	50	74.11	5.98	2.84	8

Appendix 7. Tifton-85 bermudagrass hay composition means post-soaking for up to 720 min

Treatment		DM	DM basis (%)		n
Time (min)	Temperature (°C)		TNC	Ash	
15	28	82.38	3.80	3.87	7
15	50	81.25	4.59	3.66	7
30	28	80.94	4.30	3.56	7
30	50	80.36	4.06	3.35	7
60	28	81.32	4.08	3.34	5
60	50	78.86	3.59	3.29	5
120	28	78.87	3.57	3.04	6
120	50	78.51	3.43	2.91	7
360	28	78.71	3.33	2.66	7
360	50	76.84	3.32	2.64	7
720	28	76.42	3.25	2.75	7
720	50	75.49	3.23	2.40	7

Appendix 8. Mean water temperatures for cold and hot water during hay soaking treatments through 720 min

Soaking interval (min)	Water temperature (°C)	
	Cold	Hot
0	28.5	50
15	28	48.5
30	28	46
60	28	44
120	27	40
180	27	37
360	26	30.5
720	25	26

Appendix 9. Measures of ambient temperature (°C) and relative humidity (%) during the soaking of 4 hay types in hot and cold water for up to 720 min

Date	Means	
	Ambient Temperature (°C)	Relative Humidity (%)
06/09/2016	28.73	56.58
06/10/2016	25.85	58.80
06/11/2016	25.38	59.50
06/12/2016	25.25	64.25
06/13/2016	25.65	61.75
06/14/2016	25.68	63.17
06/15/2016	24.88	62.75
06/16/2016	24.79	63.00
06/17/2016	24.95	63.88
08/12/2016	25.32	63.89
08/13/2016	24.95	62.36
08/14/2016	25.50	62.75