Assessing the reliability of plant-wax markers to delineate diet choice and feed efficiency in beef heifers

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Animal and Poultry Science

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July, 24th, 2012
Blacksburg, Virginia

Keywords: Beef cattle, diet choice, feed efficiency, grazing, plant-wax markers

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ABSTRACT

Estimating feed efficiency in grazing environments is challenging due to difficulties in quantifying food intakes and diet choices in free-grazing animals. The plant-wax marker technique may be a useful tool to redress this problem. However, its reliability needs to be validated before its wider application. This study was designed to assess the reliability of plant-wax markers for estimating botanical composition of test diets, and diet choices in beef cattle, and provided opportunities to evaluate efficiency in growing heifers. To test estimation of botanical composition, samples of red clover and fescue hay were mixed to form test diets containing 0-100% of either forage. To test estimation of diet choices, 24 heifers from large and moderate frame size lines were evaluated at two instances. Cubed red clover and fescue hays were offered ad libitum. After an acclimation period, feed intakes and body weights were collected for 10 days; fecal samples were collected for the final 5 days. Hydrocarbons and alcohols were quantified with gas chromatography. Estimates were based on least squares. Operator expertise affected measured concentrations of shorter-chained *n*-alkanes ($P<0.041$) and long-chain alcohols ($P<0.02$). Still, overall reliability of the technique was unaffected. Large and moderate framed animals did not differ in efficiency ($P>0.05$), although large framed animals had increased red clover intakes ($P<0.01$). Once corrected for fecal losses of *n*-alkanes, diet choices were estimated accurately. Plant-wax markers provided reliable estimates of botanical composition of diets, and diet choices of animals, suggesting it is a valuable tool to assess efficiencies of grazing cattle.

DEDICATION

This work is dedicated to my parents Napoleón Vargas Palacios and Mirian Jurado, who have given me their absolute support during this time. It is also lovingly dedicated to my soul mate and best friend Paulina Arboleda for her unconditional support, and is also dedicated to my brothers Andrés and Alejandro Vargas.

ACKNOWLEDGEMENTS

There are a number of people without whom this work would have not been possible and for whose guidance and help I am greatly thankful.

Above all, I would like to thank my parents, my brothers and my fiancée Paulina Arboleda for their unconditional love, support, and encouragement that helped me navigate throughout this journey. I would also like to thank my family and friends, in Ecuador, in the US and in Canada, whose kind words and thoughts I cherish.

This thesis would not have never been possible without the help, guidance, and knowledge of my advisor Dr. Ron Lewis, who kindly provided me not only with insights that allowed me to best sort out challenges that arose during the elaboration of this work, but also with tools that certainly will help me overcome future challenges in my career. Observations, comments and advice from Dr. Mark McCann, and Dr. Ben Tracy have been valuable for the development of my thesis, for which I am very grateful.

I am very grateful for the time, effort and help that I received from Amy Tanner, Sarah Blevins, Jessica Rich, during my experimental work, and for the friendship of Gabi Marquez and Joe Emenheiser.

Finally, I would like to acknowledge the financial, academic and technical support of Virginia Tech, the Graduate School and the Department of Animal Science, that provided the necessary financial support for this project. In addition I am extremely thankful for the financial support and assistantship of the Fulbright-Senescyt program in Ecuador, especially for the scholarship that I was awarded, that provided me with the financial means to complete my degree.

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LIST OF ABBREVIATIONS

Item	Term
ADF	Acid Detergent Fiber
ADF	Average daily gain
AIA	Acid Indigestible Ash
ANOVA	Analysis of Variance
ARC	Agricultural Research Council
BW	Body weight
CP	Crude protein
CWC	Cell Wall Constituents
D1	Once a day dosing
D2	Twice a day dosing
DM	Dry Matter
DMD	Dry Matter Digestibility
DMI	Dry Matter Intake
DMI1	Dry matter intake from study 1
DMI2	Dry matter intake from study 2
ERCP	Estimated Red Clover Proportion
FCR	Feed conversion ratio
FS	Frame Size
GC	Gas Chromatography
I-ADF	Indigestible ADF
KR	Kleiber ratio
KSI	Kulzcynski Similarity Index
LCFA	Long Chain Fatty Acids
LCOH	Long Chain Alcohols
MBW	Metabolic Body Weight
MPE	Mean Prediction Error
NDF	Neutral Detergent Fiber
NE	Net Metabolizable Energy
N-NO3	Nitrate nitrogen
NRC	National Research Council
ORCP	Observed Red Clover Proportion

PC1	Principal component 1
PC2	Principal component 2
PCA	Principal Components Analysis
RCP1	Red clover proportion from study 1
RCP2	Red clover proportion from study 2
RFI	Residual Feed Intake
RMPE	Relative Mean Prediction Error
SPE	Solid Phase Extraction
SRF	Standard Response Factor
SVAREC	Shenandoah Valley Agricultural Research and Extension Center
WSC	Wall Soluble Carbohydrates

CHAPTER 1

INTRODUCTION

Providing feed to cattle is the single largest expense in most commercial beef production enterprises, and thus any effort toward improving the efficiency of feed use will help reduce input costs (Arthur et al., 2001). Profitability is the main goal of an animal production system, and it is a function of both the inputs and outputs. Therefore, in order to accurately determine the profitability of any animal production system, the components of the inputs and outputs must be known. Most of the constituents related to management such as labor, facilities and equipment are easily identifiable; however, others such as feed intake are challenging to quantify.

Feed efficiency characterizes the ability of an animal to use the feed it is offered, and transform it into a valuable product, such as meat or milk. In order to effectively select for more efficient animals, feed intake must be adequately estimated. Food intake and feed efficiency have been properly assessed for some species in controlled environments, such as poultry and swine; however, under normal conditions in many beef cattle systems, both feedlot and pasture-based, animals are allowed to eat *ad libitum*. While in feedlot systems production costs can be fairly estimated, in pasture based systems it is difficult to estimate the real costs of production, as there is no accurate measure of forages being eaten. These challenges increase when there is more than one feed offered, as it is the case in animals managed under range or grazing conditions (Archer, 1999).

Genetic selection for growth in beef cattle has led to animals having larger mature sizes (live weights), therefore increasing the animals' requirements for energy and protein to meet metabolic needs. This has resulted in increased feed intakes, likely having an effect on overall efficiency (Vargas, et al. 1999; Konaroglu and Hoffman, 2010).

Efforts to assess diet intake of ruminants in free range conditions have led to the use of markers, such as chromic oxide and plant waxes. Plant cuticular waxes have been successfully used as a tool to predict feed intake. When used as fecal markers they have been shown to be valuable for estimating diet composition and digestibility of the diet in a variety of mammalian herbivores, including sheep, goats,

and dairy cattle (Dove and Mayes, 2005; Hameleers and Mayes, 2008). However, application of this technique has been limited in beef cattle.

Although there are some limitations when using this procedure, it has been shown to be robust and useful in predicting feed intake. Despite the body of literature that supports the validity of this technique, it could become meaningless if it does not incorporate conditions pertinent to those of the producers or the industry to which is aimed (e.g., plant species found in the zone, breeds of animals commonly found in production systems). It is important then to validate the accuracy of a technique that allows not only feed intake to be estimated, but also diet choice. Such validation must first be done under controlled conditions, before the technique is applied to grazing animals.

This literature review addresses the prediction of feed intake and diet choice using plant waxes, specifically *n*-alkanes and long chain alcohols, as fecal markers. In addition, aspects in both the methodology and the application of these techniques, as well as the limitations and considerations necessary for this technique to be useful, are detailed. Finally, the objectives and hypotheses pertaining to the application of this methodology to delineate feed efficiency in conditions common to beef cattle systems in the Appalachians are discussed.

CHAPTER 2

LITERATURE REVIEW

FEED INTAKE

Feed intake, along with feed quality, determines total nutrient consumption by the animal, and thus is the foundation for animal production (Vallentine, 2001).

Factors regulating feed intake

According to Dulphy and Demarquilli (1994), the two main factors determining feed intake are the characteristics of the forage offered, such as physical, chemical and organoleptic qualities, and the intake capacity of the animal. Most of the mechanisms regulating dry matter intake involve a series of metabolic pathways as well as physical constraints leading to energy balance in the animal, which can be easily affected not only by the characteristics of the diet, but also by the feeding time (Illius and Jessop, 1996; Nikkhah, 2011). Disruptions to these mechanisms may result in loss of performance or metabolic distress (NRC, 1987; Forbes, 2007). Vallentine (2001) described several factors that influence feed intake, such as (i) physiological or productive state (e.g., lactation, puberty), (ii) dietary factors (e.g., palatability, digestibility), (iii) weather, (iv) management, and (v) animal anatomical factors.

In this study, it is pertinent to discuss in more depth body size and diet quality, as these may have particular importance in the beef industry due to the trend toward increased mature weights, which in part may be redressed by improving the pastures available for grazing.

Mature Weight

Given a source of feed that is not limiting (a good quality feed *ad libitum*), the intake of grazing ruminants will be influenced by energy demands, which are dependent on the physiological state of the animal. Energy demand and feed intake are related to live weight, live weight change (growth), and milk production (Allison, 1985).

Despite several studies showing that feed intake was affected by live weight, factors other than body size such as age and previous nutrition level may have a greater impact on feed intake even in animals of the same breed (Allison, 1985). It has been hypothesized that animals of the same breed and age, and with similar nutritional backgrounds, will have similar intakes. Once accounting for these factors, the true effect of body weight on intake can be more properly assessed.

There is evidence that heavier animals will have higher total energy demands compared to lighter animals of the same type, and in the same physiological stage, thus requiring more feed to meet those demands (e.g., higher dry matter intake)(Forbes, 2007). If feed efficiency were to be assessed without accounting for differences in body, or metabolic, weight, inferences may be inaccurate if not incorrect.

Metabolic size

To account for differences in body size, several authors suggested methods to standardize and describe metabolic rate (heat production in fastened animals or basal metabolism) and body size for various species including cattle, sheep, and mice. Through various experiments, Brody and Procter (1932) obtained the following equation:

$$M = 70.4\, W^{0.734}, \qquad [1]$$

where M is metabolic size, and W represents live weight. Kleiber (1947) on the other hand, maintained that the relationship between body weight and metabolic rate was $W^{0.75}$ and that this form was preferably since it was easier to calculate. Even though initially $W^{0.734}$ was widely accepted by most scientist including the NRC, nowadays $BW^{0.75}$ is most commonly used as studies showed no systematic error when expressing body weight with either power (Blaxter and Wainman, 1966). However, the biological estimate remains 0.734, and with modern computers it is no longer a difficulty to work with logarithms, and thus the 'true' estimate should be used. It has been suggested that the energy needs per unit of weight for smaller animals are greater than those for larger animals (Allison, 1985). The use of metabolic weight therefore allows comparisons to be made regarding energy needs for animals of different body weight, which would otherwise not be possible (Kleiber, 1947).

Diet Quality

Another important factor effecting feed intake is diet quality. Digestibility of the forages, mainly neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) contents, affects intake and preference for different forages species. Cattle will select forages with a lower ADF, and thereby lower cellulose content (Allison, 1985). Having a general understanding of how these characteristics relate to feed intake is therefore of considerable importance.

ADF and NDF Content

Acid detergent fiber is a measure of a portion of plant cell walls that includes cellulose, lignin, acid insoluble ash, and silica (Van Soest, 1991; Beauchemin, 1996). Prediction of voluntary dry matter intake based on ADF alone was poor as observed by a low coefficient of determination ($r^2 = 0.40$), in studies using lambs fed mixed diets (Kursli and Russel, 2002). Indigestible ADF (**I-ADF**) has also been used to predict feed intake in grazing studies. However, due to its moderately low correlation with dry matter intake (**DMI**) ($r = 0.37$), it also is considered a poor predictor of feed intake (Mendoza et al., 1995). Neutral Detergent Fiber is a measure of the insoluble fiber found in feeds and consists of the cross linked matrix of plant cell walls and coarse fibers that forms the rumen mat that stimulates rumen function. The main components of NDF are cellulose, hemicellulose, and lignin (Van Soest et al., 1991). It traditionally has been proposed an indicator of intake and gastrointestinal fill (Mertens, 1973; Van Soest et al., 1991). However, Arelovich et al. (2008) found that NDF was not a consistent predictor of voluntary intake in dairy and beef cattle. Using regression analyses, they estimated in beef cattle that DMI increased as NDF content of the forage increased from 7.5% to 35.3% (r^2=0.965); conversely, in dairy cattle, DMI decreased as NDF content increased from 22.5% to 45.8% (r^2=0.672). These results suggested that in animals with different levels of production, and thus with different energy requirements, the effect of fiber content on DMI will differ appreciably. On the other hand, NDF appears not to be a good enough

predictor of DMI when used alone, as shown by the range of the correlation coefficient, which may range from -0.65 to -0.31 (Decruyenaere et al., 2009).

Protein Content

It has been hypothesized that animals will eat in order to maximize their production potential, although this may be limited by some constraints such as the quality of the feed and the capacity or volume of the gut (Decruyenaere et al., 2009). It follows that feeds with different levels of nutrients will affect DMI. For instance dietary protein influences digestibility of the diet because bacteria in the rumen need nitrogen to meet their particular needs (Van Soest, 1991). A relationship between the amount of protein in the diet and DMI should therefore be expected.

Nevertheless, contradictory results have been encountered. Young animals tended to select forages with higher crude protein levels (Allison et al., 1985), and in sheep feed intakes markedly decreased when crude protein levels were below 7%. Similarly, Bond et al. (1962) found that Angus cattle had higher intakes when crude protein contents increased in the diet from 5.0% to 13.4%; DMI increased from 0.44 kg of intake/kg of weight with the lowest level, to 0.76 kg of intake/kg of weight with the highest level. Comparable outcomes were observed when cattle were fed silage of Beardgrass (*Brachiaria* sp.) with a crude protein content of 90 g/kg of DM supplemented with concentrate, with crude protein contents ranging from 159.6 to 334.9 g/kg of DM. Increasing levels of concentrate resulted in increasing DMI (Pereira et al., 2008). This suggests the protein level of the diet may have a more noticeable effect on DMI when poor quality forage is the main component of the diet. On the other hand, Obeid et al. (2006) found that DMI was not affected by levels of protein in the diet ranging from 9% to 15% in young Zebu bulls.

FEED EFFICIENCY

In general, feed efficiency of an animal system could be considered as the production of a desired effect (product) with a minimum of input and measured as the ratio of output to input (Trenkle and Willham,

1977). Efficiency can be measured in a number of ways, of which gross efficiency and feed conversion ratio have been most widely used. Gross efficiency is defined as the ratio of production outputs (i.e., animal products) and feed inputs (i.e., herbage consumed), and can be expressed in terms of ratios of either fresh or dry weights of forage or some constituent of the diet (e.g. energy, nitrogen) and animal product (Hodgson et al., 1979; Archer, 1999). Partial efficiency is defined as the ratio of weight gain to feed after the maintenance requirements have been subtracted (Archer, 1999). Alternatively, feed conversion ratio, usually represents feed intake per unit of weight gain (Arthur et al., 2004).

Although other measures of efficiency have been proposed, those related to feed intake are the most pertinent to this study, and therefore will be discussed in more detail.

Feed efficiency has been shown to be correlated with other traits such as live weight and growth rate, suggesting that analysis of feed intake alone likely is not sufficient when characterizing overall efficiency. Other measures such as residual feed intake (**RFI**) and feed conversion ratio (**FCR**) have therefore been derived, which relate intake to levels of production (Arthur et al., 2004).

Residual Feed intake

Also referred to as net feed efficiency, RFI measures the difference between observed and predicted feed intake based on the requirements for body weight maintenance and level of production. It therefore allows for testing the hypothesis that feed intake can be adjusted for a determined level of production by partitioning intake into portions required for stage and level of production (Crews, 2005), and can be calculated using regression analysis as:

$$y = \beta_0 + \beta_1(ADG) + \beta_2(BW) + RFI, \quad [2]$$

where y is daily dry matter intake (**DMI**), β_0 is the intercept, β_1 is the partial regression of DMI on average daily gain (**ADG**), and β_2 is the partial regression of daily intake on body weight (**BW**). As it will be discussed later, BW can be adjusted to estimate the RFI based on metabolic weight.

Feed Conversion Ratio

One constraint when obtaining FCR and RFI is the amount of time the animals have to be measured to obtain a reliable indicator of their intakes. A period of 28 days may be necessary in order to obtain an accurate measure of dry matter intake, while for residual feed intake and conversion ratio, 84 days might be necessary since they depend not only on intake but on body measurements (i.e., body weight) that may contribute to variability (Castilhos et al., 2011). To try to reduce workload, when estimating FCR, animals and the feed they consumed usually are weighed at the beginning and end of a phase (e.g., finishing); however, this may not be an appropriate approach as any trend regarding feed intake occurring during this time would not be characterized.

Genetics of Feed efficiency

When trying to explain feed intake, animals of similar genetic background should be used in order to minimize extraneous sources of variability. Table 2.1 (adapted from Arthur et al. (2001)) shows estimates for means and heritabilities for some feed efficiency related traits in young Charolais bulls:

Please place Table 2.1 about here

Because the heritability for these traits is moderate, there is an opportunity to improve traits related to feed efficiency, such as residual feed intake, and feed conversion ratio.

In addition, there is a good body of evidence indicating that feed intake and FCR are phenotypically and genetically correlated with measures of growth and thus body size (Crews, 2005). Estimates of phenotypic correlations between FCR, weight, and weight gain range from -0.24 to – 0.75, which indicate that improving efficiency in size and performance, will result in a decrease in FCR (Koots et al., 1994).

PREDICTION OF FEED INTAKE

In order to determine the amount of feed to offer to a group of animals it is important to have a means to accurately predict what those animals will require to satisfy their needs, thus, prediction of feed intake becomes important as it allows of determination of feed utilization.(Dulphy and Demarquilly, 1994). Many methods have been developed and studied for the prediction of feed intake, but due to the variability of the source of feed (many forage species in a sward) there are challenges that need to be overcome (Fraser et al., 2006).

According to Dulphy and Demarquilly (1994), intake of forages depends largely on the capacity of the rumen, with its fill dependent on the proportion of plant cell walls, or crude fiber, found in the forages consumed. This suggests that besides rumen volume, the chemical composition of the forages could be used for prediction of feed intake.

However, in comparison to intake, lignin, ADF, and cell wall constituents have been found to be better related to digestibility. Table 2.2, adapted from Van Soest (1965), summarizes the correlation between voluntary feed intake, and nutrient characteristics and digestibility in 7 forage species.

Please place Table 2.2 about here

As mentioned earlier NDF content and crude protein alone are not good indicators of feed intake, as the strength of this relationship depends on the forage species and the physiological state of the animal. However, crude protein may have an impact on DMI when its values are lower than the needs of the rumen microbial population (Allison et al., 1985). In general, animals will tend to have higher intake of forages with higher digestibility, as denoted by lower ADF and lignin, and higher crude protein (Hadjigeorgiou et al., 2001).

Nevertheless, other prediction methods have focused on the characteristics of the animals, such as live weight, energy demands, and productive state (NRC, 1987). These methods yielded prediction models for voluntary feed intake, which will be discussed in the following sections.

Quantitative prediction of intake

Several authors have proposed mathematical models for predicting intake that take into consideration animal factors, such as body weight and level of production, and dietary factors, such as type of forage and level of concentrate. Still, Pittroff and Kothman (2001a) mentioned shortcomings for several models widely used, such as those from the ARC (Agricultural Research Council, 1980) and NRC (National Research Council, 1987). Although these models have been useful to predict DMI in controlled conditions, they seem less applicable to grazing conditions.

The following models for prediction of feed intake were developed by the NRC (1984, 1987):

$$DMI(kg/day) = W^{0.75}(0.1493NE_m - 0.046NE_m^2 - 0.0196)\text{ , and} \quad [3]$$

$$DMI(kg/day) = W^{0.75}(19.4 + 54.5NE_m)\text{ ,} \quad [4]$$

where W is the body weight of the animal, and NE_m is the net metabolizable energy. Because of their simplicity, they have been widely used. Nonetheless these models require refinement (adjustments) for frame size, sex, stage of growth, as well as some other factors, to be more accurate in their predictions. Usually tables consisting of adjustment terms are used when fitting these models, which might consider breed, sex, age, and stage of production. Those limitations may have been the reason for the development of a different model by the NRC in 1996 specific to growing animals:

$$IT = \frac{W*(0.2435\ NE_M - 0.0466\ NE_M^2 - 0.1128)}{NE_m}\text{ ,} \quad [5]$$

which leads to very different predictions when compared with that of models [3] and [4] (Pittroff and Kothman, 2001a). In their review, Pittroff and Kothman (2001a) concluded that many mathematical models have serious logical or implementation problems, and most of the models are quite variable even

when considering the same type of animal and forage. This emphasizes the need for a technique that allows a better understanding, description and prediction of feed intake.

Prediction of intake using fecal markers

Fecal markers have been widely used to estimate feed intake in grazing animals, since they allow estimates to be based on intakes over longer periods and often with minimal disruption of feeding behavior. Although many compounds have been used as fecal markers, one of the most common is chromic oxide. As an alternative, Ytterbium has been used with the same success as chromic oxide for estimation of fecal matter output and feed intake (Delagarde et al., 2010). More recently (since 1985), wax-like compounds found in plants have been introduced, validated, and successfully used to estimate and predict DMI, diet choice and digestibility in a variety of herbivore species (Dove and Mayes, 1991). While the natural occurring *n*-alkanes (odd-chain) can be used to directly estimate diet choice, the estimation of feed usually requires the administering of an even-chain *n*-alkane (external marker) to the animals. However, the major concern driving not only the *n*-alkane method, but most fecal marker methods is diurnal variation of these external markers. As a consequence, sporadic fecal samples might contain quantities of the marker with different concentrations than the mean, which may lead to inaccurate estimates of intake (Dove and Mayes, 1991). Subsequently, methods for delivering a continuous amount of the marker have been developed and analyzed, and will be discussed in following sections

Chromic Oxide

Chromic Sesquioxide (Cr_2O_3) has been traditionally accepted as one of the most reliable external indicators of digestibility and intake (Cross et al., 1973). Studies in beef steers have demonstrated that fecal recoveries of chromic oxide were high (up to 103%), and that recovery was not affected by external factors such as the amount of water offered to the animals. However, there was strong evidence of diurnal

variation in excretion, ranging from 87% to 103% of the mean concentration (Cross et al., 1973; Lobato et al. 1980).

Chromic oxide is administered daily to the animals, usually via gel capsules that contain between 2 and 20g of the marker, (Vargas Junior et al., 2011). A period of time is necessary for the marker to reach a constant rate in the rumen, usually 8 days, after which fecal samples are taken (Malossini et al., 1996). Controlled release devices for chromic oxide have been developed; however, the delivery rates between and within animals were inconsistent (Owens and Hanson, 1992).

The chrome oxide procedure has been valuable in estimating intakes of different species including sheep, and dairy and beef cattle. However several issues, which are common to most fecal markers, must be considered when applying this technique: (i) chromic oxide must be mixed uniformly into the feed (typically through some type of adherent), (ii) fecal sample collection must be continued until the entire marker has been excreted (which is not necessary for the plant-wax marker method), and (iii) a single value for digestibility of the forage must be adopted (Lobato et al., 1980; Owens and Hanson, 1992; Malossini et al., 1996). Contamination of fecal samples with soil when collected from the ground may be an issue with this technique. Ingestion of soil also may pose problems, since animals may consume up to 8% of their DMI as soil (Fries et al., 1982).

Since chromic oxide is an external marker, it is necessary to also use an internal marker for accurate prediction of feed intake. The use of Acid Indigestible Ash (**AIA**) as an internal marker, with chromic as an external marker, has shown high accuracy for predicting feed intake. Estimates of predicted feed intake for the chromic oxide/AIA method were 5.81 kg DM/day versus the measured feed intake of 5.64 kg DM/ day (Ferreira et al., 2004). This shows that the inclusion of an internal marker for the chromic oxide technique provides a reliable tool for estimating feed intake.

Ytterbium

Ytterbium is a rare element that has been used to estimate the dynamics of fecal output of grazing animals including beef cattle. Fecal recoveries of Ytterbium have ranged from 86% to 144% of the mean

concentration; like with chromic oxide, such diurnal variation potentially could affect estimates of intake. Stuth and Lyons (1999) used powdered Ytterbium acetate dosed daily in gelatin capsules to try to minimize the effect of diurnal variation, with positive results.

When compared to chromic oxide with either a once or twice a day dosing scheme, fecal outputs estimated from a twice a day dosing were more variable with Ytterbium. Predicted fecal outputs for mature beef cows from once a day dosing were 2.87 kg DM/day for Ytterbium and 2.55 kg DM/day for chromic oxide, while for twice a day dosing they were 2.81 kg DM/day for Ytterbium and 3.00 kg DM/day for chromic oxide. The measured fecal output was 2.83 kg DM/day. Although the predicted values were more variable for Ytterbium, no overall difference was detected between these two procedures (Prigge et al., 1981). However, lower accuracy of prediction of feed intake by the Ytterbium procedure was found when incorporated into a concentrate pellet. Dairy cattle were dosed with Ytterbium and *n*-alkanes under two fecal sampling methods, fecal collection at milking time and total fecal collection. Coefficients of determination for the *n*-alkane procedure were 0.97 and 0.98 for fecal sampling at milking and total fecal collection, respectively, compared to r^2 of 0.65 and 0.67 for collection at milking and total fecal collection, respectively, for the Ytterbium procedure (Perez-Ramirez et al., 2012). This reinforces the concept that for the Ytterbium technique, and other fecal markers, the delivery of the marker plays an important role in the accuracy of the prediction of feed intake.

N-alkanes, Long Chain Alcohols and Long Fatty Acids

N-Alkanes have been shown to be well suited for the identification of botanical samples because they are uniformly distributed in plants, and are highly resistant to biochemical degradation (Pietrogrande et al., 2009). Additionally, they have been useful in the prediction of feed intake for a wide range of species, most commonly ruminants such as cattle (Malossini et al., 1994; Hameleers and Mayes, 1998), sheep (Lewis et al., 2003), and goats (Dove and Mayes, 2005), but also in non-ruminant herbivores such as horses (Stevens et al., 2003) and hares (Rao et al., 2003). They have also been used to assess the digestibility of feed components in fish (Gudmundsson and Halldorsdottir, 1993). Long chain alcohols

and long fatty acids also have demonstrated to be useful for predicting feed intake and diet composition in free-ranging hervibores (Ali et al., 2005a; Ali et al., 2005b).

Since n-alkanes and long chain alcohols will be used as the basis of estimating feed intake and diet choice in the studies planned, they will be discussed in detail in the following section.

N-ALKANES AND LONG CHAIN ALCOHOLS

Plant waxes such as *n*-alkanes and long chain alcohols (LCOH) exhibit the relevant characteristics of the ideal marker remarkably well. Specifically: (i) their chemistry is fairly simple, so their analysis and identification is easier, both in feces and plants; (ii) they are largely indigestible, so they are unaltered in the digestive tract and can be fully recovered in the feces; this is especially true as their chain length increases; and, (iii) the *n*-alkane and LCOH profiles are different among plant species (Dove and Mayes, 1991; Ali et al., 2005). This last point, however, is debatable as there is evidence of variation in *n*-alkane and LCOH concentrations within different parts of the same plant, and across a season, which will be discussed later.

N-Alkanes

The use of plant waxes, for taxonomy purposes was suggested by Tulloch (1981), with particular focus on *n*-alkanes and free alcohols. These two compounds were selected due to their ease of isolation, and because the profiles of *n*-alkanes and alcohols in plants were different to allow characterization of groups or species of a certain genera (Tulloch, 1981).

The predominant *n*-alkanes in most forage species are odd-chain hydrocarbons, C_{29} and C_{31}, while the proportion of even chain *n*-alkanes represents only 3.2% to 5.6% of the total hydrocarbons found (Malossini et al., 1991). The *n*-alkane concentrations for several plant species are summarized in Table 2.3.

Please place Table 2.3 about here

Based on their profile of the *n*-alkanes C_{27}, C_{29}, C_{31} and C_{33}, which are the most abundant and often provide a unique signature even for species of the same genera, we can differentiate forage species. For instance, from Table 4, the concentrations of C_{27}, C_{29} and C_{33} for annual ryegrass are 105, 260 and 43 mg/kg DM, respectively; for perennial ryegrass the concentrations of the same *n*-alkanes are 36, 142 and 99 mg/kg DM, respectively. Such distinct patterns of *n*-alkanes make it possible to identify species of plants in a mix sward or in a mixed diet.

Long chain alcohols

The main components of plant wax for most species are wax esters of unsaturated, unbranched LCOH and long chain fatty acids (LCFA). Due to the analytical method used to extract and separate these compounds, the combined ester and free alcohol concentration is used to assess digestibility and diet composition (Dove and Mayes, 2005). Bugalho et al. (2004) mentioned challenges in effectively separating alcohols from sterols, with risk that compounds characterized by GC as 1-C_{27}-OL, and 1-C_{29}-OL may instead be sterols; that challenge may impact the utility of using LCOH to estimate diet preferences and digestibility. Alcohols have been found to be in much higher concentrations in plants than the *n*-alkanes. Kelman et al. (2005) reported that LCOH concentrations were almost an order of magnitude higher than the *n*-alkane concentration for several species of forages including *Lotus* spp., *Phalaris* spp. and *Trifolium* spp. Thus, in using compound with such difference in the magnitude of their concentrations for the estimation of diet composition, the LCOH may reduce the discriminatory power of the *n*-alkanes. In such conditions, normalization of the *n*-alkanes and LCOH may be necessary (Ali et al., 2005a)

As shown in Table 2.4 (adapted from Dove and Mayes (2005)), the most common LCOH range in length between C_{24} and C_{34}, although small concentrations of longer chains have been found (up to C_{64}). The variation found in LCOH concentrations for different species and for different chain lengths, and high recovery rates, make them a valuable tool for delineating diet composition.

Please place Table 2.4 about here

Within-plant and seasonal variation of the concentration of n-alkanes and LCOH

As mentioned previously, one of the characteristics of an ideal marker is large between species, yet small within-species, variation in its concentration. Nonetheless, several authors (e.g., Malossini, 1991; Dove and Mayes, 1991, 2005) suggest variation in *n*-alkane and LCOH concentrations exists among plant parts within a species due to phenological state (e.g., vegetative vs. reproductive). For most grasses, the onset of reproductive states involved a decrease in C_{31}, and an increase in C_{29} concentration (Dove and Mayes, 1991). This additional variability has to be taken into account when designing experiments which involve measurements that extend over time as this might lead to biased predictions. Specifically, *n*-alkanes with less discriminatory potential for spring were C_{25}, C_{26}, C_{27}, C_{28}, and C_{29}, for summer C_{26}, C_{27}, C_{28}, C_{29}, C_{30}, and for winter C_{25}, C_{26}, C_{27}, C_{28} and C_{29} (Côrtes et al., 2005). This suggests that not every *n*-alkane provides useful discriminatory information in all seasons, and that selecting a different group of *n*-alkanes for each season may be appropriate.

Moreover, beyond variation in the content of the wax constituents due to stage of development, differences exist among parts of the same plant. In tropical grasses it was found that there were significant differences between *n*-alkane concentrations in leaves *versus* stems (Dove and Mayes, 1991). Therefore, in order to address these variations within plants, an appropriate sampling and analysis of each part of the forage may be necessary.

One of the advantages of the plant wax procedure is that *n*-alkanes and LCOH in general are not readily digestible, especially as their chain length increases. Where absorption occurs, it tends to be restricted to the small intestine. Recovery rates of *n*-alkanes of different chain lengths in sheep dosed once a day with *n*-alkane shredded paper are shown in Table 6 (adapted from Dove and Mayes (1991)).

Please place Table 2.5 about here

Although losses in *n*-alkanes occur during digestion (Table 2.5), those losses decrease with an increase in chain length. Therefore, a focus on longer-chain *n*-alkanes should improve recovery rates, and increase prediction accuracy.

Dosing strategies with even-chain n-alkanes

The use of a dosed *n*-alkane as an external marker allows for feed intake to be predicted based on the ratios of odd-chain (found in forages) to even chain (dosed C_{32} or C_{36}) *n*-alkanes recovered in feces. This approach appears to provide accurate predictions, as the ratios of C_{31}:C_{32}, and C_{33}:C_{32}, seem to be more or less consistent over time. In addition, estimates of recoveries can be made from these ratios, thus there is no need for total fecal collection (Lewis et al., 2003; Charmley and Dove, 2007).

In order to minimize diurnal variation in *n*-alkane recovery rates, several methods of delivery have been suggested by Dove and Mayes (2005), which are described in Table 2.6.

Please place Table 2.6 about here

Among the methods mentioned in Table 6, several have shown positive results. Mann and Stewart (2004) suggested that dosing of C_{32} should be performed every 12 hours when used in Xanthan gum, in order to have more reliable results. According to Perez-Ramirez (2012), the use of C_{32} dissolved in heptane and incorporated into a cellulose stopper provided an effective method for obtaining a consistent recovery rate of the markers, even with varying feeding levels. However, the use of a controlled-release device for *n*-alkane dosing has been less successful, with differences between the manufacturer's release rates and the actual release rates (Ferreira et al., 2004). Thus, differences in predicted DM intake were found when using the manufacturer's release rates for C_{32}, and the calculated (actual) release rates: 6.59 kg DM/day *versus* 5.78 kg DM/day, respectively; the actual DM intake was 5.64 kg DM/day.

In relation to the use of LCOH as fecal markers, the same dosing methods as with *n*-alkanes may be used. However, instead of dosing with synthetic *n*-alkanes, Carnauba wax may be used. Carnauba wax, which

is derived from the leaves of the palm *Copernicia prunifera*, is characterized by its free even-chain alcohol content (1-C_{28}OH – 1-C_{34}OH), with 1-C_{32}OH most abundant (Regert et al., 2005). Additionally the length of time of dosing animals with the markers has been a topic of discussion and may induce some variability in the fecal output of the marker. Various experiments have demonstrated that a dosing period of 5-6 days is necessary for the concentration of the *n*-alkanes in feces to reach equilibrium (Dove and Mayes, 1991).

Fecal recovery

Although the fecal recovery of *n*-alkanes is not complete, and variable, recovery increases with the carbon chain length: 65% for C_{26} to over 90% for C_{37} *n*-alkanes. In addition, repeatability among diurnal samples increased from 0.56 for C_{31}, 0.78 for C_{32} and 0.81 for C_{33}. Therefore, longer-chain *n*-alkanes may provide a better prediction of intake, diet composition and digestibility (Malossini et al., 1994; Bovolenta et al., 1994).

Since *n*-alkane recovery in feces is incomplete, such losses must be accounted for to accurately estimate DMI (Dove and Mayes, 1991). Significant differences have been found between predicted and observed DMI in cattle fed tropical grass (*Brachiaria brizantha* cv. Marandu) for unadjusted *versus* adjusted *n*-alkane recoveries; while the observed DMI was 62.62 kg, the predicted values for unadjusted recovery ranged from 45.70 to 50.78 kg, and for adjusted recovery ranged from 62.08 to 63.17 kg (Morais et al., 2001). The ranges in predicted values were due to the *n*-alkane (C_{31}, C_{33}, and C_{35}) and sampling method (spot or total) employed. These results confirm that adjusting for fecal recovery is necessary for this method to be reliable (Morais et al., 2001).

There is considerable evidence that recoveries of adjacent *n*-alkanes become more similar as the length of the carbon chain increases. For instance, the mean values for fecal recovery of C_{32} and C_{33} are 0.868 ±0.0175, and 0.872 ±0.0125, and for C_{34} and C_{35} are 0.948 ±0.0102 and 0.947± 0.0139. However, the concentration of C_{35} in most plants is low making DMI estimation difficult using this *n*-alkane (Dove and Mayes, 1991). Because the recovery rates of adjacent *n*-alkanes are relatively high, it could be assumed

that the recovery rate of a dosed even-chain *n*-alkane would be similar to that of the adjacent odd-chain *n*-alkane, for instance the ratio of C_{32} *versus* C_{31} or C_{33} could be used as an indicator of the recovery of C_{32}, and adjustments made so that prediction of feed intake is more accurate.

Fecal sampling scheme

Diurnal variability in *n*-alkane concentrations in feces has been a concern, but usually has been overcome by increasing the frequency of sampling. However, evidence of such diurnal variation is inconsistent across species. In sheep dosed once a day with *n*-alkane paper pellets, there were no differences in the *n*-alkane output in feces (Dove and Mayes, 1991). However, significant variation in the fecal *n*-alkane output in dairy cattle was found when dosed both once and twice a day, with greater variation for the once a day dosing scheme (Dove and Mayes, 1991).

Digestibility of the diet

Digestive efficiency is an important measure, as it relates directly to the ability of an animal to effectively use the nutrients found in the diet it is offered (Vallentine, 2001). Digestibility, which is reflected by the portion of the total diet that has not been excreted in the feces, can be estimated by the plant wax procedure in a number of ways: (i) using the *n*-alkanes naturally found in plants as internal markers; and (ii) using a dosed *n*-alkane as an external marker, and the plant internal marker. The following equation can be used to determine digestibility with the use of n-alkanes (from Dove and Mayes, 2006):

$$Digestibility = \frac{Intake - Fecal\ Output}{Intake} \quad [6]$$

Fecal output can be calculated as follows:

$$Fecal\ Output\ \left(kg\,\frac{DM}{day}\right)\frac{marker\ dose\ rate\ (mg/day)}{fecal\ marker\ concentration(mg/kg\ DM)} \quad [7]$$

And feed intake can be calculated by:

$$Intake = \frac{dose\ rate_j}{\left(\frac{fecal\ content_j}{fecal\ content_i}\right) forage\ content_i - forage\ content_j} \quad [8]$$

where, *i* represent an odd-chain *n*-alkane, and *j* represents a dosed even-chain *n*-alkane.

As with the other estimates derived using the plant wax marker procedure, the reliability of the estimate of digestibility improves when based on hydrocarbons or alcohols with longer carbon chains. This is particularly important for this procedure, as errors in the estimation of digestibility will inevitably result in a reduction of the accuracy of the estimate of intake (Dove and Mayes, 2005).

The possibility to select cattle that are more efficient for turning feed into animal products depends on accurately predicting feed intake, in a large number of individuals under grazing conditions. Achieving that aim may be possible if the plant wax procedure proves to be a reliable and practical method for estimating feed intake in grazing animals. Even though many authors (Elwert et al., 2004; Charmley and Dove, 2007; Lin et al., 2012) consider this technique to be accurate under controlled feeding conditions, the challenge is to enhance these technologies so accurate estimates of individual pasture intake by cattle can be obtained. With such information, phenotypic and genetic relationships between pasture intake, growth rate, and other performance measures could be estimated, which would allow derivation of optimal selection indices to improve efficiency (Arthur et al., 2004).

FORAGE PREFERENCE/ DIET COMPOSITION

There are two main concepts that need to be distinguished in regards to diet choice, namely diet preference and diet selection (Rutter, 2006). Diet preference has been defined as 'what the animals select when given the minimum of physical constraints' (Parsons et al., 1994). Diet selection, on the other hand, is defined as 'the removal of some components of a sward or a sample of herbage rather than others, a function of preference modified by the opportunity for selection' (Hodgson, 1979).

There are a number of factors that influence the preference of an animal toward any particular source of food when given the chance to select. Most of these factors are inherent to the forage species itself such as palatability, digestibility, and quality. However, there are some additional factors that may have an effect on diet choice, and that are pertinent to the animal such as its physiological state, live weight, and genetics (Vallentine, 2001).

Pigs, poultry, and rodents have the ability to select a diet that allows them to meet their nutritional requirements when the choice was available. However, likely due to the intricacy of their digestive system, the capacity to choose a balance diet is less evident in ruminants (James et al., 2001). Still, through the analysis of various experiments, Horadagoda et al. (2009) suggest that animals tend to be proactive in balancing their nutrient intake when they have that opportunity.

Palatability is said to be the major element dictating the degree of acceptance of a particular diet over another. Palatability has been defined as 'plant characteristic or condition which stimulates a selective response in animals' (Horadagoda et al., 2009). However, given that energy and protein requirements (among others) might have an effect on selection, it seems sensible that other components of the diet may be useful as predictors of choice.

Traditionally, many experiments have focused on explaining an animal's selective choice of a diet based on its protein content, because protein is an essential nutrient for growth and production (meat and milk). In sheep, James et al. (2001) found that intake was greater for diets with a higher concentration of urea; they concluded this suggests protein was a major factors affecting diet choice.

Table 2.7, adapted from Horadagoda et al. (2009), summarizes the coefficient of determination for preferences of dairy cattle (measured in time spent grazing) for several forage species based on their water soluble carbohydrates (**WSC**), **CP**, ADF, NDF, dry matter digestibility (**DMD**), and nitrate-nitrogen (**N-NO_3**) content.

Please place Table 2.7 about here

The choice of one family of forages over another appears to depend on a number of factors, with relative the importance of each varying across forages; that is, no single forage characteristics may be a strong indicator of forage preference in ruminants.

One concept that describes the preferences of one source of feed over another, particularly in grazing animals, is the selectivity ratio. This ratio is defined as' the proportion in the animal diet of any species, species group, or plant part relative to its proportion in the available herbage', and can be obtained as follows (Vallentine, 2001):

$$Selectivity\ ratio = \frac{Proportion\ in\ the\ diet}{Proportion\ in\ the\ availabe\ herbage} \quad [9]$$

Using this ratio Rossiere et al. (1975) determined that selectivity for forage species was inconsistent although a preference index scale could be developed as a general guide (Table 2.8). Based on this scale, forage preference of cattle for clover has been estimated at 1.9, and 1.0 for brome grass (Vallentine, 2001).

Please place Table 2.8 about here

Some experiments have focused on the ratio of grass to legume consumed as a way to show the preference of animals towards a better source of protein. Table 2.9, adapted from Rutter (2006), summarizes such preferences in ruminants in different physiological stages. Regardless of species or stage of production, a clear preference for legume over grass was observed irrespective of the physiological stage of the animal.

Please place Table 2.9 about here

Correlations between sward selection indices and composition parameters, for mixed swards containing two types of perennial ryegrass (diploid and tetraploid), white clover, and weeds are presented in Table 2.10 (adapted from Stilmant et al., 2005). It can be observed that cattle and sheep prefer a mixed diet containing legumes grasses and, when given the opportunity, herbs. However, what is not clear is the reason behind the selection of a mixed diet. A number of theories have been proposed for explaining this behavior, many of which have been discounted. Still, concepts raised in these theories remain valuable considerations when attempting to understand the drive for a mixed diet, namely (i) novelty, (ii) sampling, (iii) rumen function, (iv) carbon and nitrogen balance, (v) conditioned taste aversion, and (vi) anti-predator behavior (Rutter, 2006).

Please place Table 2.10 about here

However, the estimations of preference using *n*-alkanes and LCOHs may not be entirely accurate due to the fact that the rumen would buffer (mix and retain) the forage ingested in such magnitude that the diurnal patterns that may exist about selection will be masked, being left with only a general estimate for the composition of the diet consumed (Rutter, 2006).

As noted earlier, animals tend to select one forage species over another based on its nutrient content to meet their energy demands, although such is not always the case (Emmans and Kyriazakis, 1995). However, it may be possible that animals of the same species with different requirements will have different DMI and, if allowed, diet preferences.

Using the *n*-alkane technique, and the least squares procedure, diet composition can be obtained by (Dove and Mayes, 2006):

$$Calculated\ fecal\ alkane_i = \alpha A_i + \beta B_i + \gamma C_i \quad [10]$$

where α, β, and γ represent the respective proportions of dietary components A, B, and C.

PROJECT OVERVIEW AND HYPOTHESES

Given the economic importance of feed efficiency in grazing systems, reliably predicting feed intake is central for improving the profitability of forage-based farming enterprises. In addition, diet choice may have an effect on swards and grazing management, thus impacting the overall efficiency of these grazing production systems. The use of plant-waxes used as fecal markers may provide a valuable tool for estimating both feed intake and diet selection within grazing conditions.

Two experiments were performed in the Shenandoah Valley Agricultural Research and Extension Center (**SVAREC**). Twenty-four yearling heifers of moderate and large frame score were used in each experiment. Animals were housed in a drylot and fed through a Calan-Gate system, allowing for recording of individual intakes. Two pure forages were offered in a cubed form, namely fawn fescue and red clover. In addition, animals were dosed with external markers in a supplementary feed: the synthetic *n*-alkanes C_{32} and $C_{36,}$ and Carnauba wax, as a source of LCOH. Following a 21 day adjustment period, animals were weighed, and then dosed daily for 10 days with the external markers. Feed offered and refused was also recorded daily. For the final five days of dosing, forage and fecal samples were collected in the a.m. and, with exception of the final day, p.m., and live weights recorded. Forage and fecal samples were oven dried and extracted for their *n*-alkane and LCOH content using ethanolic KOH saponification as described by Dove and Mayes (2005). Gas chromotography analysis was used to determine *n*-alkane and LCOH concentration from feces and forage samples. Diet composition was determined using non-negative least squares procedure by 'Eatwhat' Software package.

The objectives of this project are (i) to measure the feed intake and diet choice in beef cattle, and their repeatability at two physiological stages, (ii) to determine the accuracy of plant-wax markers (*n*-alkanes; LCOH) for the prediction of diet composition under lab conditions (mixed diets); and, (iii) delineate diet choice using *n*-alkanes as internal markers. Additional planned objectives are to *n*-alkanes and LCOH singly and in combination for the prediction of diet composition and food intake. However, those objectives are beyond the scope of this thesis.

The specific hypotheses to be tested were (i) heifers of larger frame score, and thereby mature weight, will have higher DMI than those of moderate frame score, (ii) heifers of larger frame score will select forage with a higher nutritional value (red clover *versus* fescue), since heavier body weights result in higher energy demands, (iii) animals in general will have higher DMI of a forage with higher nutritional value (red clover *versus* fescue); and(vi) the plant wax procedure will allow diet composition to be predicted within 95% of the observed compositions.

Table 2.1. Means and heritability for FCR and related traits

Trait	Mean	Heritability
Average daily gain	1.26	0.28 ± 0.04
Mean metabolic weight	68.77	0.40 ± 0.02
Feed intake	9.65	0.39 ± 0.03
Residual feed intake	7.79	0.29 ± 0.04
Feed conversion ratio	0.05	0.39 ± 0.03

Table 2.2. Correlations between voluntary feed intake and chemical composition of 7 forage species and digestibility

Species	Lignin	ADF	Protein	Cellulose	CWC*	Digestibility
Sudan grass	-0.89	-0.88	0.77	-0.87	-0.78	0.85
Orchard grass	-0.76	-0.74	0.79	-0.83	-0.95	0.81
Brome grass	-0.65	-0.85	0.70	-0.87	-0.77	0.62
Timothy	-0.33	-0.29	0.29	-0.47	-0.37	0.57
Alfalfa	-0.02	-0.07	0.17	-0.20	-0.29	0.45
Blue grass	0.48	0.20	-0.25	0.37	-0.06	0.18
Tall fescue	0.70	0.59	-0.46	0.54	0.57	-0.31
Total*	-0.13	-0.53	0.54	-0.59	-0.65	0.66

* CWC = Cell wall constituents

Total= Overall correlation for all of the species analyzed

Table 2.3. Forage *n*-alkane concentration (mg/kg DM) for grass and legume species (adapted from Malossini et al., 1991).

n-Alkane	Grasses			Legumes		
	Orchardgrass (*Dactylis glomerata*)	Annual ryegrass (*Lolium multiflorum*)	Perennial Ryegrass (*Lolium perenne*)	Alfalfa (*Medicago sativa*)	White clover (*Trifolium repens*)	Red clover (*Trifolium pratense*)
C_{27}	20	105	36	36	38	30
C_{28}	2	8	6	9	7	8
C_{29}	38	260	142	202	109	408
C_{30}	2	11	12	12	5	5
C_{31}	58	250	220	324	67	57
C_{32}	2	4	7	7	1	1
C_{33}	21	43	99	21	7	11
C_{35}	0	0	8	0	0	0
Total	143	681	531	611	234	523
Even chain %	4.2	3.4	4.7	4.6	5.6	3.2

Table 2.4. Mean concentration of long-chain fatty alcohols (mg/kg DM) in some forage species (adapted from Dove and Mayes (2005)).

	1-C_{24}OH	1-C_{26}OH	10-C_{29}OH	1-C_{28}OH	1-C_{31}OH
Big trefoil *Lotus pedunculatus*	28	2463	0	1327	1285
White clover *Trifolium repens*	18	143	0	61	1297
Subterranean clover *Trifolium subterraneum*	193	408	0	281	4709
Rescuegrass *Bromus catharticus*	13	85	17	4052	84
Orchardgrass *Cynodon dactylon*	31	25	-	-	0
Tall fescue *Festuca arundinacea*	27	639	10	101	58
Perennial ryegrass *Lolium perenne*	104	2628	10	446	627

Table 2.5. Recovery rates (proportion of the mean) for various *n*-alkanes in the gastro-intestinal tract, and feces in sheep (Dove and Mayes, 1991).

	Recovery					
	Duodenum		Terminal ileum		Feces	
	Mean	s.e.	Mean	s.e.	Mean	s.e.
C_{27}	1.037	0.0387	0.626	0.0250	0.594	0.0174
C_{28}	0.877	0.0424	0.759	0.0446	0.786	0.0210
C_{29}	0.997	0.0354	0.745	0.0224	0.697	0.0144
C_{31}	0.965	0.0340	0.815	0.0214	0.779	0.0095
C_{32}	0.921	0.0433	0.819	0.0329	0.859	0.0101
C_{33}	0.988	0.0348	0.875	0.0209	0.839	0.0127
C_{35}	1.013	0.0352	0.977	0.0219	0.953	0.0090
C_{36}	0.841	0.0415	0.876	0.0373	0.922	0.0115

Table 2.6. Methods for dosing *n*-alkanes for use in feeding trials.

Dosing method	Description
Paper pellet	Alkane absorbed into shredded paper, packed into a pellet, which is enclosed in a tissue paper wrapper
Gelatin capsule	Alkane suspended on cellulose powder, contained in a gelatin capsule
Paper bung	Alkane absorbed into a tissue-paper stopper designed for use with laboratory glassware
Paper filter	Alkane absorbed into a tissue paper tampon/filter designed for use in large-volume pipettors or filter cigarettes
Alkane-labeled feeds	Alkane absorbed into feeds, such as oilseed meal, pelleted cereal concentrate, sugar beet pulp or breakfast cereals
Alkane suspension	Alkane absorbed into dried grass meal and suspended in an aqueous xanthan gum solution before dosing
Alkane suspension	Alkane suspended in molasses solution and consumed 3–5 times daily from trough
Alkane emulsion	Oil-in-water emulsion of alkane, emulsifier and water and dosed in liquid form
Intra-ruminal alkane controlled-release device	Alkane in slowly dissolving matrix, within spring-loaded intra-ruminal slow-release capsule (commercial product; Captec Alkane)

Table 2.7. Coefficient of determination for preference of dairy cattle over various forage families based on chemical composition (adapted from Horadagoda et al., 2009).

Chemical Composition*	All forages	Grasses	Legumes	Herbs
WSC	0.73	0.80	0.90	0.10
CP	0.13	0.09	0.50	0.10
ADF	0.15	0.41	0.50	0.30
NDF	0.02	0.75	0.80	0.60
NO_3-N	0.30	0.31	0.50	0.20
DMD	0.05	0.18	0.20	0.10

*WSC= water soluble carbohydrate, CP= crude protein, ADF=acid detergent fiber, NDF=neutral detergent fiber, NO_3-N=nitrate nitrogen, DMD=dry matter digestibility.

Table 2.8. Preference index for selectivity of forage species

Preference Index	Meaning
2.1 or greater	Definite preference
1.4 – 2.0	Some preference
0.7 – 1.3	Same in diet as available
0.3 – 0.6	Some avoidance
0.2 or less	Avoidance

Table 2.9. Proportion of legume (%) in the diet of various species.

Animal Species	Lactating or dry	Herbage choice	% Legume	Reference
Sheep	Lactating	Perennial Ryegrass/ White clover	79.7	Parsons et al. (1994)
Sheep	Lactating	Perennial Ryegrass/ White clover	71.6	Penning et al. (1995)
Dairy sheep	Lactating	Annual Ryegrass/ Sulla	74.0	Rutter et al. (2005b)
Dairy cows	Lactating	Perennial Ryegrass/ White clover	70.0	Rutter et al. (1999)
Dairy cows	Lactating	Perennial Ryegrass/ White clover	78.0	Rutter et al. (2001)
Dairy cows	Lactating	Perennial Ryegrass/ White clover	73.8	Rutter et al. (2004a)
Sheep	Dry	Perennial Ryegrass/ White clover	65.8	Parsons et al. (1994)
Sheep	Dry	Perennial Ryegrass/ White clover	91.8	Newman et al. (1994)
Sheep	Dry	Perennial Ryegrass/ White clover	71.0	Harvey et al. (1996)
Sheep	Dry	Perennial Ryegrass/ White clover	88.4	Harvey et al. (1997)
Sheep	Dry	Perennial Ryegrass/ White clover	66.8	Harvey et al. (2000)
Sheep	Dry	Perennial Ryegrass/ White clover	70.0	Cosgrove et al. (2001)
Sheep	Dry	Perennial Ryegrass/ White clover	60.0	Rook et al. (2002)
Dairy heifers	Dry	Perennial Ryegrass/ White clover	65.0	Cosgrove et al. (1996)
Dairy heifers	Dry	Perennial Ryegrass/ White clover	68.0	Torres-Rodriguez et al. (1997)
Dairy heifers	Dry	Perennial Ryegrass/ Lotus	70.0	Torres-Rodriguez et al. (1997)
Dairy heifers	Dry	Perennial Ryegrass/ White clover	63.9	Rutter et al. (2004b)
Beef heifers	Dry	Perennial Ryegrass/ White clover	60.0	Rutter et al. (2005a)

Table 2.10. Correlation coefficients between selection index and sward composition parameters

Type/Site	White Clover	Weeds
Site 1 Low Nitrogen		
2n	0.451	-0.125
4n	0.290	-0.121
Site 1 High Nitrogen		
2n	0.684	-0.506
4n	0.689	-0.201
Site 2 Low Nitrogen		
2n	0.447	-0.116
4n	0.585	0.107
Site 2 High Nitrogen		
2n	0.565	-0.494
4n	0.483	-0.402
Means		
2n	0.537	-0.310
4n	0.502	-0.154
All	0.519	-0.232

2n= Diploid ryegrass

4n= Tetraploid ryegrass

CHAPTER 3 (Formatting of this chapter is consistent with the Journal of Grass and Forage Science to which it will be submitted)

Using *n*-alkanes and long-chain alcohols to estimate botanical composition in simple forage mixtures: effect of the operator and type of marker used

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Abstract

Feed intake and diet choice have an impact on production efficiency and management of grasslands. *N*-alkanes have been used successfully to predict both feed intake and diet choice. Still, when the hydrocarbon profiles of plants within the sward are similar, *n*-alkanes alone may not be sufficient for reliable predictions. Including long chain alcohols (**LCOHs**) as additional markers may help redress this problem. Technical expertise of operators in evaluating the *n*-alkane and LCOH concentrations of forages also may differ. As a consequence, biases may be introduced due to measurement error. In this study, two factors contributing to the reliability of predicting the botanical composition of mixed diets were tested: (i) the forage markers used; and, (ii) the expertise of the laboratory operators conducting the analyses. Two operators, with different levels of expertise, performed two separate extractions of *n*-alkanes and LCOHs from 9 mixed and 2 pure test diets of red clover and fawn fescue. Differences in measured concentrations of *n*-alkanes C_{27}, C_{29}, C_{31}, and C_{33} and alcohols C_{26}-OH, C_{28}-OH, and C_{30}-OH were tested using ANOVA to detect differences between operators, and between extracts within operator. The concentrations of C_{27} and C_{29} differed ($P < 0.041$) between extractions within operators. Between operators, only differences in C_{26}-OH and C_{28}-OH were found ($P < 0.002$). Principal components analysis suggested that for this pair of forages, C_{26}-OH did not provide useful information for estimation of botanical compositions. The slopes from the regression of estimated on actual fescue contents of the test diets did not differ from unity for any operator or marker combination. Our results suggest that, despite variability in measured concentrations due to technical expertise of the operator, prediction of forage mixtures is still reliable.

Keywords: *n*-alkanes, long chain alcohols, forage mixtures, prediction, operator

Introduction

Providing feed to animals can represent up to 65% of total production costs (Arthur et al., 2004). Because the performance of animals is affected by the nutrients they consume, an accurate measure of feed intake

is necessary to evaluate production efficiency. In addition, understanding the preferences of animals for particular forages or feeds (diet selection) can assist in developing and managing grazing systems. This is particularly important with complex sward compositions as diet selection may impact the sustainability of the vegetation (Ferreira et al., 2009). There also may be opportunity for animal genetic selection to alter diet choices (Rutter, 2010) if feed intake can be measured reliably in individual animals.

Several approaches to predict nutrient intake, including the use of fecal markers such as chromic oxide, indigestible cellulose and acid insoluble ash, have been used and are fairly accurate (Penning and Johnson, 1983; Malossini et al., 1996; Morenz et al., 2006). Still, using the markers mentioned above do not have the ability to predict diet choice. An alternative approach, using plant waxes as forage and fecal markers, appears to provide such flexibility. Among the many constituents of plant waxes, *n*-alkanes (saturated aliphatic hydrocarbons) have been used to estimate both feed intake and diet choice in many animal species (Dove and Mayes, 2005). However, the number of plant species that can be delineated in a mixed composition depends on the number of *n*-alkane markers available and their profiles in individual plants (Bugalho et al., 2004). Long chain alcohols (**LCOHs**); therefore, have been used in addition to *n*-alkanes to improve the reliability of plant species estimation (Ali et al., 2005).

The utility of *n*-alkanes and LCOHs as dietary markers relies on their marker concentrations being sufficiently different among the forage species on offer. Interestingly, the concentrations of these compounds can vary even within a plant cultivar, (Kelman et al., 2003). Therefore, the set of markers requisite to reliably delineate botanical composition may differ (Lin et al., 2012). When the aim is to estimate diet choices; techniques for identifying markers with greater discriminatory power are needed.

With the plant wax marker procedure, estimation errors may arise due to incomplete or variable recovery of plant markers in fecal samples (Oliván et al., 2007; Morais et al., 2011)., The technical expertise of the person assessing the concentrations of these compounds in forages and feces also may introduce

measurement error. Such biases may be specific to a compound class. For instance, more steps are necessary to purify LCOHs than *n*-alkanes (Dove and Mayes, 2006). Thus, in order to validate the reliability of the plant wax marker technique, the extent of measurement errors must be quantified.

This study was designed to assess the reliability of *n*-alkanes and LCOHs for estimating the composition of a two-plant mixture (red clover and fescue). Our specific objectives were to determine: (i) the effect of operator experience on measurement of *n*-alkanes and LCOHs concentrations within test diets; (ii) the impact of operator expertise on the reliability of estimating the botanical compositions of those diets; and, (iii) the value of combining different sets of markers (i.e., *n*-alkanes and LCOH) for such estimation. The overall aim was to validate the use of *n*-alkanes and LCOHs as a means for estimating the composition of a fescue-red clover diet mixture.

Materials and Methods

Since the present study did not include an animal component, no Institutional Animal Care and Use Committee approval was necessary.

Preparation of forage mixtures

Pure hay from two forage species, fawn fescue (*Festuca arundinacea*) and red clover (*Trifolium pratense*), was finely chopped, pulverized, and pressed to form 3.18 x 3.18 cm cubes, with moisture added (steam) to facilitate the binding process (McHay Company, Elgin, OK). Cubes from a single forage were sampled randomly from totes, and combined. The combined samples were oven-dried at 55 °C for 72 hours and then ground through a 1 mm mesh screen using a Wiley Mill. Test diets were prepared by weighing out dried ground forages on an analytical scale (Mettler Toledo AL204, Columbus, OH) to produce mixtures ranging from 100 % to 0% fescue, or conversely 0 to 100 % red clover, in 10 % increments. Nine mixed, and two pure, test diets were prepared (Table 3.1).

Please insert Table 3.1 about here

Operators

The experiment was designed so that two operators, one with three years of experience and one just trained, performed separate extractions and analysis of each test diet. Each operator undertook two extractions, generating a total of 44 samples (22 per operator) to be analyzed by gas chromatography (**GC**).

Alkane and LCOH extraction

Extractions were performed on each test diet in duplicate (Dove and Mayes, 2006). Briefly, 0.2 g of a test diet was weighed into 16mm x 100mm borosilicate glass tubes. Approximately 0.1g of *n*-docosane (C_{22}) and *n*-tetritriacontane (C_{34}) alkane (0.3 mg/g of each alkane), and 0.1 g of 1-heptacosanol (C_{27}-OH), 1.2 mg/g) LCOH, were added to each sample tube as internal standards. Tubes were saponified for 16 h at 90 ◦C in 2 mL of 1 M ethanolic KOH using a dry-block heater (Techne DB-3, Techne Ltd., Duxford, Cambridge, UK). Heptane (2 mL) and distilled water (0.6 mL) were added to each warmed tube (60°C). The non-aqueous layer was collected after vigorous shaking, yielding a crude extract. Hydrocarbons were collected by solid phase extraction (**SPE**) and heptane elution through a silica-gel column (bed volume 1 mL, 70-230 mesh, Fisher Scientific, Fairlawn, NJ). Crude alcohol extractions were obtained by elution with heptane/ethyl-acetate (80:20 v/v). Long-chain alcohol fractions were separated from sterols and stanols by SPE and heptane elution, and then derivatized using 2 µL of a pyridine/acetic anhydride solution (5:1 v/v). Alkane elutes and LCOH fractions were evaporated to dryness, and then re-dissolved in 200 µL of *n*-dodecane for chromatographic analysis.

GC analysis

Quantification of alkanes and LCOHs was carried out by GC, using an Agilent 6890N GC (Agilent Technologies, Inc., Santa Clara, CA). Alkane and LCOH extracts were injected (0.5µl) via a 7863 Series

auto-sampler (splitless injector) directly into a bonded-phase, non-polar column (Agilent J&W DB-1 column, 30 m, 0.530 mm internal diameter and 0.5 μm film thickness). Helium served as the carrier gas at a constant flow of 4 mL/min. Temperature programming was: 280 °C for the injector; 340 °C for the detector; and, 170 °C for 4min for the column oven followed by a first ramp of 30 °C/min to 215 °C with a 1 min hold, and then a second ramp of 6 °C/min to 300 °C with a 20 min hold.

Samples of *n*-alkanes and LCOHs standard solution mixtures (C_{10} to C_{40}; C_{20}-OH to C_{30}-OH, Sigma-Aldrich, St. Louis, MO, USA) were included in the GC analyses to identify peaks and standard response factors. Chromatograph data were analyzed using Agilent ChemStation software (Rev. B.04.02 SP1). Peak areas were determined with auto-integration and manual review of chromatograms. *N*-alkane and LCOH concentrations were calculated relative to known amounts of the internal standards (C_{22}, C_{34}, and C_{27}-OH), according to the equations outlined by Dove and Mayes (2006):

$$\text{Concentration } n\text{-alkane}_i \left(\frac{\text{mg}}{\text{kg DM}}\right) = \frac{10* \quad *\text{Conc } C_{34}\text{IS(mg/g)}*C_{34}\text{ISwt(g)}}{\text{sample wt(g)}*\text{DM content}*\text{SRF}_i*\text{FF}_i}, \quad [1]$$

$$\text{Concentration LCOH}_i \left(\frac{\text{mg}}{\text{kg DM}}\right) = \frac{10* \quad *\text{Conc } C_{27}\text{OH IS (mg/g)}*C_{27}\text{OH ISwt (g)}}{\text{sample wt (g)}*\text{DM content}*\text{SRF}_i}, \quad [2]$$

where $\text{Conc } C_{34}\text{IS}$ and $\text{Conc } C_{27}\text{OH IS}$ is the concentration of the *n*-alkane$_i$ or LCOH$_i$ internal standard, respectively, $C_{34}\text{ISwt}$ is the weight of the solution containing the internal standard, and %Area is the chromatogram peak area of *n*-alkane$_i$ or LCOH$_i$ calculated as the percent area of C_{34} or C_{27}-OH, respectively. The DM content is the sample dry weight, SRF$_i$ is the average standard response factor, calculated as the percent area of *n*-alkane$_i$ or LCOH$_i$ in the mixed standard solution divided by the percent weight of *n*-alkane$_i$ or LCOH$_i$ in the mixed standard, and FF_i is the fractionation factor.

Estimation of diet composition

Botanical composition of the forage mixtures was estimated using 'EatWhat' software, which matches the marker profile for each forage species to that in the test diet through non-negative, least squares optimization (Dove and More, 1995).

Estimation of diet composition was performed using either four *n*-alkanes (C_{27}, C_{31}, C_{33} and C_{33}), three LCOHs (C_{26}-OH, C_{28}-OH, and C_{30}-OH), or the combination of these seven compounds.

Statistical Analysis

Operator differences

Statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., Cary, NC) and R (R Development Core Team).

Potential differences between operators in their measurement of the concentration of individual *n*-alkanes and LCOHs were tested using the GLIMMIX procedure in SAS. Extract within operator was the experimental unit. The model fitted was:

$$y_{ijkl} = \mu + O_i + E_{(i)j} + S_k + \varepsilon_{ijkl}, \quad [3]$$

where y_{ijkl} is the *n*-alkane or LCOH concentration measured by operator O_i (i = 1 or 2, for the two operators) for extraction E_j (j = 1 or 2, for the two extractions by each operator) and sample S_k (k = 1, …, 11, for the two pure and nine mixed test diets), with μ the overall mean concentration. Operator one was more experienced in extracting and analyzing *n*-alkanes and LCOHs. Operator and sample were fitted as fixed effects. Random effects were extraction nested within operator ($E_{(i)j}$) and the residual (ε_{ijkl}).

Potential differences in measured concentrations of individual *n*-alkanes and LCOHs within operators were tested, again using the GLIMMIX procedure. The model fitted was:

$$y_{ijk} = \mu + O_i + S_j + (OS)_{ij} + \varepsilon_{ijk} \quad [4]$$

where y_{ijk} is the *n*-alkane or LCOH concentration measured by operator O_i (i = 1 or 2, for the two operators) for sample S_j (j = 1, …, 11, for the two pure and nine mixed test diets), with μ the overall mean concentrations. Operator and sample, and their interaction [$(OS)_{ij}$], were fitted as fixed effects. The random effect was the residual (ε_{ijk}).

Estimation of forage mixtures

Principal components analysis (**PCA**) was conducted with R using the Princomp and Biplot procedures. The PCA was performed to identify patterns of the four *n*-alkanes and three LCOHs evaluated in the two forage species. Since the concentrations of *n*-alkanes and LCOHs in both forages were similar in magnitude, no normalization procedure was performed. However, PCA was conducted using standardized variables and correlation matrices.

The estimated and actual fescue contents of the test diets were compared by regressing the natural log of predicted on the natural log of actual contents, using the REG procedure in SAS. The reliabilities of the estimates were assessed by testing the hypotheses that the slope was not different from unity, and that the intercept was not different from zero. The standard errors of the parameter estimates, 95% confidence intervals for the parameters, and 95% confidence intervals for predicted values of the fitted lines, were examined. The latter provide information on how certain the predictions of diet composition will be, given the current data (Ott and Longnecker, 2010).

Differences in the accuracy of forage mixture estimates, using the *n*-alkanes and LCOHs separately and in combination, were tested using the Kulczyński Similarity Index (**KSI)**, where a value of 0 infers total dissimilarity and a value of 100 infers total similarity (Oosting, 1956; Ferreira et al., 2009; Lin et al., 2012). The statistic was calculated as:

$$KSI = 100 \sum 2c_i / \sum (a_i + b_i), \quad [5]$$

where c_i is the lowest of the percentages of the i^{th} forage (fescue or red clover) in the estimate versus actual test diet, and is divided by $(a_i + b_i)$ which is the sum of percentages of that i^{th} forage in the estimated and actual test diet. Effects of the operator and set of markers used on the KSI values were tested using the GLIMMIX procedure in SAS. The model fitted was:

$$y_{ijk} = u + O_i + M_j + (OM)_{ij} + \varepsilon_{ijk}, \quad [6]$$

where y_{ijk} is the calculated KSI for operator O_i (i = 1 or 2, for the two operators) and set of markers M_j (j = 1, 2, or 3, for *n*-alkanes alone, LCOHs alone, or *n*-alkanes and LCOHs combined), with μ the overall mean KSI. Operator and compound were fitted as fixed effects, as well as their interaction $(OM)_{ij}$, with the residual ε_{ijk} the random effect. Tukey's test was used to perform multiple comparisons.

For all of tests, *P*-values less than 0.05 were considered indicative of significant differences among means.

Results

Mean *n*-alkane and LCOH concentrations for the two forage species used are shown in Table 3.2. The C_{27} and C_{33} *n*-alkanes were found in lower concentrations as compared to C_{29} and C_{31}. Importantly, the *n*-alkane profiles of C_{29} and C_{31} differ between the forages, with a higher C_{29} concentration in red clover and a higher C_{31} concentration in fescue. Long-chain alcohol concentrations were, in general, slightly higher than those of the *n*-alkanes. Although the C_{28}-OH and C_{30}-OH LCOH profiles for fescue and red clover differed, concentrations of C_{26}-OH were very similar for both forage species.

Please insert Table 3.2 about here

Operator effect on n-alkane and LCOH concentration

Differences in measures of *n*-alkanes and LCOH concentrations between the two operators were tested (model [3]), with corresponding *P*-values provided in Table 3.3. In addition, differences in concentrations for these compounds within an operator for separate extractions were evaluated (model [4]; Table 3.3).

Please insert Table 3.3 about here

Operators were consistent in their measures of *n*-alkanes ($P > 0.12$) but differed in their measure of C_{26}-OH and C_{28}-OH LCOHs ($P < 0.003$). The opposite was true for differences within an operator. Measured concentrations of the *n*-alkanes C_{27} and C_{29} differed between the first and second extraction for both operators, as well as C_{31} for the more experienced operator.

Principal Component Analysis

From PCA, 81.2, 15.7, and 2.7% of the variation in the pattern of concentrations of both the *n*-alkanes and LCOHs were explained by the first (PC1), second (PC2) and third principal component, respectively. Only PC1 and PC2 had eigenvalues higher than one and together explained 96.9% of the variation. The PC1 delineated the higher concentrations of C_{29}, C_{28}-OH and C_{30}-OH in red clover from the higher concentrations of C_{27}, C_{31}, and C_{33} in fescue (Figure 3.1). The PC2 was dominated by C_{26}-OH. The concentrations for this alcohol in fescue and red clover were quite similar (Table 3.2), as was the loadings of both forages along PC2. Thus it appears C_{26}-OH provides less discriminating information with regards to these forages than the other compounds.

Please insert Figure 3.1 about here

Estimation of test diet composition

Estimates of botanical compositions of the test diets for both operators, and for the three sets of markers (*n*-alkanes alone, LCOHs alone, or both), were reliable. The estimated slopes did not differ from unity in any case ($P > 0.82$), as shown in Figure 2. However, the width of the confidence limits for the slope was different for each operator and set of markers used. The intercepts of the fitted line differed from zero for the experienced operator one ($P < 0.05$), except when estimates were based on *n*-alkanes alone ($P = 0.52$). For operator two, the intercept did not differ from zero for any combination of markers ($P > 0.82$).

In general, the confidence limits for the fitted lines were much narrower for the experienced operator one than for the new operator two. Still, for operator one, estimates using the LCOH alone were least reliable, while for operator two predictions based on the *n*-alkanes alone were less reliable. For both operators, the combination of the *n*-alkanes and LCOHs improved the reliability of their predictions, as denoted by a higher coefficient of determination (r^2), and a narrower confidence interval for the slopes of the fitted line. Although the operator's technical experience affected the variability of individual estimated values, it had little effect on the overall reliability of estimation.

Please insert Figure 3.2 about here

Kulczyinski Similarity Index

The KSI values (model [5]) for the different markers and operator combinations were tested using model [6]. Least squares means for operators and sets of markers (*n*-alkanes, LCOH, *n*-alkanes and LCOH combined) for KSI are shown in Table 3.4. No significant differences were found for KSI values for any of the effects fitted in the model. However, the lowest KSI values were obtained when the LCOH were used alone, particularly by operator one, and in general when operator two performed the extractions.

Please insert Table 3.4 about here

DISCUSSION

The measured *n*-alkane concentrations for red clover were between 30 to 60% lower than those reported in the literature (Malossini et al., 1990). Concentrations for fescue were similar to those of Dove and Mayes (2006), although our measured concentration for C_{31} was around 25% lower than their value. At the time of this study, no information regarding the LCOH concentrations of red clover was found. However, the reported concentrations of C_{30}-OH for other clover species (white, subterranean, and knotted clover) are considerably higher than those obtained in this study (Bugalho et al., 2004). Variations in these measured concentrations may be due to the cultivar used or differences in methodology between laboratories. For fescue, our measured concentration for C_{26}-OH was approximately 74% lower than the reported by Dove and Mayes (2006); on the other hand, our measured concentrations for C_{28}-OH, and C_{30}-OH were around 52 and 58 % higher than their values, respectively.

Operator effect

At the time of the present study no information regarding the possible effect of operator on the measured concentration of *n*-alkanes and LCOHs was found. Although differences in mean values reported in the literature may reflect operator differences among labs, such has not been quantified. It appears that the differences between operators become less apparent with an increase in carbon-chain length of the compound. Bovolenta et al. (1991) reported that the repeatability of *n*-alkane determinations increased with the length of the carbon chain, from 0.858 for C_{27} to 0.995 for C_{33} with a reduction in the within-sample standard deviation.

Despite the difference in technical training between the operators, their estimations of the botanical composition of the test diets were reliable. However, such differences may be more important when estimating feed intakes for a particular animal, where recovery rates of longer chain compounds in feces are presumed to be similar. Error in the measurement of *n*-alkane or LCOH concentrations due to differences in operator expertise may bias estimates of feed intake (Olivan et al., 2007).

Estimation of forage mixtures

The botanical composition of a fescue-red clover test diet was reliably estimated using *n*-alkanes and LCOHs individually and in combination. The results of the current study were similar to those of Bezabih et al. (2011). They regressed proportions of pasture mixes on measured values for forage mixtures comprised of up to five species. The slope of the regression did not differ from unity, although the fit was less robust: r^2 values ranged from 0.33 to 0.99. With their evaluation of more forage species, such may be anticipated.

The addition of the LCOHs to the *n*-alkanes improved the reliability of prediction of the test diet's composition. Such an effect has been reported elsewhere. In studies with diets containing several forage components, predictions based on *n*-alkanes or LCOHs alone had reduced accuracy compared to that with a combination of markers (*n*-alkanes, LCOHs and long chain fatty acids **(LCFA)**; Lin et al., 2012). Similarly, Ferreira et al. (2009) concluded that the combination of LCFA and *n*-alkanes as markers increased the accuracy of prediction, when compared to the use of these markers alone.

Similar concentrations of C_{26}-OH in fescue and red clover suggest that this particular alcohol provides less information for estimating the botanical composition of their mixture. However, it may be useful if its profile is sufficiently different in other forage species. In other studies, different markers have been shown to be less discriminating. Lewis et al. (2003) reported that in a mixture of Lucerne and ryegrass, C_{27} appeared to be less useful for prediction of feed intake. Cortes et al. (2005) found that in tropical grasses and legumes, a number of *n*-alkanes (including C_{25}, C_{27}, and C_{29}) had different discriminatory power, depending on the season of the year. Thus, the usefulness of any particular marker might be plant, season, and climate dependent. Despite low concentrations of C_{27} and C_{33}, particularly in red clover, these two *n*-alkanes appear to provide good discriminating information, as their profiles differ from that of fescue.

Multivariate techniques, such as PCA, or discriminant analysis, provide a means to determine which set of markers are more useful for predictive purposes. Based on discriminant analysis, Bugalho et al. (2004) reported that the proportion of variation explained using *n*-alkanes, LCOHs or their combination differed among plant mixtures, suggesting that marker choices are diet dependent.

Kulczynski similarity Index Values

As reported by Ferreira et al. (2009), KSI values appeared to be higher for predictions based on *n*-alkanes. Adding LCOHs did not improve matters. In contrast, Lin et al. (2012) found that KSI values increased with combinations of *n*-alkanes, LCOHs and LCFAs, although the extent of that increase was determined by the diet. This similarity index appears to offer a means to determine if the combination of different markers provides additional discriminating information to predict diet composition. In studies assessing compositional dissimilarity of plant species between different sites, the KSI appeared to be more robust than other measures of quantitative similarity (Faith et al., 1987).

Implications

Our results suggest that for fawn fescue and red clover forages, the botanical composition of mixed diets can be reliably predicted. Therefore, we expect that the diet choice of animals when offered these forages can be successfully determined. Since fescue and red clover are commonly found in pasture systems in the eastern states (Tracy and Sanderson, 2000; Franzluebbers, 2007), this tool appears appropriate for estimating the diet choice of grazing animals within this region.

The differences in the reliability of predictions using *n*-alkanes and LCOHs between the experienced and less experienced operator were relatively small. Therefore, with only a short training period, it seems feasible to develop and implement this methodology for the analysis of plant-wax components within a laboratory.

Acknowledgements

Financial support from USDA-ARS for project 1932-21630-003-06 is greatly appreciated. We are thankful to Donald Wirth (Saddle Butte Ag Inc., Shedd, OR, USA) for supplying the forages (hays), to Phillip and Edgar McCracken (McHay Company, Elgin, OK, USA) for feed processing (cubing), and Ellie Stephens (Virginia Tech, Blacksburg, VA, USA) for technical editing.

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Table 3.1 Proportion of the two forage species in the mixed diets.

	Forage mixtures										
Species	1	2	3	4	5	6	7	8	9	10	11
Fescue	1.00	0.90	0.80	0.70	0.60	0.50	0.40	0.30	0.20	0.10	0.00
Red clover	0.00	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80	0.90	1.00

Table 3.2 Mean *n*-alkane and long chain alcohol (LCOH) concentration (mg/kg DM) for fescue and red clover (standard deviation).

	***n*-alkane**				**LCOH**		
Species	**C_{27}**	**C_{29}**	**C_{31}**	**C_{33}**	**C_{26}-OH**	**C_{28}-OH**	**C_{30}-OH**
Fescue	29.4 (4.1)	123.8 (15.8)	170.7 (12.2)	45.5 (1.1)	368.0 (48.7)	154.5 (37.1)	91.9 (28.4)
Red clover	19.3 (1.0)	299.8 (19.5)	33.8 (2.2)	6.7 (2.1)	347.2 (47.0)	340.8 (50.6)	446.2 (28.7)

Table 3.3 *P*-values from the ANOVA analysis for differences of measured *n*-alkanes and long chain alcohol (LCOH) concentrations.

	***n*-alkane**				**LCOH**		
	C_{27}	**C_{29}**	**C_{31}**	**C_{33}**	**C_{26}-OH**	**C_{28}-OH**	**C_{30}-OH**
Between	0.570	0.319	0.996	0.121	0.001	0.002	0.056
Within*							
Operator 1	0.002	<0.001	0.048	0.278	0.239	0.449	0.196
Operator 2	0.041	0.004	0.860	0.155	0.212	0.591	0.559

*****Operator one had three years of experience with extraction and analysis of *n*-alkanes and LCOHS, while operator two had little experience (just trained).

* 2 Extractions per operator

Table 4 *P*-values and least squares means associated with effects of set of markers and operator for Kulczyński Similarity Index values*.

Effect	***P*-value**	**Least Squares Means**
Operator	0.16	
1		95.46 ± 0.89
2		93.98 ± 0.89
Markers	0.36	
n-alkanes		95.79 ± 1.10
LCOH		93.01 ± 1.10
n-alkanes + LCOH		95.36 ± 1.10
Operator*markers	0.60	
1* *n*-alkanes		97.22 ± 1.55
1* LCOH		92.88 ± 1.55
1* (*n*-alkanes + LCOH)		96.27 ± 1.55
2* *n*-alkanes		94.36 ± 1.55
2* LCOH		93.13 ± 1.55
2* (*n*-alkanes + LCOH)		94.44 ± 1.55

* Kulczyński Similarity Index values were obtained using model [5] in the text.

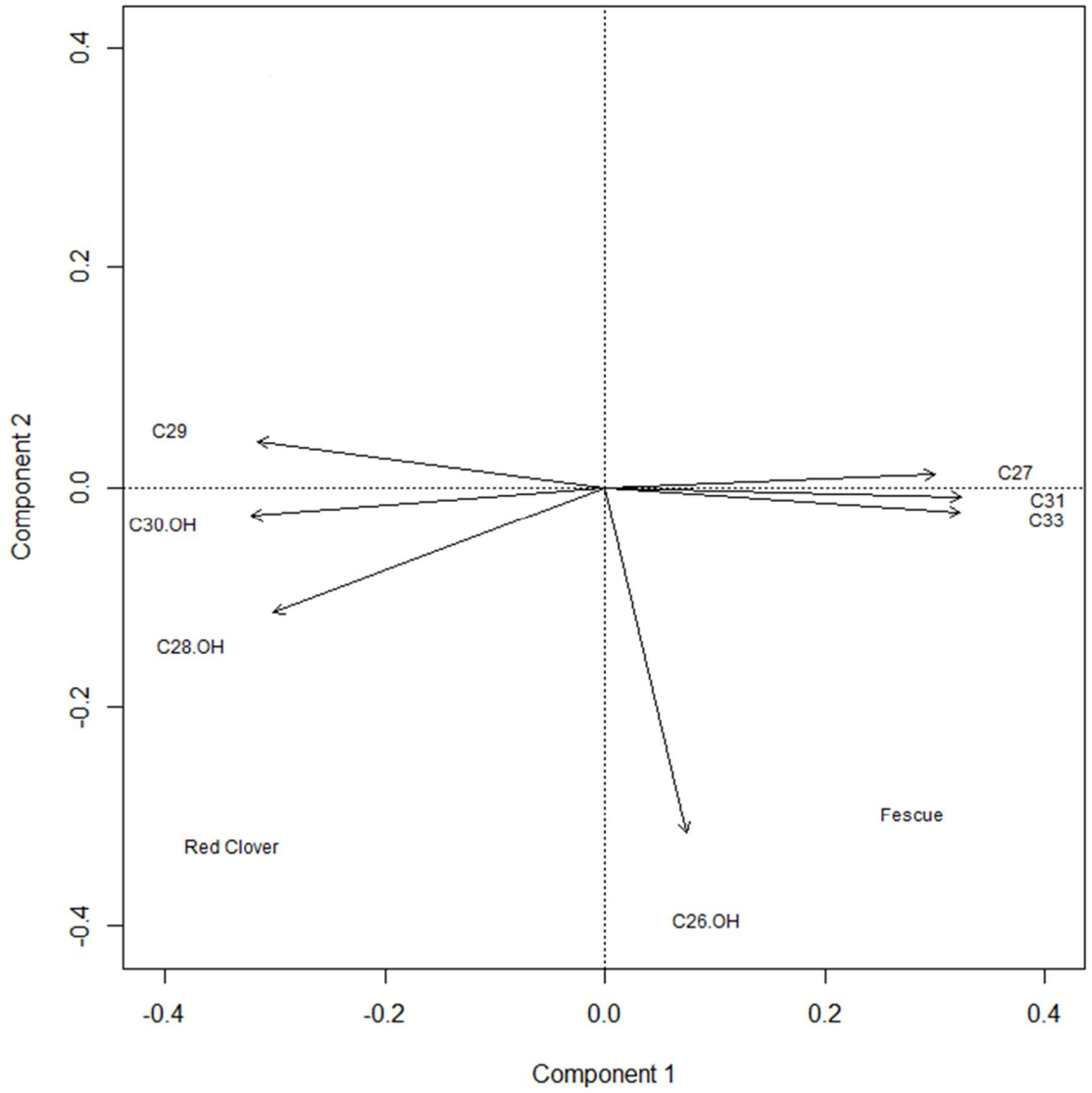

Figure 3.1 A biplot showing the two forage species on a two-dimensional space derived from principal component analyses based on *n*-alkanes (C27, C29, C31, and C33) and LCOHs (C26.OH, C28.OH, and C30.OH).

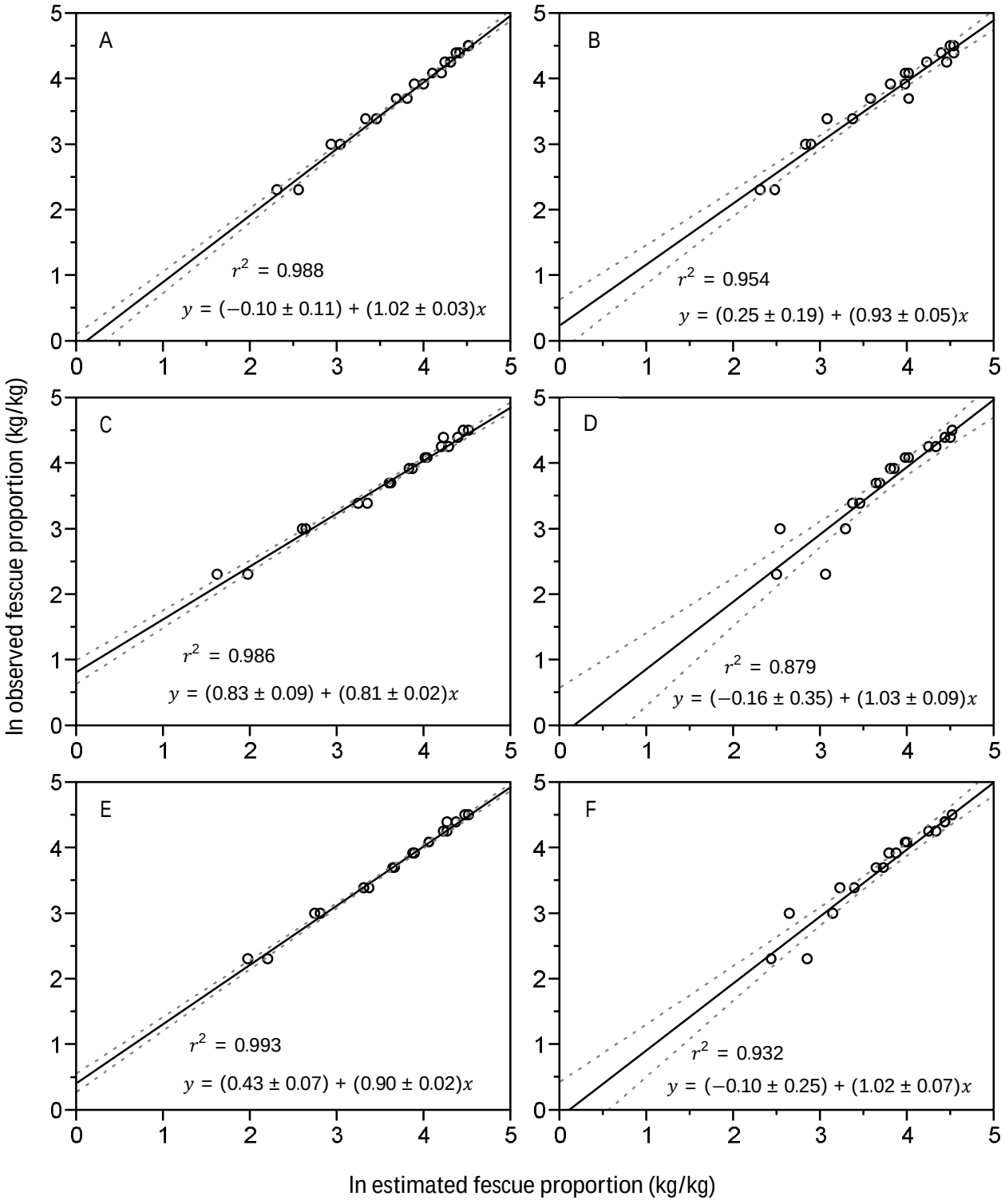

Figure 3.2 Plots of regressions of the natural logarithm of estimated on observed fescue proportions, with 95% confidence (dotted line) for operator and set of markers: operator one with test diets botanical

compositions estimated based on *n*-alkanes, LCOH and their combination (A, C, E); operator two with test diets botanical compositions estimated based on *n*-alkanes, LCOH, and their combination (B, D, F). The coefficients and R^2 values for the fit of each regression is also shown.

CHAPTER 4

Estimation of feed intake and diet choice in beef cattle using *n*-alkanes: validation of their reliability in indoor studies

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ABSTRACT

Measuring feed intake and diet choice in grazing animals is challenging, complicating the assessment of feed efficiency in pasture-based systems. Furthermore, animals may modify their intake of a particular forage species depending on its nutritive value and on their own physiological status. For instance, animals growing to larger mature sizes have higher maintenance requirements, which may affect their dietary choices. Various fecal markers have been used to estimate feed intake in grazing animals. However, plant-wax markers such as long-chain *n*-alkanes appear to provide reliable estimates of both dietary choices and intakes. Still, their use in beef cattle has been relatively limited. The present study was designed to test the reliability of the *n*-alkane technique to estimate beef heifer's diet choices. In addition, its aim was to evaluate diet choice and feed efficiency in beef heifers of large and moderate frame size (**FS**) at two ages (post-weaning at approximately 260 days of age, and as yearlings). Twenty four Angus-cross heifers were evaluated at the two ages. At each age, they were housed in a drylot equipped with a Calan-Gate system, and fed red clover and fescue hay as cubes. Following 3-week acclimation periods, DMI of each forage species was assessed daily for 10 days. During the final 5 days, fecal grab samples were collected twice daily. Body weights were recorded at least weekly, and as often as daily during the fecal collection periods. Large frame size and moderate frame size did not differ in ADG, feed conversion ratio and Kleiber ratio ($P>0.05$). However, animals from large FS had higher total and red clover DMI ($P<0.05$), and higher proportions of red clover in their diet ($P<0.001$), as yearlings. Diet choices were reliability estimated when *n*-alkane concentrations in the feces were corrected for incomplete recoveries.

Relative errors in estimation varied from 5.6 to 12.2%. It appears that the *n*-alkane technique is a useful tool for the estimation of diet choice in animals fed forages at least under controlled conditions.

KEYWORDS: *n*-alkanes, feed intake, diet choice, beef cattle, efficiency

INTRODUCTION

Quantification of feed intake by grazing animals presents a number of challenges. Although animals in grazing conditions may have *ad libitum* access to forages, their nutritive values can be limiting. Animals may modify their intake of the available forages over time in order to meet their needs. Therefore, having a measure of diet choice becomes important to estimate feed efficiency in grassland systems (Rutter, 2010; Vargas Junior et al., 2011), and in their management.

Beyond the characteristics of the forages on offer, the risk of productive and reproductive inefficiencies of larger frame size (**FS**) cattle within pasture-based systems has been raised (Vargas et al., 1999). It has been suggested that smaller FS cattle are better suited to such production systems (Taylor et al., 2008; Koknaroglu and Hoffman, 2010).

Although several approaches for the predicting feed intake have been used, fecal markers seem to provide the most reliable estimates (Prigge et al., 1981; Owens and Hanson, 1992). However, some of these techniques, such as chromic oxide, do not allow for diet choice to be predicted (Dove and Mayes, 2000). Plant-wax markers, such as *n*-alkanes (aliphatic hydrocarbons) and long chain alcohols (**LCOH**), have been demonstrated to be fairly reliable for prediction of both feed intake and diet choice (Dove and Mayes, 2006).

The majority of *n*-alkanes found in plant species are odd-chain. Therefore, even-chain *n*-alkanes have been used as external markers. Particularly when long-chained, the recovery rates of adjacent *n*-alkanes are similar, and the ratio of natural (odd-chain) and dosed *n*-alkanes have been used to estimate feed intake without the need for complete fecal collections. However, incomplete recovery of *n*-alkanes (both natural and dosed) may occur, necessitating use of a correction factor to improve the reliability of estimates of diet choice. (Dove and Mayes, 1991; Elwert et al., 2008).

Since the estimation of feed intake requires dosing with even-chain *n*-alkanes, commonly C_{32} or C_{36}, the process of dosing need to be considered. Several methodologies have been used, some requiring daily handling of animals. In studies aiming to describe feed intake in grazing conditions, minimal disturbance of feeding behavior is undoubtedly important (Charmley and Dove, 2007). Moreover, diurnal variation in the concentration of the dosed *n*-alkane in the feces needs to be considered when implementing a dosing and fecal sampling scheme (Olivan et al., 2007; Elwert et al., 2008).

The ability of the *n*-alkane technique for the delineation of feed efficiency under grazing conditions needs to be addressed and tested first in controlled conditions so extraneous sources of error can be taken into account (Lin et al., 2012). This study was designed to address the validity of the *n*-alkane technique for the prediction of feed intake and diet choice in beef heifers and to delineate the feed efficiency (actual intake and diet choice) of animals of large and moderate frame size at two physiologically immature ages (post-weaning and yearling). Two forages, fescue and red clover, commonly found in grazing systems in the Appalachian region were considered (Tracy and Sanderson, 2000; Franzluebbers, 2007).

MATERIALS AND METHODS

All of the techniques and procedures used were approved and performed in compliance with the Institutional Animal Care and Use Committee at Virginia Tech.

Animals

The experiment was performed at the Shenandoah Valley Agricultural Research and Extension Center (**SVAREC**), in western Virginia. Twenty-four spring born heifers, at least 75% Angus in breeding, were drawn from a moderate and a large frame score line at SVAREC. Measurements on these heifers were recorded at two ages or studies: (i) at approximately 260 days of age (post-weaning) at an average body weight (**BW**) of 285.3 ± 7.1 kg for the moderate line and 317.7 ± 7.4 for the larger line; and (ii) at approximately 365 days of age (yearlings) at average BW of 342.8 ± 7.8 kg, and 390.7 ± 7.8 kg for the moderate and large frame size lines, respectively (Table 4.1). During study 1 (post-weaning), one animal

was removed due to ill health and another due to its refusal to eat the matrix with the external markers. These two animals were replaced for the measurements recorded during study 2 (yearling).

Please place Table 4.1 about here

Experimental layout

All animals were housed in a drylot, in two separate pens. Each pen was equipped with 12 Calan gates (American Calan, NH) to allow measurement of individual animal intakes. For each study, animals were allowed an adaptation period of 7 days to adjust to the facilities and feeds (Figure 4.1). Gates remained unlocked, with animals having access to any feeding bunk. During this period preferences of animals for feeding in a particular gate were noted, so that assignment of the keys corresponding to individual gates best matched animal preference. After this adaptation period (on day 8), animals were acclimated to the use of the Calan gates for 14 days. Gates were locked, and only animals with the key corresponding to a particular gate had access to the individual feed bunk. During this time animals were also fed ground peanut hulls, as this was the matrix used later to dose them with external markers. At day 21 animals started receiving the labeled matrix. Dosing with this matrix lasted for 11 days, of which the last 5 days overlapped with fecal collection. Each study was 32 days.

Please insert Figure 4.1 about here

Feeding strategy

Grass cubes

In order to minimize wastage, and to more accurately weigh the amount of forage consumed, pure fawn fescue (*Festuca arundinacea*) and red clover (*Trifolium pratense*) hay was finely chopped, pulverized, and pressed to form 3.18 x 3.18cm cubes, with steam as a moistening agent to facilitate the process

(McHay Company, Elgin, OK). Cubes were bagged and stored. Both forages were analyzed for crude protein (**CP**), neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), and ash contents (Table 4.2).

Please insert Table 4.2 about here

Animals had *ad libitum* access to feed from either forage. An insert designed to hold two separate buckets was placed in each feeding bunk, with each bucket filled with a single cube-type. Each morning (8:00 a.m.), refusals from the previous day were weighed and, if dusty, discarded. The buckets were then re-filled and their weight recorded. The buckets were checked in the afternoon (3:00 p.m.) and, if necessary, additional feed weighed and added.

Bucket position

To determine if the physical location of the feed had an effect on the diet choice of an animal, buckets were switched from left to right daily in study 1 and every other week in study 2. The strategy adopted in study 2 was to allow animals more opportunity to learn the position of their preferred forage. It would be anticipated that the bucket located on the right of the feeding bunk insert may be favored as it was easier to access due to the design of the Calan-Gate system.

External markers dosing strategy

Dosing with the external markers took place from day 21 to 31 (Figure 4.1). A mixture of C_{32} (*n*-Dotriacontane; CAS# 544-85-4; Minakem, SAS, France), C_{36} (*n*-Hexatriacontane; CAS# 630-06-8; Minakem, SAS, France), and Carnauba wax (as a source of C_{32}-OH; CAS# 8015-86-9; Sigma Aldrich, St. Louis, MO, USA) were melted onto 20g of peanut hulls, previously grounded through a 5mm screen. Both C_{32} and C_{36} were supplied at 1.3mg/kg of BW, while carnauba was offered at 40mg/kg BW. The average BW of the heifers at the start of the experimental periods was predicted to be 319 kg in study 1,

and 352 kg in study 2. Thus 415mg of each *n*-alkane and 10g of Carnauba wax, or 458mg of each *n*-alkane and 14g of Carnauba wax, was fed daily in study 1 and 2, respectively.

The labeled supplement was provided to animals in two ways, either once (**D_1**) or twice (**D_2**) daily. Animals belonging to D_1 received their entire dose in the morning. Animals assigned to D_2 received half their dose in the morning and half in the afternoon. Peanut hulls – either with or without the external markers depending on dosing treatment – were mixed with 0.25 kg of sweet feed. The feed buckets were removed from the bunkers, and replaced with separate buckets containing the allotted sweet feed mixture. The bunkers were checked to ensure the sweet feed was consumed entirely before the buckets with hay cubes were reinserted. The sweet feed mixture typically was consumed quickly (within a few minutes).

Fecal collection

During the final 5 days of each study, fecal samples were collected twice a day for all animals, either by rectal grab or from the ground if the animal was identified. When possible, 200g of fecal matter was obtained from each animal. Samples were placed in individual aluminum containers, and oven dried at 65 °C for 6 to 8 days, or until 1g or less of difference in weight was noted. The dried samples were then ground on a Wiley Mill through a 1mm screen and stored until analysis.

Laboratory analyses

n-alkane and LCOH extraction

Extractions were performed in duplicate (Mayes and Dove, 2006). Briefly, 0.2g of ground forage sample, or 0.1g of ground fecal sample, from individual daily a.m. or a.m. samplings, and from pooled a.m. and pooled p.m. samples were weighed into 16mm x 100mm borosilicate glass tubes. Approximately 0.1g of *n*-docosane (C_{22}) and *n*-tetritriacontane (C_{34}) alkane (0.3mg/g of each alkane), and 0.1g of 1-heptacosanol (C_{27}-OH), 1.2mg/g) long-chain alcohol (LCOH), were added to each sample tube as internal standards. Tubes were saponified for 16h at 90◦C in 2ml of ethanolic KOH 1M for forage samples, and 1.5 mL for fecal samples, using a dry-block heater (Techne DB-3,Techne Ltd., Duxford, Cambridge, UK). Heptane

(2mL for forage samples, or 1.5 mL for fecal samples) and distilled water (0.6mL for forage samples and 0.5 for fecal samples) were added to each warmed tube (60°C). The non-aqueous layer was collected after vigorous shaking yielding a crude extract. Hydrocarbons were collected by solid phase extraction and heptane elution through a silica-gel column (bed volume 1ml, 70-230 mesh, Fisher Scientific, Fairlawn, NJ). Crude alcohol extractions were obtained by elution with heptane/ethyl-acetate (80:20 v/v). Long-chain alcohol fractions were separated from sterols and stanols by solid phase extraction and heptane elution, and then derivatized using 2µl of a pyridine/acetic anhydride solution (5:1 v/v). Alkane elutes and LCOH fractions were evaporated to dryness, and then re-dissolved in 200µl of *n*-dodecane for chromatographic analysis.

Gas chromatography analysis

Quantification of *n*-alkanes and LCOH was carried out by gas chromatography (GC), using an Agilent 6890N GC (Agilent Technologies, Inc., Santa Clara, CA). Alkane and LCOH extracts were injected (0.5µl) via a 7863 Series auto-sampler (splitless injector) directly into a bonded-phase, non-polar column (Agilent J&W DB-column, 30-meter, 0.530mm internal diameter and 0.5µm film thickness). Helium served as the carrier gas at a constant flow of 4ml/min. Temperature programming was: 280°C for the injector; 340°C for the detector; and, 170◦C for 4min for the column oven followed by a first ramp of 30°C/min to 215°C with a 1min hold, and then a second ramp of 6°C/min to 300°C with a 20 min hold. Samples of an *n*-alkane and LCOH standard solution mixture (C_{10} to C_{40}; C_{20}-OH to C_{30}-OH, Sigma-Aldrich, St. Louis, MO) were included in the GC analyses to determine peak identification and standard response factors. Chromatograph data were analyzed using Agilent ChemStation software (Rev. B.04.02 SP1). Peak areas were determined with auto-integration and manual review of chromatograms. *n*-alkane and LCOH concentrations were calculated relative to known amounts of the internal standards (C_{22}, C_{34}, and C_{27}-OH), according to the equations outlined by Mayes and Dove (2006):

$$Concentration\ n-alkane_i\ \left(\frac{mg}{kg\ DM}\right) = \frac{10*\%Area*Conc\ C_{34}IS(mg/g)*C_{34}ISwt(g)}{sample\ wt(g)*DM\ content*SRF_i*FF_i}, \quad [1]$$

$$Concentration\ LCOH_i\ \left(\frac{mg}{kg\ DM}\right) = \frac{10*\%Area*Conc\ C_{27}OH\ IS\ (mg/g)*C_{27}OH\ ISwt\ (g)}{sample\ wt\ (g)*DM\ content*SRF_i}, \quad [2]$$

where $Conc\ C_{34}IS$ and $Conc\ C_{27}OH\ IS$ is the concentration of the *n-alkane*$_i$ or *LCOH*$_i$ internal standard, respectively, $C_{34}ISwt$ is the weight of the solution containing the internal standard, and %Area is the area of *n-alkane*$_i$ or *LCOH*$_i$ calculated as the percent area of C_{34} or C_{27}-OH, respectively. The DM content is the sample dry weight, *SRF*$_i$ is the average response factor, calculated as the percent area of *n-alkane*$_i$ or *LCOH*$_i$ in the mixed standard solution divided by the percent weight of *n-alkane*$_i$ or *LCOH*$_i$ in the mixed standard, and FF_i is the fractionation factor.

Derived variables

Feed efficiency

Efficiency of feed utilization was assessed through average daily gain (**ADG**), feed conversion ratio (**FCR**), and Kleiber ratio (**KR**). The ADG and FCR were obtained as the regression of body weight on days, and as the regression of cumulative feed intake on body weight, respectively. Kleiber ratio was obtained as the ratio between ADG and mean metabolic body weight (**MBW**) and multiplied by 100 (Arthur et al., 2001). The values of these variables were obtained from performance levels over the last 10 days of the experiment.

Estimation of diet composition

The amount of fescue and red clover in the diet of each animal was estimated from four *n*-alkanes found in the forages and feces: C_{27}, C_{29}, C_{31}, and C_{33}. Estimates were obtained using 'Eatwhat' software, which uses non-negative least squares optimization. This technique best matches the *n*-alkane profile in the forages to that of a fecal sample (Dove and Moore, 1995; Newman et al., 1995). These estimates were

obtained in three ways: from pooled (combined) fecal samples over the a.m. or the p.m. collections; from averaging the measured *n*-alkane concentrations from the individual a.m. and p.m. collections; or, from averaging the measured concentrations from the a.m. and p.m. pooled samples. Three correction factors were used to account for losses of *n*-alkanes in fecal samples due to their incomplete recovery (Table 4.3).

Please place Table 4.3 about here

Statistical analysis

Statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., Cary, NC).

Observed feed intake

For each study, feed intake was measured over the last 10 days of the experiment. Potential differences between animals from the two different frame sizes were tested using the GLIMMIX procedure in SAS. The model fitted was:

$$y_{ijkl} = u + S_i + H(S)_{(i)j} + D_k + (SD)_{ik} + P_l + (PD)_{lk} + \varepsilon_{ijkl}, \quad [3]$$

where y_{ijkl} is either fescue, clover, or total daily DMI (kg), u is the overall mean DMI, S_i is the i^{th} FS (i=1 or 2, for moderate or large), $H(S)_{(i)j}$ is the effect of the j^{th} heifer belonging to the i^{th} FS (j=1, 2,…,12), D_k is the effect of the k^{th} day (k=1, 2, …, 10), $(SD)_{ik}$ is the interaction between FS and day, P_l is the effect of bucket position (l=1 or 2, for left or right), $(PD)_{lk}$ is the interaction between day and bucket position, and ε_{ijkl} is the residual. Frame score, day, position and their interactions were fitted as fixed effects. Heifer within FS, which was used to test the effect of FS, and residual were fitted as random terms.

Repeatability of feed intake and diet choice

In order to determine if the patterns of dietary choice remained the same at the two ages, the repeatability of DMI was determined by regressing the natural logarithm of DMI from study 1 (**DMI1**) on the natural logarithm of DMI from study 2 (**DMI2**). Natural logarithms were used because of the wide range in DMI, it also makes the errors proportional to an observation Similarly, the repeatability of diet choice was tested by regressing the natural logarithm of proportion of clover in the diet in study 1 (**RCP1**) on the natural logarithm of proportion of clover in the diet from experiment 2 (**RCP2**). Only data on the 22 animals included in both studies were used.

For individual animals within each study, the regression of cumulative daily DMI on the cumulative difference between daily DMI of red clover and fescue was fit to determine the persistency of preference for one or the other forage.

Feed efficiency

Potential differences in these measures of efficiency between animals belonging to the two different FS were assessed by ANOVA. A simple model was fitted to analyze the three measures of efficiency:

$$y_{ij} = \mu + S_i + \varepsilon_{ij}, \qquad [4]$$

where, y_{ij} is either FCR, ADG, or KR, μ is the overall mean for these measures, S_i is the effect of the i^{th} FS (i=1 or 2, for moderate or large), and ε_{ij} is the residual.

Estimation of diet choice with the n-alkane technique

Differences in the accuracy of the estimates of diet choice were assessed by the Kulczynski Similarity Index (**KSI**; Oosting, 1956; Ferreira et al., 2009a), where a value of 0 represents complete dissimilarity, and a value of 100 represent total similarity. The statistic was calculated as:

$$KSI = 100\frac{\sum 2c_i}{\sum(a_i+b_i)}, \quad [5]$$

where c_i is the lowest of the percentages of the i^{th} forage (fescue or red clover) in the estimate versus the known diet, and is divided by $(a_i + b_i)$, which is the sum of percentages of that i^{th} forage in the estimated and known diet. Potential differences between the estimates of diet choice from the four different scenarios of fecal recovery correction factors (4^{th} scenario was no correction factor), and four scenarios for obtaining *n*-alkane fecal concentrations (pooling and averaging samples) on the KSI values were tested with ANOVA, using the GLIMMIX procedure. The model fitted was:

$$y_{ij} = \mu + C_i + F_j + (CF)_{ij} + \varepsilon_{ij}, \quad [6]$$

where, y_{ij} is the KSI value, μ is the overall mean KSI, C_i is the i^{th} effect of the fecal correction factor (i=1,…,4, for no correction factor, and 3 correction factors drawn from literature) F_j is the j^{th} effect of the type of fecal sample used for diet choice estimation (j=1,…,4, for pooled a.m. samples, pooled p.m. samples, average between a.m. and p.m. pooled samples, and average from daily a.m. and p.m. fecal samples), $(CF)_{ij}$ is their interaction, and ε_{ij} is the residual. Correction factor and scenario were fitted as fixed effect as well as their interaction, and the residual was the random term.

In addition, to assess the accuracy of the estimates of diet composition, mean prediction error (**MPE**: Elwert et al., 2004) were obtained as follows:

$$MPE = \sqrt{\frac{1}{n} \cdot \sum(\hat{y}_i - y_i)^2} \quad [7]$$

where $\hat{y}_i$ refers to the estimated red clover proportion, and y_i is the observed (known) red clover proportion. Also, because the intakes of red clover or fescue in our case are different for each animal, the Mean Relative Predicted Error (**MRPE**: Elwert et al., 2005) was considered helpful in providing an estimate of the relative mean discrepancy between estimated and known proportions. The statistic was calculated as:

$$RMPE = \sqrt{\frac{1}{n} \cdot \sum\left(\frac{\hat{y}_i - y_i}{y_i}\right)^2}, \quad [8]$$

where $\hat{y}_i$ represents the estimated red clover proportion and y_i refers to the known red clover proportion. Lastly, the estimated (**ERCP**) and observed (**ORCP**) proportions of red clover in the diet were compared by regressing the natural logarithm of ERCP on the natural logarithm of ORCP. The hypotheses tested were that the slope was not differed from unity, and that the intercept was not differed from zero.

RESULTS

n-alkane concentration

The *n*-alkane concentrations of the forages, and other feedstuffs, used are presented on Table 4.4. In the forages, four *n*-alkanes occurred in the highest concentrations, C_{27}, C_{29}, C_{31} and C_{33}. Fescue has higher concentration of C_{27}, C_{31} and C_{33}, while clover presents higher concentrations of C_{29}. In addition, while

sweet feed (grain), molasses and peanut hulls have lower concentrations for the *n*-alkanes analyzed, carnauba wax has high *n*-alkane concentrations.

Please place Table 4.4 about here

Observed feed intake

Differences in average daily intake between FS for study 1 and 2 are presented in Table 4.5. At both ages, moderate FS animals ate less total food than large FS animals ($P<0.001$), and had lower red clover DMI ($P<0.03$).

Please place Table 4.5 about here

The physical position of the bucket seemed to influence fescue DMI in both studies, and clover DMI in study 1, but it did not have an effect on total DMI in either study.

Repeatability of feed intake

The slope of the regression of DMI1 on DMI2 differed from unity ($P<0.01$), suggesting that the DMI of animals post-weaning (study 1) did not reliability predict their DMI as yearlings (study 2), as shown in Figure 4.2.

Please place Figure 4.2 about here

Repeatability of diet choice

Patterns of diet choice were not stable, with the proportion of red clover in the diet differing at the two ages in large and moderate FS animals (Figure 4.3). There was a tendency for large FS animals to increase the proportion of red clover in their diet as yearlings as compared to post-weaning (0.320 (±

0.113) kg/kg; $P < 0.02$; $r^2 = 0.411$). The pattern of red clover intake was more erratic in moderate FS animals, although they tended to reduce the proportion of red clover in their diet at the older age (-0.501 (± 0.339) kg/kg; $P = 0.178$; $r^2 = 0.116$).

Please place Figure 4.3 about here

During study 1 (post-weaning), there was no clear preference for red clover in either FS category across days (Figure 4.4). Through day 5, cumulative DMI of red clover was higher than that of fescue; thereafter that trend reversed. Conversely, during study 2 (yearling), large FS heifers persistently ate more red clover daily, while the relative intakes of the two forages remained constant in the moderate FS heifers.

Please place Figure 4.4 about here

The apparent difference in forage preferences between FS at yearling age was tested by regressing the cumulative difference between red clover and fescue DMI on cumulative DMI. As shown in Figure 4.5, there was no clear preference for either forage in large (P=0.456; $r^2 = 0$) or moderate ($P = 0.626$; $r^2 = 0$) FS heifers post-weaning (study 1). At yearling age, such remained the case in moderate FS heifers ($P = 0.177$; $r^2 = 0.117$). However, large framed heifers preferentially increased their DMI of red clover over that of fescue by 0.149 (±0.007) kg per kg total DMI ($P < 0.001$; $r^2 = 0.978$). Thus, as their intakes increased, animals with large FS increasingly preferred red clover over fescue in their diets.

Please place Figure 4.5 about here

Feed efficiency

There were no substantial differences in the measures of efficiency between moderate and large FS animals in either of the two studies (P>0.09; Table 4.6). However, while FCR from heifers of large FS

(4.20 (± 0.22) kg/kg) was almost identical to those of moderate FS (4.21 (± 0.23) kg/kg) post-weaning, the large FS heifers tended toward poorer FCR (5.47 (± 0.28)) kg/kg) than their moderate FS (4.77 (± 0.28) kg/kg) counterparts as yearlings, suggesting a trend towards decreased efficiency for large framed animals.

Please place Table 4.6 about here

Estimated diet choice

When based on average *n*-alkane concentrations from fecal samples collected over the 5 days of sampling – either the mean of pooled a.m. and p.m. samples, or the mean of individual daily samples – diet choices were estimated reliably (Table 4.7). When *n*-alkane concentrations were corrected for fecal recovery losses, regardless of the set of correction factors used, the MPE and MRPE were lower than when no corrections were made. The 'best' estimates were obtained using the correction factors of Elwert et al. (2004) in combination with mean *n*-alkane concentrations from individual daily a.m. and p.m. fecal samples, as both the MPE and RMPE were the lowest of the four scenarios used.

Please place Table 4.7 about here

The estimated proportions of red clover in the diets were regressed on the observed proportions (Table 4.7). When no fecal correction factor was used, the estimated and observed red clover proportions differed for all scenarios considered ($P<0.001$). However, with the exception of when the correction factors of Brosh et al. (2003) were used, correction of fecal *n*-alkane concentrations for incomplete recovery resulted in reliable estimates of diet choice. When there was no correction for incomplete fecal recovery, observed values were under-predicted (Figure 4.6).

Please place Figure 4.6 about here

Least squares means for KSI values with respect to fecal sampling scenario and fecal recovery correction are presented in Table 4.8. Correcting for incomplete fecal recovery improved the KSI values ($P<0.001$). However, fecal sampling scenarios did not affect KSI values ($P=0.516$) nor was there an interaction between the two factors ($P=0.999$). In addition, there was no difference in the KSI calculated values among the three correction factors used ($P > 0.478$).

Please place Table 4.8 about here

DISCUSSION

Observed feed intake. Daily intake of fescue and red clover individually and in combination were higher at yearling age (study 2), as would be expected based on an increase in BW. In addition, while red clover intake was only marginally higher between FS categories post-weaning (study 1), it was much higher in the large as compared to moderate FS heifers at yearling age. That difference likely corresponds to the large FS heifers being heavier in observed and metabolic BW, thereby having higher nutritional demands, and choosing to eat more of a more digestible food.

Daily DMI values for the heifers in study 1 were consistent to those reported by Durunna et al. (2012), given that their age and breed-type (Angus crossbreds) were similar. By yearling age, their daily DMI values were over 1.2 times higher.

Observed diet choice. During study 1, large and moderate FS animals did not appear to discriminate between the forages, as both ate approximately equal proportions of both foods. The daily change in the physical position of the feed bucket may have contributed to the apparent lack of discrimination between forage species. However, in study 2 the red clover content of the diet of large FS heifers increased appreciably – on average to 0.56 kg red clover DMI per kg total DMI intake – perhaps reflecting higher

maintenance requirements. Still, the proportion of red clover in their diets was lower than that reported by Rutter (2006), in which beef heifers chose 60% legumes in their diet. Also, although the large FS heifer expressed a clear tendency to increase their clover intake in study 2, forage choices overall were somewhat inconsistent. Van Dorland et al. (2007), working with dairy cows, found their preferences for ryegrass, and white and red clover, were not constant during the timeframe of their experiment. They suggested this may be due to the relatively small differences in the nutritive value between the forages on offer, which also is our case (Table 4.2). The main difference between fescue and red clover in our study is the NDF content, which may not be sufficient to cause substantial differences in their respective palatability. In many other studies differences in crude protein seemed to be the main driver for diet selection (Newman, 1992; James et al., 2001).

Feed efficiency. Similar to Corbet et al. (2006), ADG and FCR did not differ between post-weaned animals from the two different FS; however, our ADG and FCR values were around 30% higher likely due to breed differences between these experiments. On the other hand, our ADG were very similar to those of Schutt et al. (2009), although our FCR only half as much. Such difference may be due to their animals being substantially older (average age of 598 days) than those in our experiment. In addition, KR values obtained in this study are much higher (more than twice) than those reported by Arthur et al. (2001), Schutt et al. (2009), and Durunna et al. (2012). While the age of the animals evaluated by Arthur et al. (2001) and Schutt et al. (2009) may account for the differences among these studies, Durunna et al. (2012) used animals of similar weight and age as in our experiment.

A possible reason for discrepancies between our and other studies may be their durations. Although the length of this experiment was sufficient to allow animals to settle and adapt to their feeds and management conditions, Archer and Berghman (2000) and Castilhos et al. (2011) suggested that the minimum trial duration for reliable estimate of DMI should be 28 days for DMI, and 70 to 84 days for ADG and FCR. However, given that the primary intent of our study was to determine if the plant-wax

marker component had the ability to accurately reflect the observed intakes and diet choices, an increased duration of the trial was not necessary.

n-alkane content. The concentration of the major *n*-alkanes in the forages used appears to be different enough to allow for the estimation of diet choice. With the exception of carnauba wax, all of the feedstuff used has low odd-chain *n*-alkane concentration. Furthermore, since the amounts that these feedstuffs were included in the diet of the animals were comparatively small, their odd-chain *n*-alkane contribution were fairly negligible, thereby not affecting the estimates of diet choice.

Estimated diet choice. Of the four scenarios used for collecting and combining *n*-alkane concentrations across fecal samples, diet choices were most reliably estimated by averaging those concentrations across the 5 days of collection. The KSI values for our study were extremely high (> 90%), indicating accurate estimates, even when fecal *n*-alkane concentrations were not corrected for fecal losses. This contrasts with Lin et al. (2012) where KSI values ranged from 67.8 to 92.3, and were dependent on the maker used (*n*-alkanes, long chain alcohols, long chain fatty acids or combinations of the three) and the diet. However, their diet mixtures were more complicated, with up to 8 components.

Correcting for fecal losses of *n*-alkanes improved our estimates of diet choice, and reduced MPE and MRPE. These results are in accordance with Brosh et al. (2003) who reported more accurate estimates of diet composition once correcting for incomplete fecal recovery. However, Ferreira et al. (2009b) reported that the fecal recoveries for individual alkanes may not only be specific to an animal species (i.e., sheep, goats, cattle), but also depends on the diet. The decision, and conditions, to correct for fecal losses therefore must be considered carefully.

Based on the MPE, without the use of correction factors the difference between observed and estimated red clover proportion in the diet ranged from 53 to 57 g/kg DMI; once correcting for fecal losses that

difference reduced to between 23 and 32 g/kg DMI (Table 6). Those results are similar to those of Charmley and Dove (2007), who reported that MPE from sheep fed different diets ranged from 0.60 to 106 g/kg DMI. In addition, our MRPE halved once correcting for incomplete fecal recovery of *n*-alkanes. Without the use of such correction factors the MRPE ranged from 11 to 12.2%, and were reduced to 5.6 to 7.7% in our best scenario. This is in accordance to Elwert et al. (2005), who reported MRPE of 1.4 to 7.7% for sheep depending on their diet.

CONCLUSION

Although clear differences in intake between animals from the two FS were observed at yearling age, high FCR but similar ADG suggest lower efficiency in large FS cattle. However, a strong tendency from large FS animals to increase their red clover intake may suggest that they have higher nutritional requirements that need to be fulfilled by including more of a nutritious feed in their diets.

The *n*-alkane technique provided a useful and reliable tool for the estimation of diet choice, at least with a simple diet. With the application of appropriate correction factors to account for incomplete fecal recovery of *n*-alkanes, and with pooling of fecal samples collected over several days, this technique offers clear opportunity to determine diet choice in wider contexts. Although, prediction errors were relatively minor when estimating diet choice with our simple diets, it would be useful to expand these investigations to more complex diets, or even grazing situations.

AKNOWLEDGEMENT

Financial support from USDA-ARS for project 1932-21630-003-06 is greatly appreciated. We are thankful to Donald Wirth (Saddle Butte Ag Inc., Shedd, OR, USA) for supplying the forages (hays), to Phillip and Edgar McCracken (McHay Company, Elgin, OK, USA) for feed processing (cubing), and Ellie Stephens (Virginia Tech, Blacksburg, VA, USA) for technical editing.

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Table 4.1 Least squares for body weight (BW) and metabolic body weight (MBW) for animals corresponding to moderate and large frame size at two ages (post-weaning, at approximately 260 days of age, and yearling)

Age	Variable	Frame size		*P*-value
		Moderate	Large	
Postweaning	BW	285.3 ± 7.1	317.7 ± 7.4	0.0048
	MBW	63.3 ± 1.2	68.8 ± 1.2	0.0049
Yearlings	BW	342.8 ± 7.8	390.7 ± 7.8	<0.0001
	MBW	72.5 ± 1.2	79.9 ± 1.2	<0.0001

n=22 at post-weaning

n=24 as yearlings

Table 4.2 Chemical composition (expressed as percentages) of the forages and feed used

Foodstuff	DM	CP	NDF	ADF	Ash
Fawn fescue	90.6	12.2	60.5	32.3	8.5
Red clover	89.8	13.1	45.1	33.0	9.1
Peanut hulls	94.8	17.8	43.2	32.1	6.6
Sweet feed	87.7	14.4	16.7	6.1	6.9

DM= Dry matter content (%), CP= Crude protein, NDF= Neutral detergent fiber, ADF= Acid detergent fiber.

Table 4.3 Fecal correction factors (proportion recovery) used for the estimation of diet choice using the *n*-alkane technique

	n-alkane			
Correction factor	C_{27}	C_{29}	C_{31}	C_{33}
Brosh et al. (2003)[1]	0.642	0.729	0.815	0.901
Dove and Mayes (1986)	0.714	0.745	0.848	0.894
Elwert et al. (2003)	0.677	0.816	0.878	0.935

[1]Obtained through regression.

Table 4.4 *n*-alkane concentration (mg/kg DM) for forages and feeds used in the experiments

Foodstuff	C_{27}	C_{29}	C_{31}	C_{33}
Fescue	34.3	142.6	193.5	47.8
Red clover	19.7	314.2	34.9	6.0
Sweet feed + molasses[1]	4.5	4.9	14.4	5.9
Peanut hulls	0.0	3.4	4.9	3.3
Carnauba wax	95.8	207.9	310.1	73.6

[1] 0.25 kg of sweet mixed with 10 g of molasses.

Table 4.5 Least squares means for daily total dry matter intake (DMI, red clover plus fescue), and red clover and fescue DMI separately, for beef heifers from moderate and large frame sizes at two ages (post-weaning, at approximately 260 days of age, and yearling), and physical position of the feeding bucket containing red clover.

		Frame size			Bucket position		
Age	Variable	Moderate	Large	*P*-value	Left	Right	P-value
Post-weaning	Total DMI	6.60 ± 0.11	7.31 ± 0.11	<0.0001	6.87 ± 0.09	7.03 ± 0.09	0.0642
	Fescue DMI	3.31 ± 0.15	3.58 ± 0.15	0.1855	3.55 ± 0.11	3.33 ± 0.11	0.0066
	Clover DMI	3.29 ± 0.13	3.73 ± 0.13	0.0343	3.33 ± 0.11	3.69 ± 0.11	<0.0001
Yearlings	Total DMI	8.07 ± 0.09	9.19 ± 0.09	<0.0001	8.68 ± 0.08	8.57 ± 0.08	0.3496
	Fescue DMI	4.13 ± 0.11	4.01 ± 0.11	0.4577	4.24 ± 0.09	3.90 ± 0.09	0.0076
	Clover DMI	3.94 ± 0.10	5.18 ± 0.10	<0.0001	4.44 ± 0.10	4.68 ± 0.10	0.0691

n=22 at post-weaning age

n=24 at yearling age

Table 4.6 Least squares means for three efficiency traits, feed conversion ratio (FCR), average daily gain (ADG), and Kleiber ratio (KR) in beef heifers from moderate and large frame scores at two ages (post-weaning, at approximately 260 days of age, and yearling)

		Frame size		
Variable	Age	Moderate	Large	*P*-value
FCR	Post-weaning	4.21 ± 0.23	4.20 ± 0.22	0.966
	Yearlings	4.77 ± 0.28	5.47 ± 0.28	0.091
ADG	Post-weaning	1.45 ± 0.11	1.55 ± 0.10	0.519
	Yearlings	1.49 ± 0.10	1.57 ± 0.10	0.568
KR	Post-weaning	2.31 ± 0.16	2.27 ± 0.15	0.871
	yearlings	2.08 ± 0.15	1.99 ± 0.15	0.668

n=22 at post-weaning.

n=24 at yearling age.

Table 4.7 Parameter estimates for the regression of estimated on observed red clover proportion based on three different correction factors for incomplete fecal recovery, and without corrections for incomplete fecal recovery (no correction).

		Intercept		Slope			*P*-value				
Correction factor	Scenario[1]	Estimate	s.e.	Estimate	s.e.	r^2	a=0	b=1	x=y	MPE[2]	MRPE[3]
No correction	Pooled AM	-0.089	0.071	0.796	0.076	0.839	0.227	0.014	<0.001	0.049	11.5
	Pooled PM	-0.099	0.075	0.778	0.079	0.820	0.204	0.011	<0.001	0.053	12.3
	AM+PM	-0.040	0.060	0.847	0.064	0.892	0.520	0.027	<0.001	0.047	11.9
	Mean	-0.044	0.064	0.843	0.068	0.878	0.498	0.034	<0.001	0.049	11.0
Brosh et al. (2003)	Pooled AM	-0.085	0.071	0.954	0.090	0.841	0.247	0.618	0.011	0.036	8.8
	Pooled PM	-0.091	0.074	0.937	0.093	0.828	0.234	0.508	0.037	0.034	8.5
	AM+PM	-0.037	0.060	1.013	0.076	0.895	0.543	0.865	0.004	0.031	7.6
	Mean	-0.041	0.065	1.010	0.082	0.878	0.530	0.906	0.005	0.032	8.1
Dove and Mayes. (1986)	Pooled AM	-0.082	0.071	0.936	0.088	0.842	0.261	0.478	0.101	0.030	7.5
	Pooled PM	-0.092	0.085	0.893	0.105	0.771	0.293	0.322	0.557	0.034	8.3
	AM+PM	-0.035	0.060	0.993	0.074	0.894	0.570	0.930	0.075	0.026	7.5
	Mean	-0.040	0.065	0.989	0.080	0.878	0.544	0.893	0.079	0.029	7.0
Elwert et al. (2004)	Pooled AM	-0.083	0.071	0.884	0.084	0.841	0.257	0.180	0.273	0.028	6.7
	Pooled PM	-0.089	0.074	0.868	0.086	0.826	0.244	0.143	0.145	0.032	7.7
	AM+PM	-0.033	0.060	0.940	0.070	0.894	0.580	0.402	0.295	0.023	5.6
	Mean	-0.038	0.065	0.937	0.076	0.878	0.562	0.416	0.401	0.027	6.0

[1]AM+PM= average between pooled AM fecal sample and PM fecal sample *n*-alkane concentration for each animal; Mean= average of daily fecal sample *n*-alkane concentration for each animal.

[2]MPE= Mean prediction error (kg/kg).

[3]MRPE= Mean relative prediction error (%).

Table 4.8 Mean Kulczynski Similarity Index (KSI) values and significance for the scenarios used, and correction factors for incomplete fecal recovery.

Effect	Level	Mean	s.e.	*P*-value
Scenario[1]				0.512
	Pooled AM	96.340	0.281	
	Pooled PM	96.377	0.283	
	AM+PM	96.820	0.284	
	Mean	96.744	0.284	
Correction factor				<0.001
	No correction factor	94.858	0.284	
	Brosh et al, (2003)	96.804	0.284	
	Dove and Mayes, (1986)	97.247	0.283	
	Elwert et al., (2004)	97.373	0.281	

[1] Pooled AM= pooled a.m. fecal samples

Pooled PM= pooled p.m. fecal samples

AM+PM= average of n-alkane concentration from pooled a.m. and p.m. samples

Mean= Average of individual daily fecal samples, for both a.m. and p.m. samplings

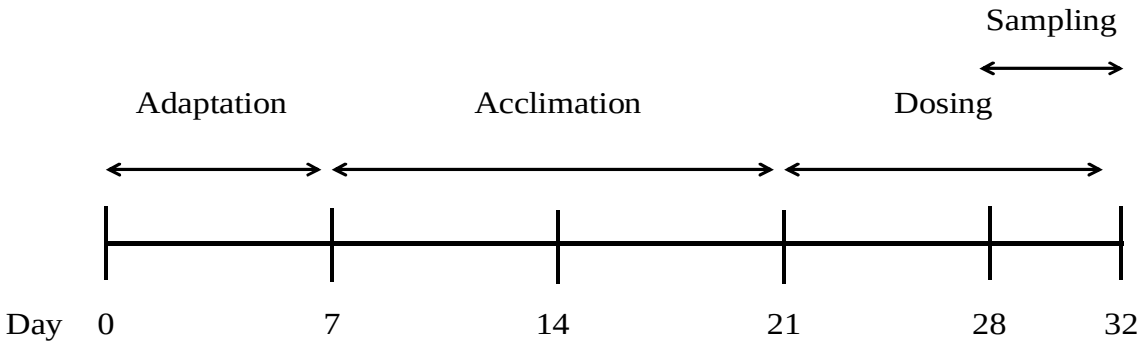

Figure 4.1 Schematic of the experimental period.

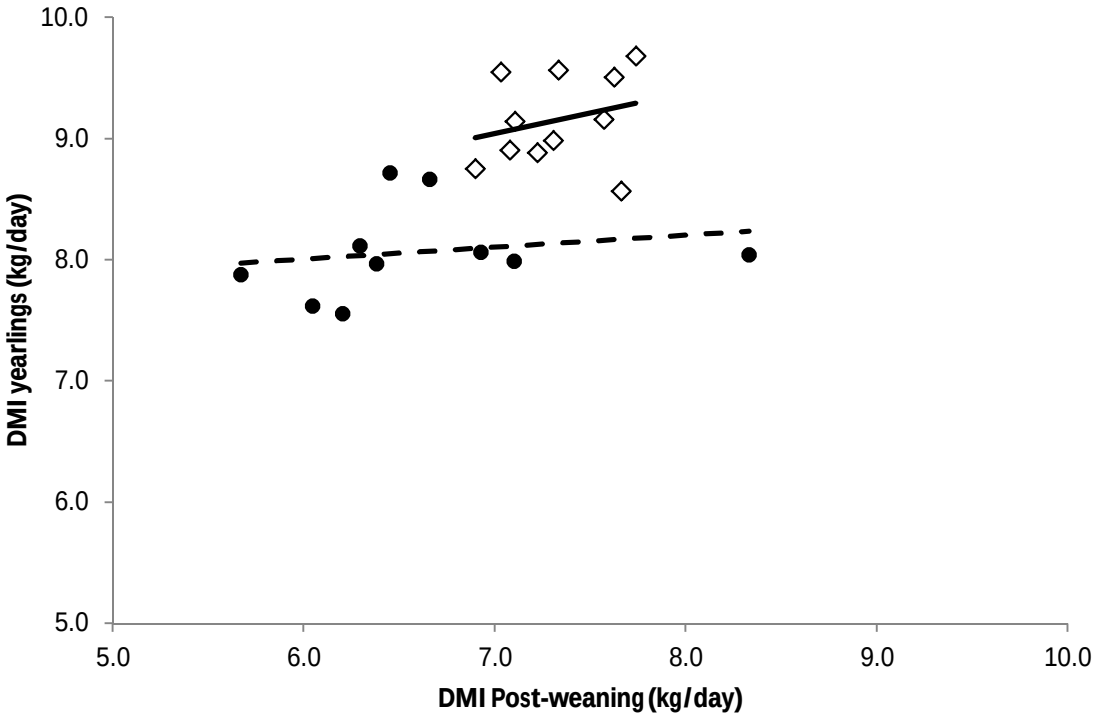

Figure 4.2 Observed DMI at post-weaning vs. yearling age, in large (white diamonds) and moderate (black circles) frame size heifers. The solid lines represent the linear fit for large frame size, and the broken line represents the linear fit for moderate frame size.

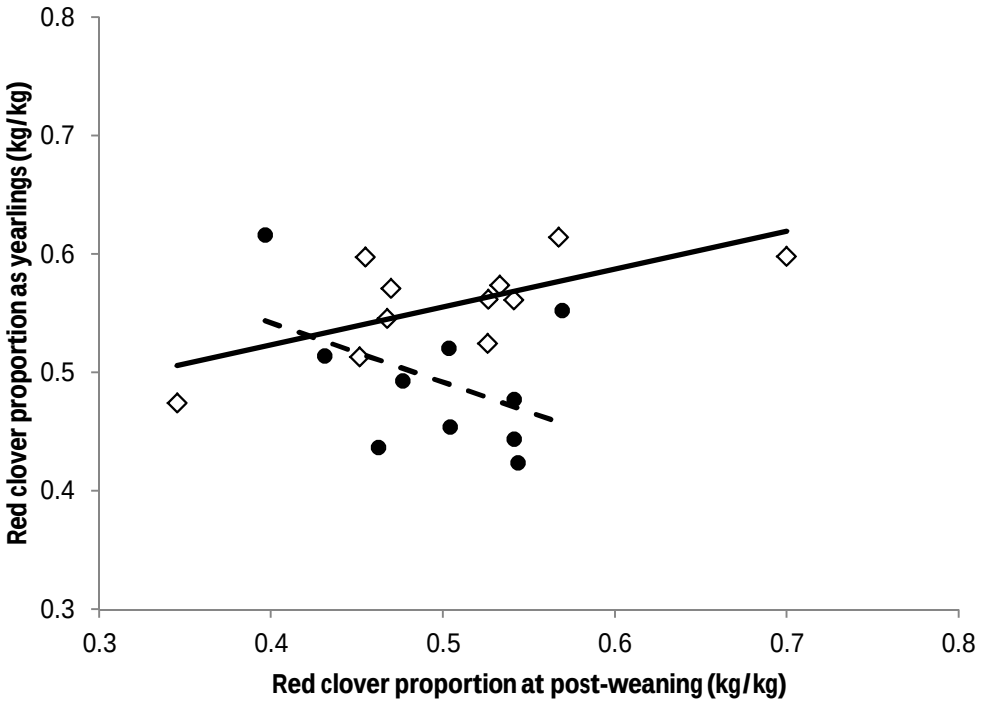

Figure 4.3 Observed proportions of red clover in diet post-weaning vs. yearling age, in large (white diamonds) and moderate (black circles) frame size heifers. The solid lines represent the linear fit for large frame size, and the broken line represents the fit for moderate frame size.

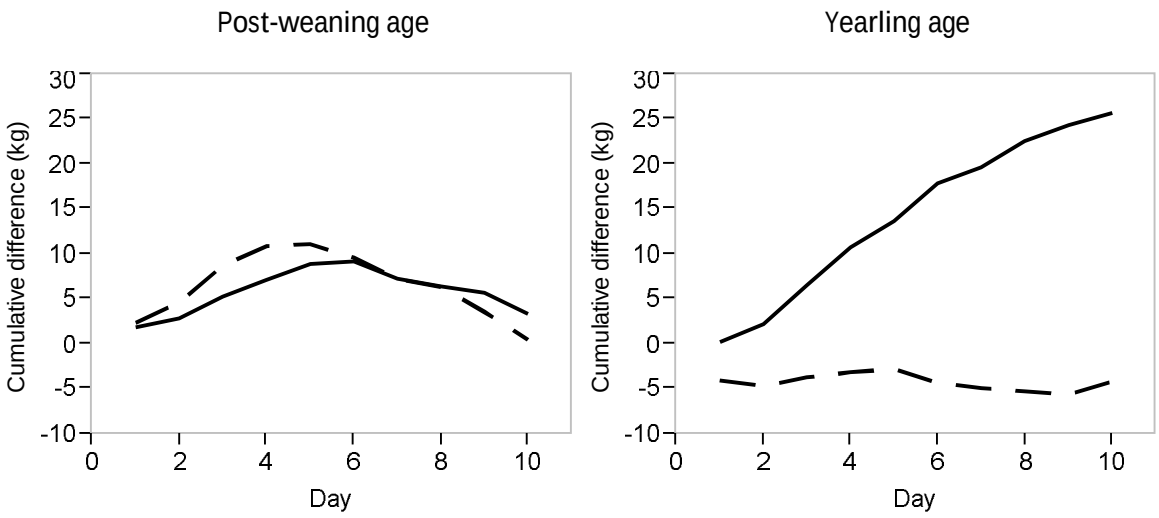

Figure 4.4 Cumulative difference in DMI (red clover DMI minus fescue DMI) of red clover and fescue vs. day at two ages (post-weaning, at approximately 260 days of age, and yearling). The solid line is for the large frame size, and the broken line for the moderate frame size, heifers.

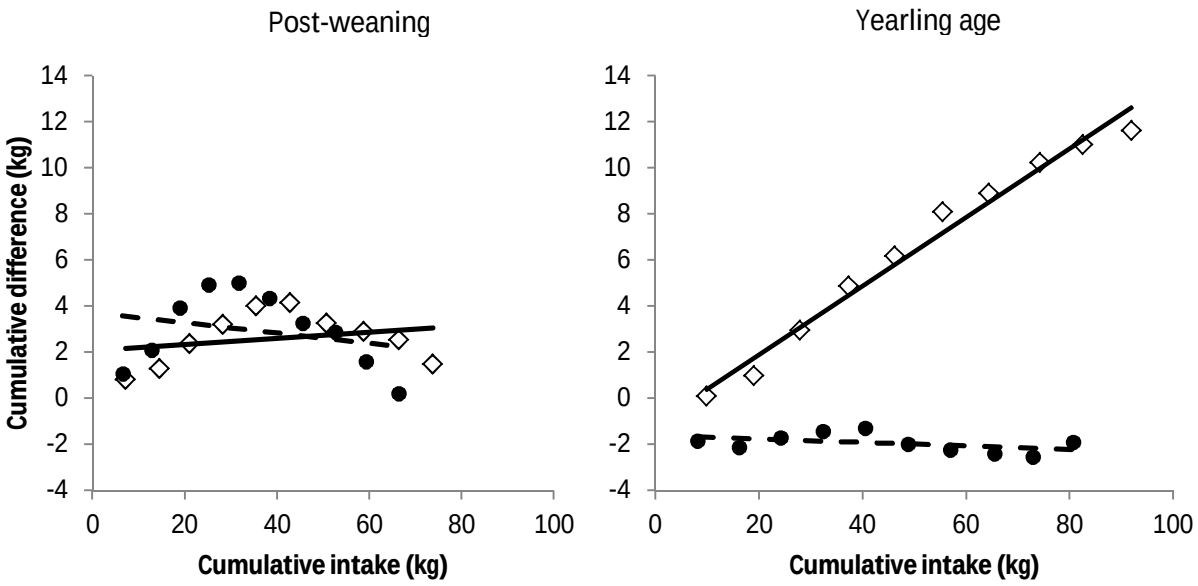

Figure 4.5 Cumulative DMI vs. cumulative difference in DMI between red clover and fescue (red clover DMI minus fescue DMI), from animals belonging to large (white diamonds and solid line) and moderate (black circles and broken line) frame size categories. Results from the two ages (post-weaning, at approximately 260 days of age, and yearling) are shown separately.

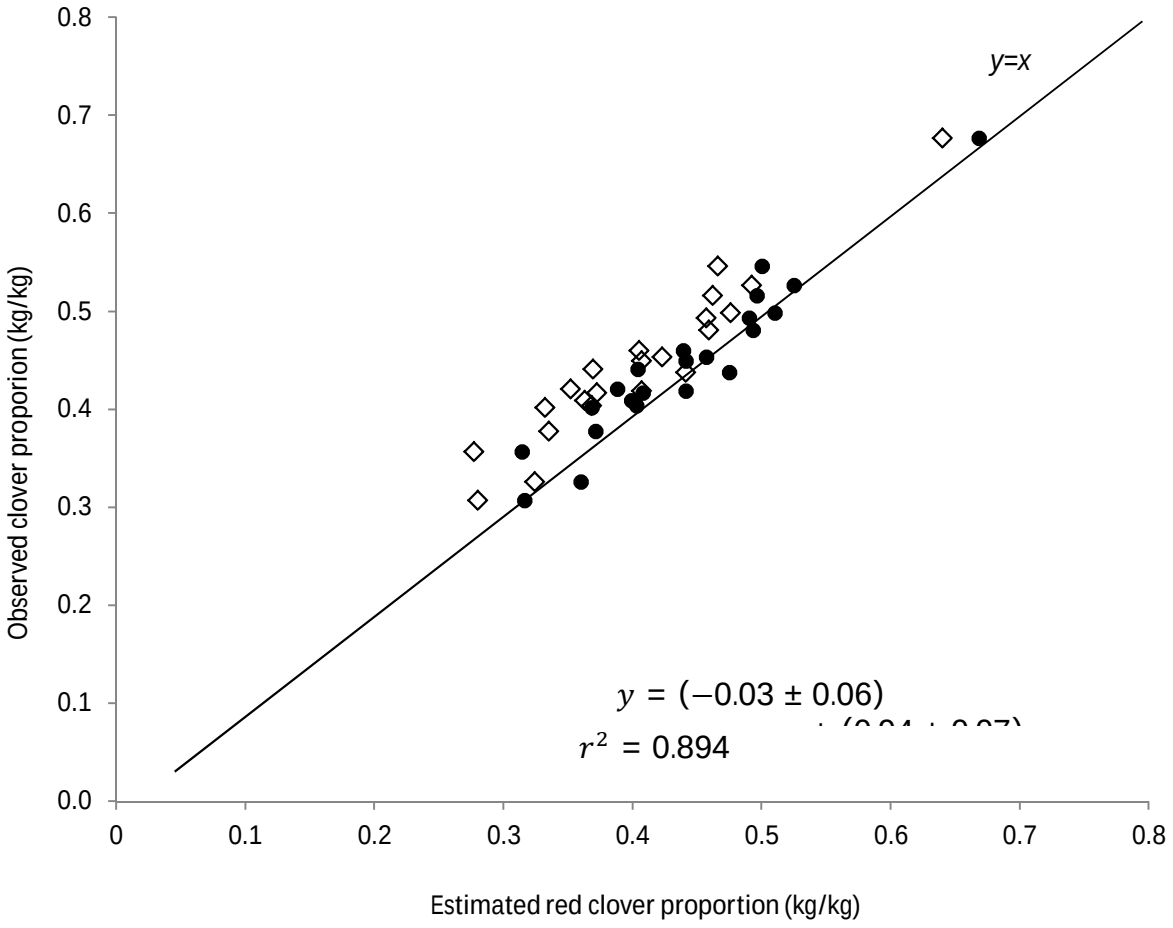

Figure 4.6 Observed vs. estimated red clover proportions in the diet selected by heifers offered a choice of cubed red clover and cubed fescue. The estimated values were based on *n*-alkane concentrations averaged across a.m. and p.m. fecal samples. The white diamonds show values without correction for incomplete fecal recovery, while the black circles show values with correction for incomplete fecal recovery of the n-alkanes based on Elwert et al. (2004). The solid line shows where observed and estimated proportions of red clover in the diet are equal.

CHAPTER 5

CONCLUSIONS

Summary

The results from the present work confirm that the plant wax marker technique is useful in determining botanical composition in, at least, simple test diets. The highly consistent estimates obtained by, even, an operator with limited expertise in preparing samples and in conducting gas chromatography suggest that the technique is reliable, however due to our limited sample sizes more work is needed to confirm this holds true. Although variability in measured concentrations of the markers (both *n*-alkanes and LCOH) between operators and across duplicate extractions did not appreciably affect the reliability of the estimates of botanical composition, it may play a role when dealing with more complicated plant mixtures. Since more complicated swards (higher number of species) will require the addition of other types of markers, such as long chain fatty acids or include shorter-chained compounds, such as C_{25}, C_{22}-OH, or C_{24}-OH, sources of error may be introduced. Thus, further work is needed to determine if the technique remains robust with such modifications.

Similarly, estimates of diet composition from fecal samples using *n*-alkanes alone appear to be reliable. Although the KSI values seemed to indicate high reliability of diet choice estimates even when no correction factors were used, accounting for incomplete fecal recovery of these markers improves the accuracy of these estimates. In the present study, using corrections factors reported in other studies increased the accuracy of the estimates; however, improved accuracy may be obtained if correction factors from our own experiments were used, as these factors seem to be diet and species dependent. In addition, given that in the present study the reliability of the technique was assessed by comparing estimates of diet choices (with or without correction for incomplete *n*-alkane recovery) to known diet choices, in situations in which the actual diet choice is not known, such as in grazing conditions, such

comparisons of reliability are not possible. Therefore, the use of correction factors needs to be carefully considered.

Differences in measures of efficiency between moderate and large frame size cattle were not clear; however, daily dry matter intake, as well as red clover intake, was substantially higher for large frame size animals. The inconsistent patterns of red clover intake for animals from both frame size categories at post-weaning age suggests that at that age, these animals had similar nutritional requirements. In addition, the change in the physical position of the feeding buckets may have contributed to these inconsistent patterns. However, since in the second study the feeding buckets remained in their positions for a longer time, a definite conclusion is not possible at this time. At yearling age, there was a continuous increase in the red clover content of the diets of cattle of large frame size. This indicates that as animals of larger mature size continue to grow their higher nutritional demands need to be fulfilled by increased consumption of a more nutritious food, apparently even when its nutritive quality is only slightly higher. Further work is needed to achieve more conclusive results on this matter.

Although the laboratory techniques used were fairly accurate in measuring the concentration of odd-chain *n*-alkanes in forages and feces, issues arose in the extraction and measure of the dosed even-chained *n*-alkanes used as external markers. Therefore, I was unable to include estimates of feed intake in the present work. The problem appears to be unanticipated contamination of some of the materials and equipment used for extracting the *n*-alkanes despite careful adherence to established protocols. The dosed *n*-alkanes were longer-chained than other compounds analyzed and have higher boiling points. It is suspected that their residues have proven more difficult to remove from the laboratory equipment used. Addressing this issue is clearly of high priority, since reliably quantifying these compounds is essential for estimating feed intake with the *n*-alkane technique. Also, since evaluating feed efficiency in grazing conditions may well entail the use of fecal markers to estimate feed intake, resolving these laboratory issues is prerequisite to applying this technique in cattle grazing more complex swards.

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