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**Evaluation of internal and external markers for estimating dry matter intake and
digestibility in cattle**

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Evaluation of internal and external markers for estimating dry matter intake and digestibility in cattle

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CERTIFICADO

Certificamos que o Projeto intitulado "Avaliação de indicadores internos e externos na estimativa do consumo e digestibilidade da matéria seca em bovinos", protocolado sob o CEUA nº 2442060314, utilizando 29 Bovinos (20 machos e 9 fêmeas), sob a responsabilidade de Romualdo Shigueo Fukushima, foi aprovado na reunião de 13/08/2014, e está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo.

We certify that the Research "Evaluation of internal and external markers for estimating dry matter intake and digestibility in cattle", protocol number CEUA 2442060314, utilizing 29 Bovines (20 males and 9 females), under the responsibility Romualdo Shigueo Fukushima, was approved in the meeting of day 08/13/2014, and agree with Ethical Principles in Animal Research adopted by Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo.

Atenciosamente,

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

*To my parents, Edgar and Gloria, and to my
family I dedicate this manuscript. For the
example and support given*

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To my dear mother, for teaching me to thrive on challenges

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To Brazil, amazing country full of opportunities

RESUMO

VELASQUEZ, A.V. **Avaliação de marcadores internos e externos para estimar o consumo e a digestibilidade da matéria seca em bovinos.** [Evaluation of internal and external markers for estimating dry matter intake and digestibility in cattle]. 2017. 87 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2017.

A determinação do consumo de alimentos é uma valiosa ferramenta na hora da tomada de decisões de manejo do rebanho. O uso de marcadores, sejam internos ou externos, é atualmente a técnica mais utilizada para estimar o consumo em animais de produção. Três experimentos foram conduzidos. Em vacas de leite alimentadas com silagem de milho com 45% de concentrado, a recuperação fecal (RF) média de TiO_2 foi maior do que a RF de Cr_2O_3 e ambas foram maiores do que a unidade. A RF não foi afetada pelo método de coleta de fezes, mas, as estimativas de digestibilidade da matéria seca e produção fecal (PF) foram. A dupla de marcadores Cr_2O_3 +i fibra detergente ácido indigestível (iADF) produziu estimativas de consumo de matéria seca (CMS) acuradas. Em novilhos alimentados com diferentes fontes de forragem, a RF do TiO_2 foi próxima a 1 e este marcador produziu estimativas de PF adequadas. A RF foi afetada pela fonte de forragem que compunha a dieta e nenhum dos marcadores produziu estimativas de CMS acuradas. Em tourinhos Nelore alimentados com dietas compostas de silagem de milho ou feno de Tifton-85 e diferentes relações de volumoso:concentrado, a RF média dos marcadores internos lignina brometo de acetila (LBA), fibra detergente ácido indigestível (iFDN), (iFDA) e cutina foi diferente de 100%. Os marcadores Cr_2O_3 e TiO_2 tiveram RF menor e maior que 100%, respectivamente. A combinação de marcadores internos e externos para estimar o CMS produziu resultados acurados e satisfatórios, principalmente as duplas Cr_2O_3 e iFDN ou iFDA. Os marcadores internos LBA e cutina merecem mais estudos. O método de coleta GRAB produziu estimativas de DMS, PF e CMS acuradas, as quais foram iguais ou melhores que as produzidas pelo método de coleta BULK. Independentemente do método de coleta de fezes e da composição da dieta, é necessário estabelecer a RF real dos marcadores que serão usados em experimento para obter estimativas corretas. Nenhum dos marcadores teve RF completa e consistente nos 3 experimentos. A cutina e LBA tiveram RF menor do que a unidade em todas as dietas e falharam em produzir estimativas acuradas de DMS. Quando qualquer dos dois foi pareado com Cr_2O_3 ou TiO_2 as estimativas de CMS também não foram acuradas. Das fibras indigestíveis, iFDN e iFDA, a segunda foi mais acurada em estimar o CMS quando pareada com Cr_2O_3 . As estimativas de PF corrigida foram acuradas para Cr_2O_3 em diferentes dietas sob metodologia de coleta GRAB. O mesmo não foi

observado para o TiO_2 , que produziu estimativas enviesadas inclusive após a correção. O método de dosagem dos marcadores externos é extremadamente importante já que afeta e determina em grande medida os resultados. Qualquer que seja o método, este deve permitir ao animal comportar-se e alimentar-se normalmente sem comprometer seu desempenho. Os métodos de coleta GRAB podem substituir a coleta total de fezes (CTF) (após determinação da RF) o que poderá abrir novas possibilidades para estudos com animais em pastagem ou alojados em baias coletivas.

Palavras-chave: Cutina. Dióxido de titânio. Fibra indigestível. Lignina. Óxido de cromo.

ABSTRACT

VELASQUEZ, A.V. **Evaluation of internal and external markers for estimating dry matter intake and digestibility in cattle.** [Avaliação de marcadores internos e externos para estimar o consumo e a digestibilidade da matéria seca em bovinos]. 2017. 87 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2017.

Feed intake assessment is a valuable tool for herd management decisions. The use of markers, either internal or external, is currently the most used technique for estimating feed intake in production animals. Three experiments were conducted with beef and dairy cattle. In dairy cows fed a corn silage based diet with 45% concentrate, the average fecal recovery (FR) of TiO_2 was higher than FR of Cr_2O_3 and both FR were more than unity. The FR was unaffected by the fecal sampling procedure but, estimates for dry-matter digestibility (DMD) and fecal output (FO) were affected. The Cr_2O_3 + indigestible acid detergent fiber (iADF) marker pair produced accurate dry-matter intake (DMI) estimates. In steers fed diets with different forage sources FR of TiO_2 was close to 1 and this marker produced adequate FO estimates. The FR was affected by the forage source that composed the diet and none of the marker pairs produced accurate DMI estimates. In young Nelore bulls fed corn silage or Tifton-85 hay based diets with varying F:C ratios, the average FR of internal markers acetyl bromide lignin (ABL), indigestible neutral detergent fiber (iNDF), iADF and cutin was different from 100%. Markers Cr_2O_3 and TiO_2 had FR lower and higher than 100%, respectively. The combination of internal and external markers to estimate DMI produced satisfactory and accurate results, particularly Cr_2O_3 based pairs with iNDF and iADF. Internal markers ABL and cutin deserve more attention. The GRAB sampling procedures yielded accurate DMD, FO and DMI estimates, which were as good as or better than BULK sampling procedures. Regardless of the fecal sampling method and the composition of the diet, it is necessary to establish the FR of the markers to be used in each experiment, in order to obtain correct estimates. None of the markers had complete FR consistent throughout the 3 experiments. Cutin and ABL had lower than unity FR on all diets and failed to produce accurate DMD estimates. When either one was paired with Cr_2O_3 or TiO_2 , DMI estimates were also not accurate. Of the indigestible fibers, iNDF and iADF, the later was more accurate in estimating DMI when paired with Cr_2O_3 . Consistently throughout the experiments, FR was lower than and higher than unity for internal and external markers, respectively. Corrected FO estimates were accurate for Cr_2O_3 on various diets under GRAB sampling. The same was not true for TiO_2 ,

which even after FR correction was applied failed to produce accurate FO estimates consistently. The method of dosing the external markers is extremely important and greatly affects and determines results. Whichever the method, it must allow the animals to display normal feeding behavior and not affect performance. The GRAB sampling procedures can replace TFC (once FR is established) which may open new possibilities for pasture based or collectively housed animals.

Keywords: Cutin. Titanium dioxide. Indigestible fiber. Lignin. Chromic oxide.

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Introduction

Feed intake assessment is an aid for herd management decisions. It is also the starting point for interpretations about animal nutrition. Today, this topic has more relevance in relation to evaluation of the environmental impact caused by livestock production, which requires accurate estimates of how much forage animals consume when they are on pasture. Also, to determine ration composition to meet animal requirements.

Scientists have been trying to estimate feed intake in ruminants for many years and several strategies have been made available. Among these strategies is the use of prediction equations with multiple regression analysis between observed intake and variables that are supposed to affect it. These equations are based on feed composition but, organoleptic characteristics, weather, management and learning among others, can alter voluntary intake. The use of markers, either internal or external, is currently the most used technique for estimating feed intake in production animals.

Even though the use of internal and external markers may be the most adequate for estimating intake, the method has also received criticisms. Theoretically, all markers should be indigestible, not secreted by the host, have passage characteristics similar to those of the feed being consumed, be completely recovered in feces and allow for precise and practical analysis. According to Merchen (1993), none of the substances that are used as markers fulfill all of these characteristics, but some of them are adequate enough to produce important data. For this reason, the search for an “ideal” marker is a topic that has been given attention by the scientific community.

A reliable method for estimating intake should produce results with small variation and high repeatability (SMITH et al., 2005). An “ideal” method should have minimal influence on animal behavior (LE DU & PENNING, 1982), avoiding decreases in intake and consequently in performance. Information about the currently available methods for measuring intake in ruminants will allow the proposal of guidelines for the use of these tools.

General Overview

Approximately half of the meat produced around the world comes from tropical and subtropical countries (JANK et al., 2005). In these countries, production systems are mostly pasture based and are subject to strong variations in quantity and quality of pastures due to seasonal changes. Through supplementation, these effects can be countered allowing for the evolution of national beef and dairy herds raised under these conditions. The main difficulty in doing so, is that in order to correctly, precisely and economically supplement an animal we must first have an accurate idea of how much feed it can consume and the quality of the feed that it will be consuming. When animals are individually housed (show animals, tie-stall or experimental conditions) or with electronic feed bunks it is possible to determine how much an animal eats per day. Under normal farm conditions, where animals are either on pasture or housed in groups within free-stalls or feed-lots, individual intake can only be calculated as the average of the group's intake or inferred by measuring available forage before and after the animals enter the paddocks. Although, these intake estimates may be of some use, they are not adequate enough to allow for decision making, supplement formulation and balancing or even to determine efficiency in the use of nutrients.

Voluntary intake depends on ruminal capacity (volume) and the chemical composition of the feed which will determine the extent of ruminal fermentation. Thus, an increased rate of ruminal fermentation results in faster passage rate which leads to increased voluntary intake. Ruminal fermentation causes feed particles to diminish in size and increase in density. Once the particle is small enough, it exits the rumen through the millimetric reticulo-omasal orifice and “opens space” for new feed to be consumed. Mechanic receptors on the ruminal wall detect changes in ruminal distension and signal the hypothalamus, causing the animal to start another feeding episode. This mechanism causes that increased ruminal degradation results in shorter interval between feeding episodes (BERCHIELLI et al., 2011).

Feed intake can be defined as the amount of dry matter (DM) ingested over 24-hours (kg DM/day) and it involves a **number of feeding episodes**, **duration of these episodes** and the respective **rate of ingestion**. Any change in intake is necessarily associated to one or more of these three variables. Direct methods for measuring intake have tried to determine the average values for each variable but, the variation among individuals and animal categories *plus* the many factors that can affect each one have steered research towards more indirect approaches like the use of markers.

Since intake is associated to the fraction of feed which is less digestible (ELLIS et al., 1994), a hypothetical increase in passage rate could reduce total digestibility. According to Demment and Van Soest (1985), longer periods of ruminal retention result in higher digestibility, especially for the structural parts of forages. Therefore, increasing rate of ruminal fermentation has a positive effect on dry matter intake (DMI) but needs to be balanced according to the composition of the diet, so that feed efficiency is not compromised.

Considerations on behavior and physiology of feed intake

Studies with domestic animals give emphasis to factors that drive intake, such as maintenance, growth, pregnancy and lactation. This compartmentalized approach produces fixed levels of energy or nutrients believed to be optimal for each factor. The problem is that no such levels exist in nature and therefore, the term “requirement” should be used very carefully or not at all. The biologically correct approach would be to express these drivers in terms of response to nutrients. This way, biological and economic relationships between intake of nutrients and results such as growth, weight gain, reproduction and lactation could be determined (FORBES, 2010).

Under natural grazing conditions, animals adjust intake of available forages to meet their nutritional needs, without necessarily maximizing intake. When animals are collectively housed or receive supplements on pasture, they adjust intake to what is being offered and this explains the variable individual results observed (BOWMAN AND SOWELL, 1997). This is especially important when animals are transferred to new environments (buy/sell, weaning, pre-partum, lactation groups, feed-lots, etc.) because not only will the animal need to adjust to the new feed offer but also its new surroundings. An adaptation period is necessary, in which learning about the new feed will take place and will be associated to post ingestion effects (FORBES, 2007; FORBES, 2010). If post ingestion effects do not cause discomfort then intake will gradually increase until the limit is imposed by the predominant regulation factors (physical capacity of the rumen, distension and blood metabolites). Therefore, animals are conditioned by association of feed properties and post intake changes caused by digestion and metabolism of the feed.

Another aspect that should be considered is social interaction among animals. Dairy cows are social animals that form classes according to dominance hierarchy, especially at the feed bunk (FRIEND and POLAND, 1974). Dominance and competition are accentuated during the first 30-45 minutes after feed is offered (GRANT AND ALBRIGHT, 1995) causing that non-dominant

animals consume less feed, with different composition than what was formulated which will ultimately compromise animal performance.

Some breeds of cattle, especially *Bos t. indicus* and their crosses, tend to be more nervous than others. This may be caused by minimum human contact or incorrect handling of animals during routine management. Either way, nervous animals are easily stressed resulting in high adrenaline and cortisol levels which negatively affect intake. It is important to consider this when working with these breeds, to allow adaptation to humans, machinery and every-day activities.

Methods for estimating voluntary intake

Diverse approaches have been used when trying to solve the intake issue, especially with animals on pasture. Methods can be divided into direct or indirect depending if the focus is on the animal or on what it is consuming. It is possible to directly determine or indirectly estimate fecal output (FO), dry matter digestibility (DMD) and dry matter intake (DMI), although any procedure that is chosen will have advantages and disadvantages when compared to the others.

Direct measurements are collected by weighing all feed that is offered, orts and total feces collection (TFC). This procedure is the one that most reliably represents reality; on the other hand, collection of this data is labor intensive, expensive and hard to obtain (BERCHIELLI et al., 2000). According to Barbosa (2005), estimating DMD is the main method used to determine nutritional value of feeds. For this, the most commonly used procedure is TFC. Considering the elevated cost and labor required for TFC, the use of markers has become a convenient alternative for this type of studies. Even though the use of markers might be the easiest and most adequate way of estimating intake, the method has also received criticism.

Several alternative methods have been proposed with the objective of overcoming these difficulties. Approaches that have been contemplated: differences in weight of feed (CORBETT, 1980; GARDNER, 1986 and BURNS et al., 1994), differences in body weight (HORN et al., 1979), feeding behavior (LUGINBUHL et al., 1990), mathematical predictions or regression equations (POPPI, 1996) and, more recently, biomass volume measurements through satellite images (EDIRISINGHE et al., 2012). All of these methods have also received criticism for reasons that vary from low reliability of results to excessively high cost of analysis, which prevents their routine adoption.

Electronic methods are available, such as the Calan-gates®, GrowSafe® and Kloppen® systems, allowing group housing with individual intake measurements. With these systems, the feed bunk recognizes a magnetic collar or ear-tag on the animal and stores intake information from each animal in the group. Each system has its own variations but the major problems with these technologies is their high cost and the difficulty of receiving good technical support outside the USA or Europe.

The use of markers to estimate intake is based on the principle that fecal output (FO) is inversely proportional to dry matter digestibility (DMD) and directly proportional to dry matter intake (DMI) (PENNING and JOHNSON, 1983), being calculated by the formula:

$$DMI = \frac{FO}{(1 - DMD)}$$

Estimating FO by the marker method consists of using a reference substance that allows quantitative recovery (OWENS and HANSON, 1992). The premise of efficiency for markers is the capacity of being recovered in feces. Knowing the amount of marker consumed by an animal allows for estimates of FO from fecal marker concentration.

One of the difficulties associated to the marker method for estimating FO is the means of dosing the animals with the external markers. Various approaches have been employed for this task and all of them have advantages and disadvantages. The most important consideration is choosing a form of delivery that minimizes handling of animals and stressful conditions, but at the same time it must guarantee that an exact and known amount of marker is delivered. Working with ruminally cannulated animals can be easier and allow for precise dosing but this type of animal is usually used only for experimental purposes and not easily available in practical conditions. Even with ruminally cannulated animals there is a need to use a chute to safely deliver the markers, so this does not completely solve the issue of the excessive need for human interaction with the animals. More handling results in stress for the animals and less time spent feeding which may influence intake results.

The main difficulty associated to the use of markers is feces collection. Individually housed animals are easier to work with but require constant monitoring to avoid loss or contamination of samples. Collectively housed or pasture based animals pose a bigger challenge to avoid cross contamination of feces and because the use a containment structure (chute) is necessary for sampling. The sampling design to be used is very important and will determine how many days of sampling and the interval between each sampling episode. About the length of the sampling

period, Schneider and Flatt (1975) reported that a minimum of 7-10 days of sampling were necessary to avoid errors in FO estimates. Barbosa (2005) did not observe significant differences between 5 or 6 days of sampling, but increasing the number of days produced more precise estimates. Ferreira et al., (2008) reported no significant differences between 3 and 5 sampling days for estimating digestibility in beef and dairy cattle.

Research has shown that just as the number of sampling days can be reduced, collection of various sub-samples throughout the day can represent TFC. These GRAB samples are collected after spontaneous excretion or manually, directly from the rectum. These subsamples are then pooled by animal, day and/or period for later analysis. The more recent results show opportunity of collecting feces samples a few times a day without compromising results, which opens new possibilities for this method. Therefore, it is possible to propose sampling designs that occur only during the day or that are easily adaptable to everyday activities of research centers and maybe even commercial operations.

After review, Valadares Filho et al., (2010) recommended that a 3 day feces sampling period was enough and concluded that with the use of 1 internal and 1 external marker it is possible to accurately estimate individual intake in collectively fed animals. Among the markers they studied, chromic oxide (Cr_2O_3) and titanium dioxide (TiO_2) worked best for estimating individual supplement intake and, indigestible neutral detergent fiber (iNDF) and indigestible acid detergent fiber (iADF) produced the best estimates of individual forage intake.

It is possible to estimate the indigestible fraction of a feed by laboratory analysis based on its chemical composition. From this indigestible fraction, it is possible then calculate how much intake would be. This seems easier and practical yet studies have shown that in vitro dry matter digestibility (IVDMD) overestimated intake when compared to iNDF (DETMANN et al., 2001). Therefore, they advised that IVDMD should not be used for intake estimates in ruminants.

Internal markers

Fecal recovery (FR) of indigestible feed fractions is the basis for using internal markers to estimate DMD (VAN SOEST, 1994). Internal markers use is advantageous when compared to external markers because they are already naturally present in the feed and they distribute uniformly in digesta (PIAGGIO et al., 1991). On the other hand, they require prolonged incubation periods, minimum of 144 hours (BERCHIELLI et al., 2000), to be considered an indigestible fraction, such as iNDF and iADF. More so, longer incubation times have been suggested recently, 288 hours (KRIZAN and HUHTANEN, 2013), due to variations among

different feeds. With grazing animals (DETMANN et al., 2001) or feeding sugarcane as forage to heifers (FERREIRA et al., 2008), iNDF produced better estimates than iADF, but the opposite happened when the same authors fed corn silage to lactating dairy cows.

According to Casali et al., (2008) there is no consensus among researchers on the incubation time for indigestible fibers and published reports vary from 98 to 288 hours. In order to obtain relevant data from the use of these markers it is necessary to standardize one procedure that is adopted by the scientific community. This will validate results and allow for comparisons between different studies.

Lignin is considered the primary limiting factor of cell wall degradation by constructing a physical barrier that denies access to rumen microorganisms and their enzymes (BUXTON and RUSSELL, 1988). Lignin, determined as acid detergent lignin (ADL), has been used as an internal marker in digestion studies and is considered indigestible because there are no known mammalian enzymes that can degrade it (VAN SOEST, 1994). Meanwhile, reservations exist and no consensus is found in literature. Studies dated as far back as 1920 already showed that lignin was not an inert part of animal diets. Thonney et al., (1979) used potassium permanganate lignin as a marker for steers consuming 20, 30, 60, 80% concentrate and observed that the marker underestimated digestibility, when compared to TFC, by 23.9 percentage points. Crampton (1940) reported that even if digested, lignin, did not have any nutritional role in the animal because the small quantities that could be absorbed were excreted via urine. Results are conflicting and point to problems with FR. Also, methods for quantification of this cell wall component are controversial.

While much of the research is focused on modifications of the lignin molecule as it passes through the GIT, experimental results justify the use of this compound as a marker. The observations of Porter and Singleton (1971) showed that the chemical composition and amount of lignin that passed through the duodenum was almost the same as fecal lignin. Since lignin is intimately linked to the nutritive value of feeds, it deserves more attention to better understand its role and how it can be used.

One of the main reasons for conflicting results observed for lignin is, as with iNDF and iADF, the availability of several different methods to determine lignin and the proven flaws that most of them have shown. Lignin procedures that use 72% sulfuric acid, such as ADL and Klason lignin, can also measure cutin or some Maillard compounds as being lignin. On the contrary, part of the lignin can be destroyed or solubilized during the procedure, either by the acid detergent

solution or during hydrolysis with 72% sulfuric acid. Fukushima et al., (2015) backed up this observation by verifying ABL concentrations twice as high as ADL in grasses, but not in legumes. This had already been reported by Kondo et al., (1987), who declared that this effect was stronger on grasses.

The alternative method acetyl bromide lignin (ABL) calculates the concentration of this compound by spectrophotometry at UV 280 nm. The lignin values obtained by this method have presented strong correlation with IVDMD and in vitro NDF digestibility in studies conducted by our research team. Thus, it seems interesting to use ABL for estimating DMD, FO and DMI.

Another compound with potential for being used as an internal marker is cutin. Cutin is a polymer of esterified fatty acids linked to the aerial parts (leaves, fruits and stems) of some plants. Silva et al., (2006) used it as an internal marker to calculate digestibility of nutritional components in supplements for cattle, and their results indicated potential for this use. Siqueira et al., (2009) used cutin as a marker to estimate apparent digestibility in horses and observed that FR differed ($P < 0.05$) from FR of ADL and Cr_2O_3 . Studies that have used cutin as a marker are rare.

External markers

External markers can be used to determine DMD, FO and other intake measurements when feces sample collection is not a practical option. In this category, Cr_2O_3 has been widely studied for estimating FO due to easiness of incorporating it into diets, relatively low cost and simplicity of analysis (VALADARES FILHO et al., 2006). Titgemeyer (1997) under extensive review, verified that FR was not complete, especially on pasture. On the other hand, Paixão et al., (2007) reported inert passage through the GIT and complete FR of the administered dose, without significant difference between 1 and 5 sampling days. Penning (2004) review literature data and concluded that for cattle, the recommended daily dose of Cr_2O_3 was between 5 to 10g, divided into two doses administered with intervals between 8 and 16 hours, concomitant with feces sampling. Prigge et al., (1981) used Cr_2O_3 to estimate FO and compared it to TFC in confined dairy cows. The authors concluded that Cr_2O_3 given in two daily doses of 5g each, for twelve days (7 days for adaptation and 5 days for sampling) was an efficient procedure to estimate FO.

As an alternative to Cr_2O_3 , TiO_2 which was approved for legal use as a coloring additive for supplements, (AAFCO, 1996), may be used as an external marker. During the last decade, TiO_2

has received more attention by researchers and therefore it is important to collect information that can allow progress to be made in the use of this external marker in ruminant studies.

Other markers such as Lithium (LiCl), Ytterbium (Yb), LIPE® (isolated, purified and enriched *Eucalyptus grandis* lignin), and more recently alkanes, have all been subject of study. Results are not conclusive and more research is necessary. Some of the results obtained from alkanes are promising but the extremely high cost of this analysis is still the main limiting factor for wider use.

Chapter 1.

Evaluating internal and external markers versus fecal sampling procedure interactions when estimating intake in dairy cows consuming a corn silage-based diet

Introduction

Dry matter intake (DMI) determines nutrient intake and may be an index of the efficiency with which nutrients are metabolized, therefore it is the main factor influencing animal performance. Many different strategies have been used over the years to estimate voluntary DMI in ruminants. Behavioral studies (time and rate of intake, mass of bite) (SILVA *et al.*, 2004; COSTA *et al.*, 2011), before and after grazing pasture measurements (POPPI *et al.*, 1987) and multiple regression equations that consider different variables believed to affect intake, have been commonly used approaches.

The use of markers is currently the most widely used technique to estimate DMI in ruminants. Markers are indigestible substances that are not secreted by the animal, have passage rates similar to feeds, can be recovered completely after ingestion and allow for practical and precise chemical analysis (FAHEY and JUNG, 1983), among others characteristics. According to Merchen (1993), none of the substances that are used as markers in ruminant digestion studies meet all of the requirements but, some are adequate enough to produce important data for the advance of research.

The marker technique to estimate intake uses an external marker to estimate fecal output (FO) and an internal marker (naturally occurring in feedstuffs) to estimate dry matter digestibility (DMD). Intake is then calculated by dividing FO by the indigestibility ($1 - \text{DMD}$) of the feed. The main criticisms to the technique are the difficulty and labor of feeding the external markers twice a day, for several days; the need for excessive interaction between animals and people which may negatively affect intake and the intensive labor and high cost of feces sample collection.

Total feces collection (TFC) is regularly used in intake studies as the standard to which estimates are compared to and as the source of BULK samples for later analysis. The recommended number of days for TFC ranges from 7-10 days (SCHNEIDER & FLATT, 1975) to 3-5 days (FERREIRA *et al.*, 2008), with authors reporting no significant differences for digestibility estimates. Just as number of days for TFC can be reduced, different fecal sampling designs have been proposed in order to reduce the short term bias of estimates (LANGLANDS *et al.*, 1963), which accounts for the inconsistencies of obtaining the feces samples. In this approach, GRAB samples collected at specific times during the day, either after spontaneous excretion or collected directly from the rectum, are composited to make up the daily samples. Sampaio *et al.*, (2011) compared 9 different fecal sampling designs and reported that four daily GRAB samples distributed along the whole day or just during the diurnal period will produce accurate estimates.

From the above, it can be hypothesized that the combination of internal and external markers, with fecal sampling designs that produce composite GRAB samples over 3-4 days of collection period, should result in accurate estimates for DMD, FO and DMI in dairy cows consuming a corn silage based diet.

The objective of the experiment was to test the accuracy of internal markers, acetyl bromide lignin (ABL), cutin, indigestible neutral detergent fiber (iNDF) and indigestible acid detergent fiber (iADF) for estimating DMD; of external markers, chromic oxide (Cr_2O_3) and titanium dioxide (TiO_2) for estimating FO; the combination of internal and external markers for estimating DMI and to compare 3 different sampling designs against TFC.

Material and Methods

The experiment was conducted at the Dairy Cattle Research Laboratory of the Animal Nutrition and Production Department at the University of São Paulo. The experiment was approved by the Ethics Committee for Animal Use (CEUA) of the University of São Paulo (Approval number: CEUA 24420603114). No animals were harmed or fell ill during the experiment.

Animals, diet and experimental design

Ten multiparous Holstein cows (average $\pm$ SD; parity: 1.2 ± 0.4 lactations; Days in milk: 123 ± 53.7 d; milk yield; 25.5 ± 3.8 kg/d; BW: 616.7 ± 67.2 kg), were used for the study. The mouth, tongue and teeth of all cows were examined prior to starting the experiment in order to guarantee absence of wounds or abnormalities that could compromise feed intake. Cows were housed in a covered, concrete floor free-stall, divided by gates into individual stalls, each of which had a feed bunk, access to clean water and a sand covered bed. Cows were milked twice a day (07:00 and 15:00) and milk yield was recorded during the experimental period.

All cows received a corn silage-based diet, with 55:45 forage to concentrate ratio (F:C). Daily allowances of the total mixed ration (TMR), plus 10% of calculated daily natural matter intake (to guarantee orts), were fed in two equal portions at 08:00 and 16:00, to represent a natural diurnal feeding pattern of cattle. Feed subsamples were taken on Mondays, Wednesdays and Fridays for dry matter (DM) determination (drying in a forced draft oven (SOLAB Cientifica®) at 55°C for 3 days). Orts were collected daily so that *ad libitum* intake could be determined as the difference between the DM offered and refused. The concentrate was composed of coarsely-ground corn grains, soybean meal and whole roasted soybeans, cottonseed meal and mineral mix. Cottonseed meal was added to replace part of the soybean meal, due to its high contents of cutin (from added cotton hulls). Increasing the cutin concentration of the diet was desirable in order to facilitate analysis and decrease variability of results. Composition of silage, concentrate and TMR are shown in Table 2.

The experiment ran for 19 days (day regarded as the 24 h period from 8am to 8 am). The experimental period consisted of three phases: d1-d5 were allocated to adaptation of the animals to the diet; ten days (d5-d15) of marker excretion stabilization and the final 4 days (d16-19) were for sample collection. The animals were adapted to the experimental conditions during 14 days prior to the experimental period. The 10 cows were distributed into a Split-plot design, with the main plot (markers) being completely randomized and the sampling designs as sub-plots.

Markers

Four internal markers (ABL, cutin, iNDF and iADF) were used to estimate DMD and two external markers (Cr_2O_3 and TiO_2) were used to estimate FO. The eight combinations (1 internal marker and 1 external marker) derived from these six markers were used to estimate voluntary DMI.

Estimates of DMD, FO and DMI were compared against their reciprocal TTAD, RFO and RDMI values, respectively.

Internal markers are naturally occurring indigestible fractions of feedstuffs. External markers, in this case Cr_2O_3 and TiO_2 , need to be fed to the animals exogenously, be it orally or via ruminal cannula. For the experiment, capsules containing 0.75 g of Cr_2O_3 or 1 g of TiO_2 were prepared at a local pharmacy. Cows received 15 g of Cr_2O_3 and 15 g of TiO_2 daily. This dose was divided into two equal parts and the animals were fed the capsules (mixed with a small amount of concentrate and molasses) before receiving the rest of the meal (08:00 and 16:00). The animals were dosed from d5 to d18 of the experimental period and had been receiving molasses with concentrate before each meal during the 14 days prior to the start of the experiment.

Feces

On d15, all stalls were thoroughly cleaned (pressure hosed). On d16 at 06:00 stalls were cleaned of what was excreted overnight and at 08:00 the 72 h TFC period started. Two people teams constantly watched the animals, in 8 hour shifts, and collected all feces immediately after excretion to avoid contamination with bedding sand or partial loss of material. Cows were slowly and carefully followed to the milking parlor to collect any fecal matter excreted during this procedure. Collected feces were placed into individual (1 per cow) plastic containers (previously weighed empty) which were kept sealed and away from direct sunlight. At 07:30 of d17 to d19 the containers were weighed and the daily RFO recorded for each animal. Fecal matter in each container was homogenized (mixed thoroughly with a ladle) and an approximately 500 g sample was taken for each animal daily, during the sampling period. These BULK samples constituted the 72-hour continuous sampling design.

Two GRAB sampling designs were evaluated during this study. Sampaio *et al.*, (2011b), compared 9 different sampling design protocols and reported that 4 samples collected during daylight hours were enough to obtain a representative sample of the daily excretion profile. A commonly used design in marker studies is the 3-day sampling period with samples being taken every 9 hours. This way, at the end of the three days, the 24 hours of a day will be represented by the various samples. Table 1 illustrates the fecal sampling design protocols used in this study.

Table 1. Sampling design protocols for BULK and GRAB feces samples*Figure 1*

Design	Sampling	Duration – Sampling Times
A	BULK 72h	72 hours (d16-d18) - Continuous
B	GRAB 9x3	3 days - d16 = 8:00, 17:00, 2:00; d17 = 11:00, 20:00, 5:00; d18 = 14:00, 23:00, 8:00
C	GRAB 4x4 Daylight	4 days (d16-d19) - 8:00, 11:00, 14:00, 17:00

Chemical analysis and measurements

For quantification of voluntary intake, feed supplied and feces collected between d15-d18, and orts collected between d16-d19 were measured. Composite samples were elaborated per animal and frozen at -20°C. Later, feces, feed and ort samples were thawed at room temperature, oven-dried at 55°C and processed in a MA-048 knife mill (MARCONI®, São Paulo, Brazil), with a 2 mm mesh screen. Preference was given to the 2 mm screen because, according to Froidmont *et al.*, (2008), fine grinding can cause loss of small particles (containing lignin and cutin) which would have a significant impact on our expected results.

Feed and TMR samples were analyzed for natural dry-matter (DM) and mineral matter (MM) by incineration in a muffle furnace (MARCONI®, São Paulo, Brazil). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the fiber bag procedure (VAN SOEST, ROBERTSON & LEWIS, 1991), with addition of heat-stable amylase and without sodium sulfite (MERTENS, 2002), in a TE-149 Fiber Analyzer (TECNAL®, São Paulo, Brazil).

Cell wall (CW) preparation was obtained according to the procedure proposed by Fukushima and Hatfield (2001) and later used as substrate for acetyl bromide lignin (ABL) determination in feed, orts and feces samples, according to Fukushima and Kerley (2011). Lignin absorbance was determined with a Libra S80 spectrophotometer (BIOCHROM®; United Kingdom) at a wavelength of 280 nm. Soluble fiber was calculated as the difference between CW and NDF contents, composed mainly of pectin. Indigestible neutral detergent fiber and indigestible acid detergent fiber (iNDF and iADF, respectively) were obtained by weighing silage and feces (0.5 g), and concentrate (1.0 g) samples into F57 bags (ANKOM Technology Corp.®, Fairport, NY). Samples were incubated inside the rumen of a cannulated cow for 288 hours (CASALI *et al.*,

2008; KRIZAN and HUHTANEN, 2013), washed with abundant water and treated according to Van Soest and Robertson (1985) for determination of these indigestible fractions.

Cutin contents in feed, TMR and feces samples were determined in non-woven textile (NWT) fiber bags according to the procedure described by Fukushima (personal communication). Briefly, dry matter samples are sequentially treated with neutral detergent and acid detergent solutions to obtain an acid detergent residue (ADR). Fiber bags containing ADR were treated with potassium permanganate solution (KMnO_4) in order to oxidize lignin and afterwards treated with 72% sulfuric acid solution (H_2SO_4) to hydrolyze cellulose, leaving behind cutin and mineral residue. Bags were dried in a forced draft oven at 105°C for 12 hours and weighed. Finally, the residue containing bags are incinerated at 450°C in a muffle furnace and cutin contents is calculated as:

$$\text{Cutin\%} = ((\text{WaH} - (\text{Wal} - \text{Wc}) \times 100) \div \text{SiW})$$

where: WaH = weight after sulfuric acid hydrolysis, Wal = weight after incineration, Wc = weight of crucible and SiW = sample initial weight.

Crude protein ($\text{N\%} \times 6.25$) was determined in feed and TMR samples by the micro-kjeldahl procedure in a TE 036/1 Nitrogen Distiller (TECNAL®, São Paulo, Brazil). Ether extract (Ee) content of feeds and TMR was determined by petroleum ether extraction using a XT10 Extractor (ANKOM Technology Corp.®, Fairport, NY).

The quantification of Cr_2O_3 in feces samples was performed by visible light spectrophotometry (550 nm) according to the procedure proposed by Kimura *et al.*, (1957) with the modifications proposed by Graner (1972). Standard samples containing 0, 2, 4, 6, 8 and 10 mg of chromium per g DM were analyzed in triplicate, totalizing 18 standard samples. Pure Cr_2O_3 (99% trace metals basis; Solumix®) was employed to produce the standards. The concentration of Cr_2O_3 was analyzed and compared with the added amount of Cr_2O_3 . The results of the regression are shown in Figure 1.

The quantification of TiO_2 in feces samples was performed by visible light spectrophotometry (410 nm) according to Myers *et al.*, (2004). To validate the accuracy of the TiO_2 analysis, a regression equation was created using the method proposed by Glindemann *et al.*, (2009). Standard samples containing 0, 1, 2, 3, 4, 5 and 6 mg of titanium per g DM were analyzed in triplicate, totalizing 21 standard samples. Pure TiO_2 (99.3% trace metals basis; Dinâmica®

#1093) was employed to produce the standards. The concentration of TiO_2 was analyzed and compared with the added amount of TiO_2 . The results of the regression are shown in Figure 2.

Figure 1. Relationship between Cr_2O_3 added and Cr_2O_3 analyzed

Figure 2. Relationship between TiO_2 added and TiO_2 analyzed

Calculations

Real DMI (RDMI) was calculated as daily feed offer minus daily orts (Equation 1) and Real FO (RFO) was obtained by 3 days (d16-d18) of TFC (24-hour collection and daily weighing of amount excreted). Total-tract apparent digestibility (TTAD) was calculated as RDMI minus RFO (Equation 2):

$$\text{RDMI(g/day)} = \text{daily feed offered(g/day)} - \text{orts collected(g/day)} \quad (1)$$

$$\text{TTAD (g/kg DM)} = \frac{\left[\text{RDMI}\left(\frac{\text{g}}{\text{day}}\right) - \text{RFO}\left(\frac{\text{g}}{\text{day}}\right) \right] \times 1000}{\text{RDMI}} \quad (2)$$

Equations (3) and (4) were used to estimate marker excretion and recovery rate. These equations derived from both BULK and GRAB samples. The overall recovery rate of markers was calculated from the total amount of marker given and the amount recovered during the fecal sampling period (d15-d19). Daily recovery rate of markers was calculated from d16 to d19:

$$\text{marker excretion}\left(\frac{\text{g}}{\text{day}}\right) = [\text{fecalmarker}] \left(\frac{\text{g}}{\text{g}}\right) \times \text{RFO}\left(\frac{\text{g}}{\text{day}}\right) \quad (3)$$

$$\text{recovery rate of the marker} = \frac{\text{marker excreted}\left(\frac{\text{g}}{\text{day}}\right)}{\text{marker ingested}\left(\frac{\text{g}}{\text{day}}\right)} \quad (4)$$

Equation (5) was used to estimate FO using external markers and Equation (6) was used to estimate DMD using internal markers:

$$\text{FO estimated by external marker}\left(\frac{\text{g DM}}{\text{day}}\right) = \frac{\text{daily marker dose}\left(\frac{\text{g}}{\text{day}}\right)}{[\text{fecalmarker}]\left(\frac{\text{g}}{\text{g DM}}\right)} \quad (5)$$

$$\text{DMD estimated by internal marker}\left(\frac{\text{g}}{\text{kg DM}}\right) = 100 - \left(100 \times \left(\frac{[\text{marker in feed}]\left(\frac{\text{g}}{\text{g}}\right)}{[\text{fecalmarker}]\left(\frac{\text{g}}{\text{g DM}}\right)}\right)\right) \quad (6)$$

Equation (7) was used to estimate total voluntary intake from FO and DMD estimates obtained by equations (5) and (6). Total DMI was estimated for both BULK and GRAB samples:

$$\text{DMI estimated by markers}\left(\frac{\text{g}}{\text{day}}\right) = \frac{\text{FO(kg}\frac{\text{DM}}{\text{day}}\text{)}}{1 - \text{diet DMD}} \quad (7)$$

Statistical Analysis

Results were analyzed with SAS® 9.3 (SAS®, 2011), after verifying normality of residuals and homogeneity of variance. With normally distributed TTAD values and DMD estimates, a 4x4

factorial arrangement was adopted (4 markers and 4 sampling procedures) and data was analyzed using PROC-MIXED, at 0.05 significance level, according to the model:

$$Y_{ijk} = \mu + T_i + M_j + TM_{ij} + A(T)_k + \mathcal{E}_{ijk},$$

where Y_{ijk} = is the DMD estimated in animal k, by marker i, under sampling procedure j; μ = is the general constant; T_i = is the fixed effect of internal marker; M_j = is the fixed effect of sampling procedure; TM_{ij} = is the interaction between internal marker i and sampling procedure j (fixed); $A(T)_k$ = is the random effect of animal k nested to marker i; and $\mathcal{E}_{ijk}$ is the sampling error supposed to be normal and independently distributed.

After verifying normal distribution of RFO values and FO estimates, a 2x4 factorial arrangement was adopted (2 markers and 4 sampling procedures) and data was analyzed using PROC-MIXED, at 0.05 significance level, according to the model:

$$Y_{xyz} = \mu + T_x + M_y + TM_{xy} + A(T)_z + \mathcal{E}_{xyz},$$

where Y_{xyz} = is the FO estimated in animal z, by marker x, under sampling procedure y; μ = is the general constant; T_x = is the fixed effect of external marker; M_y = is the fixed effect of sampling procedure; TM_{xy} = is the interaction between external marker x and sampling procedure y (fixed); $A(T)_z$ = is the random effect of animal z nested to marker x; and $\mathcal{E}_{xyz}$ is the sampling error supposed to be normal and independently distributed.

Values of RDML and DML estimates were checked for normal distribution and an 8x4 factorial arrangement was adopted (8 marker pairs (1 internal + 1 external) and 4 sampling procedures). Data was analyzed using PROC-MIXED, at 0.05 significance level, according to the model:

$$Y_{bcd} = \mu + T_b + M_c + TM_{bc} + A(T)_d + \mathcal{E}_{bcd},$$

where Y_{bcd} = is the DML estimated in animal d, by marker b, under sampling procedure c; μ = is the general constant; T_b = is the fixed effect of marker pair; M_c = is the fixed effect of sampling procedure; TM_{bc} = is the interaction between marker pair b and sampling procedure c (fixed); $A(T)_d$ = is the random effect of animal d nested to marker b; and $\mathcal{E}_{bcd}$ is the sampling error supposed to be normal and independently distributed.

Linear regression analysis and Student's paired t-test were used to compare estimates against real observed values (those derived from TFC) for all variables being studied.

Results

Chemical composition of feeds and TMR diets are shown in Table 2. Mean RDMI (average $\pm$ SD) was 24.1 ± 4.0 kg/animal/day, equivalent to $3.9 \pm 0.4\%$ of BW. Mean RFO (average $\pm$ SD) was 6.9 ± 1.1 kg DM/animal/day, equivalent to $1.1 \pm 0.2\%$ of BW. The chemical composition of the feeds and TMR were in accordance to the diet formulated for the experiment. The values for TTAD (average $\pm$ SD) 710.7 ± 41.1 g/kg DM were higher than expected, as were those for Ee (average $\pm$ SD) $9.4 \pm 0.4\%$ DM, considering the average quality of the corn silage which composed 55% of the diet.

Fecal recovery of markers

Mean fecal recovery (FR) of markers varied widely among them and are shown in Table 3. A highly significant effect for marker ($P < .0001$) was observed. No effect for sampling procedure ($P = 0.325$) or for the marker $\times$ sampling procedure interaction ($P = 0.126$) was detected. Cutin produced the lowest FR results among all the markers, always well under 100% for the 3 sampling procedures. Oppositely, TiO_2 FR was unexpectedly high (around 200%) independently of the sampling procedure. Indigestible NDF and ADF produced similar FR, around 150% for the 24-hour period and ABL had a FR around 100% similar in all sampling procedures. The FR of Cr_2O_3 differed ($P = 0.044$) between the 4x4 and 9x3 GRAB sampling procedures, but neither one was different ($P = 0.344$ and $P = 0.285$, respectively) from FR of the 72h sampling procedure.

Table 2. Chemical composition of feeds and total mixed ration (TMR), total-tract apparent digestibility (TTAD), real fecal output (RFO) and real dry matter intake (RDMI) of TMR

	Silage	Concentrate	TMR
DM ¹ (g/kg)	288.4	822.4	528.7
MM ² (g/kg DM)	43.0	56.9	49.3
CP ³ (g/kg DM)	94.0	265.9	171.4
Ee ⁴ (g/kg DM)	66.0	127.9	93.9
NDF ⁵ (g/kg DM)	560.0	457.6	535.7
CW ⁶ (g/kg DM)	736.9	763.9	749.1
SF ⁷ (g/kg DM)	137.4	306.3	213.4
ADF ⁸ (g/kg DM)	348.1	97.7	235.5
iNDF ⁹ (g/kg DM)	247.2	29.6	155.7
iADF ¹⁰ (g/kg DM)	160.0	20.0	99.6
ABL ¹¹ (g/kg DM)	95.8	13.0	58.5
Cutin (g/kg DM)	3.5	7.6	5.3
TTAD (g/kg DM)	-	-	710.7
RFO (g DM/day)	-	-	6900.3
RDMI (g DM/day)	-	-	24078.1

¹ dry matter; ² mineral matter; ³ crude protein (N% $\times$ 6.25); ⁴ ether extract; ⁵ neutral detergent soluble fiber; ⁶ cell wall; ⁷ SF = CW – NDF; ⁸ acid detergent soluble fiber; ⁹ indigestible neutral detergent soluble fiber; ¹⁰ indigestible acid detergent soluble fiber; ¹¹ acetyl bromide lignin

Table 3. Twenty-four hour fecal recovery (FR) (mean $\pm$ SE) of markers under two GRAB (4x4 and 9x3) and one BULK (72h) fecal sampling procedures

Marker	Method		
	4x4 GRAB	9x3 GRAB	72h BULK
ABL	0.98 ^{cd} $\pm$ 0.09	0.95 ^d $\pm$ 0.08	1.11 ^c $\pm$ 0.13
Cutin	0.81 ^d $\pm$ 0.14	0.67 ^e $\pm$ 0.09	0.80 ^d $\pm$ 0.11
iADF	1.47 ^b $\pm$ 0.08	1.55 ^b $\pm$ 0.08	1.60 ^b $\pm$ 0.08
iNDF	1.43 ^b $\pm$ 0.07	1.50 ^b $\pm$ 0.07	1.53 ^b $\pm$ 0.08
Cr ₂ O ₃	1.01 ^{Bc} $\pm$ 0.06	1.23 ^{Ac} $\pm$ 0.06	1.12 ^{ABc} $\pm$ 0.06
TiO ₂	1.95 ^a $\pm$ 0.16	1.99 ^a $\pm$ 0.14	1.83 ^a $\pm$ 0.14

Within a line, mean values with common upper case superscript are not significantly different by Fisher's LSD ($P < 0.05$). Within a column, mean values with common lower case superscript are not significantly different by Fisher's LSD ($P < 0.05$).

Prediction of DMD based on fecal concentration of internal markers

The mean DMD estimates are shown in Table 4. Significant effects for marker, sampling procedure and a tendency on their interaction were observed ($P < .0001$, $P = 0.003$ and $P = 0.060$, respectively). Estimates of DMD from iADF and iNDF were not significantly different from TTAD, independently of the sampling procedure. Cutin and ABL estimates of DMD were only accurate under the 72h sampling procedure ($P = 0.479$ and $P = 0.654$, respectively).

Table 4. Mean total tract apparent digestibility (TTAD) (g/kg DM) and dry matter digestibility (DMD) (g/kg DM) estimates derived from internal markers on two GRAB (4x4 and 9x3) and one BULK (72h) fecal sampling procedures

Marker	Sampling Procedure			
	4x4 (GRAB)	9x3 (GRAB)	72h (BULK)	TTAD
ABL	660.6 ^{Bab} $\pm$ 20.8	663.5 ^{Bb} $\pm$ 17.0	698.9 ^{AB} $\pm$ 17.5	710.7 ^A $\pm$ 6.6
Cutin	621.1 ^{Bb} $\pm$ 38.3	572.5 ^{Bc} $\pm$ 39.0	690.2 ^A $\pm$ 33.9	710.7 ^A $\pm$ 6.6
iADF	703.8 ^a $\pm$ 13.7	720.9 ^a $\pm$ 11.7	728.6 $\pm$ 12.2	710.7 $\pm$ 6.6
iNDF	710.5 ^a $\pm$ 12.7	726.0 ^a $\pm$ 10.6	728.5 $\pm$ 11.7	710.7 $\pm$ 6.6

Within a line, mean values with common upper case superscript are not significantly different by Fisher's LSD ($P < 0.05$). Within a column, mean values with common lower case superscript are not significantly different by Fisher's LSD ($P < 0.05$).

Prediction of FO based on fecal concentration of external markers

The mean FO estimates are shown in Table 5. Significant effects for marker, sampling procedure and their interaction were observed ($P < .0001$, $P = 0.002$ and $P = 0.006$, respectively). Estimates for FO produced by TiO₂ were not accurate when compared to RFO. Estimates of FO from Cr₂O₃ concentrations were only accurate for the 9x3 and 72h fecal sampling procedures ($P = 0.206$, $P = 0.306$, respectively).

Table 5. Mean real fecal output (RFO) (g DM/day) and fecal output (FO) (g DM/day) estimates derived from external markers on two GRAB (4x4 and 9x3) and one BULK (72h) fecal sampling procedures

Marker	Sampling Procedure			
	4x4 GRAB	9x3 GRAB	72h BULK	RFO
Cr ₂ O ₃	7971.0 ^{Aa} ± 286.2	6455.1 ^{Ca} ± 208.3	7260.1 ^{Ba} ± 293.3	6900.3 ^{BC} ± 204.9
TiO ₂	5015.3 ^{Bb} ± 223.3	4823.8 ^{Bb} ± 202.2	5403.6 ^{Bb} ± 307.5	6900.3 ^A ± 204.9

Within a line, mean values with common upper case superscript are not significantly different by Fisher's LSD ($P < 0.05$). Within a column, mean values with common lower case superscript are not significantly different by Fisher's LSD ($P < 0.05$).

Prediction of DMI based on internal and external marker pairs

The mean DMI estimates are shown in Table 6. Highly significant effects for marker and sampling procedure ($P < .0001$) were observed, yet no effect ($P = 0.447$) for their interaction was detected. Most DMI estimates from TiO₂ paired with any of the internal markers differed significantly from RDMI. When TiO₂ was paired with iNDF and iADF, under the 72h sampling procedure, estimates were accurate ($P = 0.145$ and $P = 0.146$, respectively), and the same was observed when it was paired with cutin under the 4x4 sampling procedure ($P = 0.080$). Estimates of DMI from Cr₂O₃ paired with any of the internal markers were mostly accurate. When paired with iADF, DMI estimates from Cr₂O₃ did not differ from RDMI independently of the sampling procedure used.

Table 6. Mean real dry matter intake (RDMI) (g DM/day) and dry matter intake (DMI) (g DM/day) estimates derived from 1 internal + 1 external marker pairs on two GRAB (4x4 and 9x3) and one BULK (72h) fecal sampling procedures

Ext. marker Int. marker	Sampling Procedure			
	4x4 GRAB	9x3 GRAB	72h BULK	RDMI
Cr ₂ O ₃				
ABL	26381.0 ^A ± 1815.8	21520.6 ^B ± 1881.3	26585.8 ^A ± 1724.4	24078.1 ^{AB} ± 633.8
Cutin	28558.8 ^A ± 4496.9	19038.7 ^B ± 1978.1	25344.5 ^A ± 3839.0	24078.1 ^A ± 633.8
iADF	28258.8 ± 1394.4	24435.0 ± 1423.4	28116.2 ± 1634.1	24078.1 ± 633.8
iNDF	28711.9 ^A ± 1339.8	24626.1 ^{AB} ± 1334.6	28045.7 ^{AB} ± 1621.2	24078.1 ^B ± 633.8
TiO ₂				
ABL	16656.0 ^B ± 1231.3	15706.8 ^B ± 1126.3	19726.3 ^{AB} ± 1397.6	24078.1 ^A ± 633.8
Cutin	19661.2 ^{AB} ± 3764.6	13798.8 ^C ± 1461.4	17244.7 ^{BC} ± 1951.2	24078.1 ^A ± 633.8
iADF	17793.4 ^B ± 984.8	17966.7 ^B ± 896.3	20410.7 ^{AB} ± 1111.3	24078.1 ^A ± 633.8
iNDF	18157.5 ^B ± 1007.7	18170.0 ^B ± 852.7	20406.0 ^{AB} ± 1125.7	24078.1 ^A ± 633.8

Within a line, mean values with common upper case superscript are not significantly different by Fisher's LSD ($P < 0.05$).

Discussion

Fecal recovery of markers

Fecal recovery varied widely among markers and ranged from 0.67-0.81 g/g DM for cutin to 1.83-1.99 g/g DM for TiO_2 . No reports were found on the use of cutin as a marker in ruminant digestion studies and therefore our data is novel to this field of research. Siqueira *et al.*, (2009) used cutin as DMD marker in horses consuming four different diets and observed FR around 1.51 g/g DM. The FR in horses was almost double of what our study observed for ruminants. Obvious and known differences in digestive anatomy and physiology of both species could account, at least partially, for these FR differences. Liquid and solid phase stratification of contents in the rumen compared to the herbivore monogastric stomach are totally different. The differences in these phases will directly influence how the cutin particle interacts and binds to digesta around it. This interaction will in turn, determine the retention time (T_R) and passage rate (Q_P) of cutin and ultimately affect what fraction of ingested cutin will be excreted in feces over a 24-hour period. Njaa (1961) reported that mean FR less than one could be attributed to inaccuracy of the analysis. Since cutin makes up such a small fraction of DM, gravimetric errors tend to be more likely to happen.

Several authors (KOTB & LUCKEY, 1972; FAHEY & JUNG, 1983) have long described what the properties of the ideal marker should be. Among these properties, one of the most important ones is that the daily ingested/dosed amount of marker can be totally recovered in feces within a 24-hour period. Many studies have been conducted over the last 3 decades using TiO_2 and the FR observed for it has been around 100%. Based on these findings the routinary use of TiO_2 in ruminal digestion trails has been suggested. Our findings do not support the use of TiO_2 as an external marker to estimate FO in ruminants, since we observed FR that ranged from 1.83 to 1.99 g/g DM. These findings were contrary to those of Brandt and Allam, (1987); Hafez *et al.*, (1988) and Titgemeyer *et al.*, (2001), who observed mean FR for TiO_2 of 0.96-0.99 in sheep, 0.96-1.02 and 0.90-0.95 in dairy cattle, respectively. Glindemann *et al.*, (2009) found FR higher than 1 and attributed this to possible ingestion of soil which contained TiO_2 and therefore would increase the fecal concentration. Our diet was composed of corn silage and concentrate, the animals were kept in stalls with concrete flooring, so the risk of soil contamination is low.

Mean FR of Cr_2O_3 varied from 1.01 to 1.23 g/g DM in this study. Benvenuti *et al.*, (2014) worked with Brahman steers fed leafy or steamy grass hay and observed FR of 0.92 to 1.03 g/g DM. Since Cr_2O_3 has been widely used as a marker in digestions studies, various FR have been reported for it: in horses FR ranged from 0.712 to 1.031 g/g DM (OLIVEIRA *et al.*, 2003;

SIQUEIRA *et al.*, 2009; LANZETTA *et al.*, 2009), in sheep from 0.80 to 0.92 g/g DM (RODRIGUES *et al.*, 2010), in dairy cattle from 0.87 to 0.89 g/g DM (DELAGARDE *et al.*, 2000) and in steers from 0.85 to 1.05 g/g DM (SAMPAIO *et al.*, 2011). Paixão *et al.*, (2007) fed Cr_2O_3 to steers and observed total fecal recovery of the administered dose. Our results are in accordance to their findings and suggest that Cr_2O_3 is an appropriate external marker for estimating FO in cattle.

Lignin, determined as acid detergent lignin (ADL), has been used as an internal marker for digestibility studies and is considered indigestible because there are no microbial or mammalian enzymes that can degrade it (VAN SOEST, 1994). The ADL method has received criticism over the years because it can measure cutin and Maillard's compounds as being lignin. Additionally, this method has also been criticized because of lignin solubilization or destruction during hydrolysis with 72% H_2SO_4 solution. According to Lowry *et al.*, (1994) this loss of lignin during analysis can be upwards of 50% in some tropical grasses. Because of this lignin had not been extensively used as an internal marker and the few published reports, account for under estimation of DMD. Goachet *et al.*, (2009) used ADL to estimate digestibility coefficients in horses and observed FR of 0.90 and 0.86 g/g DM for 3 and 4 days of fecal sampling, respectively. This study measured lignin as ABL, a procedure in which no lignin loss occurs during sample preparation and that is less subject to gravimetric errors because of its spectrophotometric nature. Mean ABL FR ranged from 0.95 to 1.11 g/g DM in our study, with the lower values corresponding to GRAB sampling procedures. These results suggest that fecal recovery of lignin is complete when measured by ABL and that it is an appropriate internal marker for estimating DMD in cattle. Our findings also support the observations of the above-mentioned authors that, when ADL is used, loss of lignin during ADF preparation (LOWRY *et al.*, 1994) alters FR results and subsequent calculations.

Indigestible neutral detergent and acid detergent fibers are two of the most extensively used internal markers today. Mean FR ranging from 0.50 to 1.21 (BARROS *et al.*, 2009; KOZLOSKY *et al.*, 2009; RODRIGUES *et al.*, 2010; SAMPAIO *et al.*, 2011a and, LEE and HRISTOV, 2013) and from 0.80 to 1.21 g/g DM (OLIVEIRA Jr. *et al.*, 2004; RODRIGUES *et al.*, 2010; SAMPAIO *et al.*, 2011a) for iNDF and iADF, respectively. Oliveira *et al.*, (2003) worked with horses and observed FR of 1.08 to 1.34 g/g DM and up to 1.54 g/g DM for iNDF and iADF, respectively. In this study, mean FR for iNDF and iADF were 1.43 to 1.53 g/g DM and 1.47 to 1.60 g/g DM, respectively. These results were higher than expected and partially agree with previous findings of other authors. The disparity of results for different experiments is, at least in part, due to

differences in incubation time of the samples for obtaining the fibrous fractions. Our study followed the procedure proposed by Krizsan & Huhtanen (2013) who compared various incubation times (144h, 240h and 288h) and reported that at least 12 days of incubation were necessary to isolate the completely indigestible fiber pool in feeds fed to dairy cows. The results in this study suggest that iNDF and iADF would not be adequate internal markers for ruminant digestion studies, because higher than 1 FR lead to underestimation of DMD.

Prediction of DMD based on fecal concentration of internal markers

Values for DMD were under-estimated and differed significantly from TTAD when GRAB sampling procedures and ABL or cutin were used. When fecal concentration was measured in BULK samples, both markers produced accurate DMD estimates in comparison to TTAD. Cutin is not a part of lignin but it is measured as such by the acid detergent lignin method. A possible explanation for the low DMD estimates produced by these two markers under the GRAB sampling procedures would be their low concentration in both diet and feces samples. Lower concentrations make chemical analysis less reliable because of increased difficulty to detect differences in such small amounts of residue that are left behind for weighing after the procedures are performed. In this study, lignin was measured as ABL and thus gravimetric related issues would not be a problem. The cutin procedure that is being proposed is gravimetric and subject to these limitations. When BULK sampling was used, both markers produced accurate DMD estimates. From our observations, neither ABL or cutin could be suggested as accurate internal makers for determination of DMD in dairy cows if GRAB sampling procedures were to be used. With the use of BULK sampling procedures, these two markers deserve further attention to better understand how they behave. From what we know after researching literature of the subject, nor ABL or cutin had ever been used in ruminant digestion studies and therefore more research is necessary before discarding them as possible markers.

Mean DMD estimates produced by iNDF and iADF fecal concentrations did not differ from TTAD under any of the sampling procedures. Estimates were not different between the two indigestible fractions when compared within the same sampling procedure, evidencing the close relationship between both fibrous fractions. Using animals on pasture, Detmann *et al.*, (2001) reported more accurate DMD estimates when using iNDF compared to iADF. Berchielli *et al.*, (2005) reported conflicting results when using these indigestible fractions, in response to the incubation procedure as was mentioned before when discussing FR. With heifers fed fresh sugar-cane iNDF produced better DMD estimates than iADF but, when corn silage was fed to

dairy cows, iADF estimates were more accurate than those of iNDF (FERREIRA *et al.*, 2008). Our findings suggest that both iNDF and iADF are suitable internal markers for estimating DMD.

Prediction of FO based on fecal concentration of external markers

Both Cr_2O_3 and TiO_2 were unable to produce accurate FO estimates when compared to RFO values. Only under the 72h BULK sampling procedure, Cr_2O_3 estimates did not significantly differ ($P=0.306$) from RFO, with a mean estimate bias (residual error) of 5%. Fecal recovery of Cr_2O_3 , under the 4x4 GRAB sampling procedure, was 100% and therefore, over-estimated FO was unexpected. No consistency was observed for Cr_2O_3 estimates as values were over-estimated when 4x4 GRAB and 72h BULK sampling was used but under-estimated under 9x3 GRAB sampling procedure. When comparing Cr_2O_3 and TiO_2 in steers, Titgemeyer *et al.*, (2001) observed that both markers significantly underestimated FO. Delagarde *et al.*, (2000) reported accurate FO estimates, derived both from BULK and GRAB sampling procedures, positively correlated to RFO (R^2 from 0.86 to 0.96) when using Cr_2O_3 as a marker in dairy cows. A more recent study that fed Cr_2O_3 to steers, reported that FO estimates from GRAB samples were significantly higher than RFO (BENVENUTTI *et al.*, 2014). Our results suggest that Cr_2O_3 could be used as a marker for estimating FO but, since FR differs from 100%, there is a need for TFC and the best results derive from the 72h BULK sampling procedure.

During the last decade or so TiO_2 has received increasing attention by researchers in response to the potential health threats that have been attributed to Cr_2O_3 (MYERS *et al.*, 2006). Studies have reported that FO estimates are overestimated, not significantly different from RFO (TITGEMEYER *et al.*, 2001) or under-estimated (GLINDEMANN *et al.*, 2009). The variety of results appear to be caused by differences in FR which directly affects the FO estimates. In our study, all of the TiO_2 derived FO estimates were significantly underestimated, under all sampling procedures, when compared to RFO. This is a reflex of the unusually high FR observed in our experiment since higher than 100% FR lead to lower FO estimates.

Prediction of DMI based on internal and external marker pairs

Estimates of DMI varied widely among marker pairs and sampling procedures. Only the Cr_2O_3 +iADF pair produced accurate DMI estimates under all the sampling procedures. Not significantly different DMI estimates were also observed for Cr_2O_3 +cutin under 4x4 GRAB and 72h BULK sampling, as well as for Cr_2O_3 +iNDF under 9x3 GRAB and 72h BULK sampling procedures. Estimates of DMI for the Cr_2O_3 +ABL pair did not differ significantly from RDMI but the observed values were different between the sampling methods. Also, although not

significantly different from a statistical point of view, Cr_2O_3 +ABL DMI estimates differed by an average of 2456 g/day when compared to RDMI. This difference is equivalent to ± 10 percentage units error in DMI estimates, which may be manageable for practical situations but not acceptable for research studies.

When TiO_2 was paired with internal markers, accurate DMI estimates were only obtained under 4x4 GRAB sampling for cutin and under 72h BULK sampling for ABL, iADF and iNDF. Once again, although estimates were “accurate”, the mean difference 4027 g/day or 17 percentage units less than RDMI. This much of a difference would not be acceptable under any conditions.

Previous studies with TiO_2 (TITGEMEYER *et al.*, 2001) and Cr_2O_3 (RODRIGUES *et al.*, 2006 and BENVENUTTI *et al.*, 2014) observed that DMI estimates were lower than or equal to RDMI for these markers, respectively. In their studies, the authors used each marker to estimate both DMD and FO, in order to, estimate DMI from these values. What they reported as the main cause for the inaccuracy of DMI estimates was FR different than 100% which resulted in under or over estimation of FO. The higher the FR the lower FO estimates that will be obtained and *vice-versa*. Given an accurate DMD estimate (or TTAD), if FO is underestimated then DMI will also be lower than RDMI. The same thing happens when DMD is underestimated at a given or accurate FO (or RFO). The degree of recovery of these markers has been variable (BERCHIELLE *et al.*, 2005 and RODRIGUES *et al.*, 2006) and this happens because these fractions do not constitute uniform chemical units that are constant throughout feeds.

The results of our study were in accordance with the observations of the above-mentioned authors. Almost all TiO_2 pairs derived estimates were lower than RDMI and this can be explained by the unexpectedly higher FR for this external marker. Then, when these FO estimates were paired with underestimated DMD estimates from the internal markers, the error effect was additive. On the contrary, when estimates from Cr_2O_3 pairs were used the errors seem to cancel each other out, as generally FO was a little over estimated and DMD estimates were lower than TTAD (ABL and cutin) or more accurate (iNDF and iADF).

Conclusions

In dairy cows fed a corn silage based diet with 45% concentrate, the average FR of TiO_2 was higher than FR of Cr_2O_3 and both FR were more than unity. With internal markers, ABL and cutin FR was lower than unity and average FR for indigestible NDF and ADF was 1.5. The FR was unaffected by the fecal sampling procedure and appears to be an intrinsic property of each molecule and how it interacts with digesta. Of the two external markers, only Cr_2O_3

produced accurate FO estimates and the same happened to DMD when iNDF and iADF were used. Estimates for DMD and FO were affected by sampling procedure, being that 72h BULK consistently produced accurate results. GRAB sampling procedures were accurate when using either of the indigestible fibers (iNDF or iADF) to estimate DMD. As a result of these observations, we can only recommend GRAB sampling procedures when concomitant TFC is performed on at least 1 animal per treatment in order to determine FR. We can conclude that Cr_2O_3 is a suitable marker for estimating FO and that iNDF and iADF are adequate for estimating DMD. Moreover, the Cr_2O_3 +iADF marker pair produces accurate DMI estimates and deserves further attention for research in ruminant nutrition studies.

Chapter 2.

Forage *versus* marker interactions when estimating intake in collectively housed steers using Grow Safe® technology

Introduction

The use of markers is currently the most widely used technique to estimate DMI in ruminants. Markers are indigestible substances that are not secreted by the animal, have passage rates similar to the feed, can be recovered completely after ingestion and allow for practical and precise chemical analysis (FAHEY & JUNG, 1983), among others characteristics. According to Merchen (1993), none of the substances that are used as markers in ruminant digestion studies meet all of the requirements but, some are adequate enough to produce important data for the advance of research.

The use of dietary markers to study feed utilization was proposed as far back as 1874 (KOTB & LUCKEY, 1972). Daily dry matter intake (DMI) can be estimated by means of digestion markers. The most frequently used method estimates fecal output (FO) and dry matter digestibility (DMD) (PENNING, 2004).

Estimating the digestibility of a diet is based on its apparent indigestibility as a starting point. One basic parameter of indigestibility is FO since it represents the portion of what was ingested that was not used during passage through the gastrointestinal tract (GIT) (DETMANN *et al.*, 2004). Estimation of FO is obtained through cause/effect relationships between the diet and the

events throughout the GIT (DETMANN *et al.*, 2007), which cause increase in marker concentration of digesta as other compounds are removed by digestion and absorption (ASTIGARRAGA, 1997).

The quality of forage consumed by animals affects the passage rate (Q_p) (POPPI *et al.*, 1981 a,b) and thus the retention time (T_r) (DIAS *et al.*, 2011) of digesta in the GIT, therefore influencing the rate of change of marker concentration in feces. The concentration of the marker in feces will ultimately determine its fecal recovery (FR) which is a crucial characteristic, if not the most, that any marker should possess in order to be free of recovery bias (DETMANN *et al.*, 2007). According to Langlands *et al.*, (1963), FR appears to be an inherent characteristic of each marker and how it interacts with feed particles.

Indigestible residues are the most commonly used internal markers in ruminant digestion studies. These residues are commonly represented by indigestible neutral detergent fiber (iNDF) and indigestible acid detergent fiber (iADF). Other indigestible, more specific, feed fractions have also been used as internal markers, such is the case of lignin, mainly as acid detergent lignin (ADL).

Several substances, especially rare earth materials have been used as external markers. Titanium dioxide (TiO_2) has been proposed as an alternative to chromic oxide (Cr_2O_3) for ruminant studies (TITGEMEYER *et al.*, 2001; MYERS *et al.*, 2006; GLINDEMANN *et al.*, 2009; SAMPAIO *et al.*, 2011a), in light of legal aspects regarding the use of Cr_2O_3 because of possible carcinogenic properties (MYERS *et al.*, 2006).

The marker technique to estimate intake uses an external marker to estimate FO and an internal marker (naturally occurring in feedstuffs) to estimate (DMD). Intake is then calculated by dividing FO by the indigestibility ($1-DMD$) of the feed. The main criticisms to the technique are the difficulty and labor of feeding the external markers at least twice a day, for several days; the need for excessive interaction between animals and people which may negatively affect intake and the intensive labor and high cost of total feces sample collection (TFC). Sampling protocols with GRAB feces sampling (directly from the rectum or upon spontaneous excretion) have been tested in order to decrease the labor of TFC and some have reported satisfactory results. Still, even though there is evidence to support the use of internal and external markers in ruminant digestion studies, results are conflicting and further understanding is necessary.

From the above, it can be hypothesized that the combination of internal markers and TiO_2 should result in accurate estimates for DMD, FO and DMI in steers consuming diets with different forage sources. The objective of the experiment was to test the accuracy of internal markers, acetyl bromide lignin (ABL), iNDF and iADF for estimating DMD, TiO_2 for estimating FO and the combination of these estimates for estimating DMI and compare it to real DMI (RDMI) from data obtained by the Grow Safe® System.

Material and Methods

The experiment was conducted at the UM Beef Research and Teaching Farm of the Animal Science Research Center (ASRC) at the University of Missouri (UM). The experiment was approved by the Ethics Committee for Animal Use (CEUA) of the University of São Paulo (Approval number: CEUA 24420603114). No animals were harmed during the experiment. Two animals fell ill to respiratory disease during the sampling period so their data was not used in the analysis.

Animals, diets and experimental design

Thirty Angus and 6 Charolais-cross steers (average $\pm$ SD; BW: 316.4 ± 18.6 kg) were randomly allocated to 12 covered pens (8m x 6m) with concrete flooring. The mouth, tongue and teeth of all steers were examined prior to starting the experiment in order to guarantee absence of wounds or abnormalities that could compromise feed intake. Each pen had a Grow Safe® feed bunk and allowed the animals access to clean water. The Grow Safe® System is an electronic method that allows group housing with individual intake measurements. In this system, the feed bunk recognizes a magnetic ear-tag on the animal and stores intake information from each animal in the group. Two pens were assigned at random to each of the 6 experimental diets.

All diets were composed of a 70:30 forage to concentrate with a 20% corn-silage base. The remainder of the forage portion of each diet was made up of one of six forage sources: 1) *Medicago sativa* (**Alfalfa hay**); 2) *Festuca arundinacea* Schreb. ("**Texoma**" **MaxQ II haylage**"); 3) *Avena sativa* (**Forage Oats haylage**); 4) *Sorghum bicolor* ssp. Drummondii (**Sorghum Sudan haylage**); 5) *Festuca arundinacea* Schreb mixture (varieties: Bar Optima, MaxQ Jesup, Martin Free, K31, Protek Martin 2, Estancia, Protek Tower) (**Variety Plot haylage**) and 6) *Thinopyrum intermedium* (**Wheatgrass hay**). Diets were formulated so that rumen fill, and not metabolic satiety, would limit intake.

Daily allowances of the total mixed ration (TMR), plus 10% of calculated daily natural matter intake (to guarantee orts), were fed once a day at 10:00. Feed subsamples were taken on

Mondays, Wednesdays and Fridays for dry matter (DM) determination (drying in a forced draft oven (55°C for 3 days). Pen orts and individual intake were registered daily by the Grow Safe® software. The concentrate was composed of dried distiller's grains with solubles (DDGS), AMINOPLUS®, minerals and vitamin premixes. Composition of supplements and TMR diets are shown in Table 1.

Table 1. Composition of supplements and experimental diets

Ingredient, DM (%)	Concentrates					
	S1 Alfalfa	S2 II	MaxQ	S3 Oats	S4 SS ^e	S5 V.Plot ^f S6 WG mix ^g
Amino Plus® ^a	8.2	13.3		11.9	11.9	13.8
DDGS ^b	23.9	18.9		19.4	20.2	18.3
ADE Nutra MIX® ^c	0.1	0.1		0.1	0.1	0.1
Custom Mineral Premix ^d	0.03	0.03		0.03	0.03	0.03
Vitamin E	0.2	0.2		0.2	0.2	0.2
Lime	0.6	0.6		1.5	0.6	0.6
NaCl	0.3	0.3		0.3	0.3	0.3
Ingredient, DM (%)	Diets					
	D1	D2	D3	D4	D5	D6
Treatment Forage	55.1	55.0	55.0	55.0	55.1	55.1
Corn Silage	11.6	11.6	11.6	11.6	11.6	11.6
Supplement	33.3	33.4	33.4	33.4	33.3	33.3

^a 52% CP; ^b distiller's dried grains with solubles; ^c vitamin mix (4,000,000 UI/lb vitamin A, 800,000 UI/lb vitamin D3, 500 UI/lb vitamin E); ^d mineral mix (24,8 – 28,8% Ca, 100 ppm Co, 1% Cu, 2,5% Fe, 500 ppm I, 2% Mn, 100 ppm Se, 3% Zn); ^e sorghum Sudan haylage; ^f variety plot haylage; ^g Wheatgrass hay.

The experiment ran for 19 days (day regarded as the 24 h period from 8 am to 8 am). The experimental period consisted of three phases: d1-d5 were allocated to adaptation of the animals to the diets; ten days (d5-d15) of marker excretion stabilization and the final 4 days (d16-19) were for sample collection. A completely randomized block design was used, with animals being stratified by weight and allocated to two blocks per treatment.

Markers

Three internal markers (ABL, iNDF and iADF) were used to estimate DMD and one external marker (TiO₂) were used to estimate FO. The 4 combinations (1 internal marker and 1 external marker) derived from these markers were used to estimate voluntary DMI. Values for DMD and FO were estimated and used to estimate DMI which was compared to its RDMI obtained from the Grow Safe® system.

Internal markers are naturally occurring indigestible fractions of feedstuffs. External markers, in this case TiO_2 , need to be fed to the animals exogenously, be it orally or via ruminal cannula. For the experiment, a water based suspension containing 2% carboxy-methyl cellulose (CMC) and 0.25 g/mL of TiO_2 was prepared. This suspension was fed twice daily (07:00 and 16:00), via oral cannula connected to a pressure pump, with the animal contained inside a chute. Each steer received 15 g of TiO_2 per day, divided into two equal parts. Given the concentration of the suspension, animals received 30 ml (7.5 g of TiO_2) each time they were dosed.

Feces

From d15 to d19, fecal GRAB samples were obtained twice daily, directly from the rectum, when the animals were contained at the chute for dosing. Composite samples were elaborated per animal and frozen at -20°C for later analysis. Estimates for DMD, FO and DMI were then grouped by pen and diet for statistical analysis.

Chemical analysis and measurements

For quantification of digestibility, feed samples were collected between d15-d18, and orts collected between d16-d19. Composite samples were elaborated per pen and frozen at -20°C . Later, feed, orts and feces samples were thawed at room temperature, oven-dried at 55°C and processed in a Wiley mill (Arthur H. Thomas Company®, Philadelphia, PA) with a 2 mm mesh screen. Preference was given to the 2 mm screen because, according to Froidmont *et al.*, (2008), fine grinding can cause loss of small particles (containing lignin) which would have a significant impact on our expected results.

Forage, concentrate and TMR samples were analyzed for dry-matter (DM) and mineral matter (MM) by incineration in a muffle furnace. Ash and protein-free neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the fiber bag procedure (VAN SOEST, ROBERTSON & LEWIS, 1991), with addition of heat-stable amylase and without sodium sulfite (MERTENS, 2002), in a A-200 Fiber Analyzer (ANKOM Technology Corp.®, Macedon, NY).

Cell wall (CW) preparation was obtained according to the procedure proposed by Fukushima and Hatfield (2001) and later used as substrate for acetyl bromide lignin (ABL) determination in feed, orts and feces samples, according to Fukushima and Kerley (2011). Lignin absorbance was determined with an Evolution 201 spectrophotometer (Thermo Scientific®; Waltham, MA) at a wavelength of 280 nm. Indigestible neutral detergent fiber and indigestible acid detergent fiber (iNDF and iADF, respectively) were obtained by weighing forages and feces (0.5 g), and concentrate (1.0 g) samples into F57 bags (ANKOM Technology Corp.®, Macedon, NY).

Samples were incubated inside the rumen of a cannulated steer for 288 hours (CASALI *et al.*, 2008; KRIZAN and HUHTANEN, 2013), washed with abundant water and treated according to Van Soest and Robertson (1985) for determination of these indigestible fractions.

Crude protein (N% x 6.25) was determined in forage, concentrate and TMR samples by N-analyzer (Elementar Americas®, Mt. Laurel, NJ) analysis. This method is a fast, safe and environmentally friendly alternative to the classical Kjeldahl procedure because it does not use such strong acids.

The quantification of TiO₂ in feces samples was performed by visible light spectrophotometry (410 nm) according to Myers *et al.*, (2004). To validate the accuracy of the TiO₂ analysis, a regression equation was created using the method proposed by Glindemann *et al.*, (2009). Standard samples containing 0, 1, 2, 3, 4, 5 and 6 mg of titanium per g DM were analyzed in triplicate, totalizing 21 standard samples. Pure TiO₂ (99% trace metals basis; ALDRICH Chemistry®) was employed to produce the standards. The concentration of TiO₂ was analyzed and compared with the added amount of TiO₂. The regression equation obtained was $y = 8.856x + 0.10$ ($r^2 = 0.987$).

Calculations

Real DMI (RDMI) was calculated from the intake data from the Grow Safe® software. Equations (1) and (2) were used to estimate marker excretion and recovery rate. The overall recovery rate of markers was calculated from the total amount of marker given and the FO estimated by TiO₂ concentration in feces during the fecal sampling period (d15-d19). Daily recovery rate of markers was calculated from d16 to d19:

(1)

$$\text{marker excretion} \left(\frac{\text{g}}{\text{day}} \right) = \text{fecal marker concentration} \left(\frac{\text{g}}{\text{g}} \right) \times \text{RFO} \left(\frac{\text{g}}{\text{day}} \right)$$

(2)

$$\text{recovery rate of the marker} = \frac{\text{marker excreted} \left(\frac{\text{g}}{\text{day}} \right)}{\text{marker ingested} \left(\frac{\text{g}}{\text{day}} \right)}$$

Equation (3) was used to estimate FO using TiO₂ and Equation (4) was used to estimate DMD using internal markers:

(3)

$$\text{FO estimated by external marker} \left(\frac{\text{g DM}}{\text{day}} \right) = \frac{\text{daily marker dose} \left(\frac{\text{g}}{\text{day}} \right)}{\text{fecal [marker]} \left(\frac{\text{g}}{\text{g DM}} \right)}$$

(4)

$$\text{DMD estimated by internal marker} \left(\frac{\text{g}}{\text{kg DM}} \right) = 100 - \left(100 \times \left(\frac{\text{feed [marker]} \left(\frac{\text{g}}{\text{g DM}} \right)}{\text{fecal [marker]} \left(\frac{\text{g}}{\text{g DM}} \right)} \right) \right)$$

Equation (5) was used to estimate total voluntary intake from FO and DMD estimates obtained by equations (3) and (4). Total DMI was estimated from GRAB samples:

(5)

$$\text{DMI estimated by markers} \left(\frac{\text{g}}{\text{day}} \right) = \frac{\text{FO} \left(\frac{\text{kg DM}}{\text{day}} \right)}{(1 - \text{diet DMD})}$$

Statistical Analysis

Results were analyzed with SAS® 9.3 (SAS®, 2011), after verifying normality of residuals and homogeneity of variance. With normally distributed RDMI values, DMI, FO and DMD estimates a 6x4 factorial arrangement was adopted (6 forages and 4 markers (3 internal and 1 external)) and data was analyzed using PROC-MIXED, at 0.05 significance level, according to the model:

$$Y_{hijk} = \mu + P_h + F_i + M_j + FM_{ij} + A(P)_{hk} + \varepsilon_{hijk},$$

where Y_{ijk} = is the RDMI value, DMI, FO or DMD estimated in animal k, by marker i, under sampling procedure j; μ = is the general constant; P_h is the random effect of pen; F_i = is the fixed effect of forage source; M_j = is the fixed effect of internal marker; FM_{ij} = is the interaction

between forage source i and internal marker j (fixed); $A(P)_k$ = is the random effect of animal k nested to pen h ; and ε_{hijk} is the sampling error supposed to be normal and independently distributed.

Linear regression analysis and Student's paired t -test were used to compare estimates against real observed values (those derived from TFC) for all variables being studied.

Results

Mean RDMI (average $\pm$ SD) was 6.7 ± 1.2 kg/animal/day, equivalent to $2.2 \pm 0.1\%$ of BW; 11.3 ± 2.4 kg/animal/day, equivalent to $3.5 \pm 0.5\%$ of BW; 14.7 ± 1.5 kg/animal/day, equivalent to $4.7 \pm 0.3\%$ of BW; 12.2 ± 4.2 kg/animal/day, equivalent to $4.0 \pm 0.3\%$ of BW; 10.6 ± 1.5 kg/animal/day, equivalent to $3.2 \pm 0.1\%$ of BW; 6.3 ± 1.1 kg/animal/day, equivalent to $2.0 \pm 0.4\%$ of BW; for Alfalfa hay, MaxQ II haylage, Forage Oats haylage, Sorghum Sudan haylage, Variety Plot haylage and Wheatgrass hay, respectively.

The chemical composition of the forages, concentrates and TMR diets were in accordance to the diet formulated for the experiment. Chemical composition of forages and supplements is shown in Table 2. Chemical composition of TMR diets is shown in Table 3.

Table 2. Chemical composition (g/kg of DM) of forages and supplements

	Forages							Supplements				
	Corn Silage	Alfalfa	MaxQ II	Oats	SS	V. Plot	WG mix	S1	S2/S5	S3	S4	S6
DM ¹	473.0 ^c	839.2 ^a	401.1 ^d	314.1 ^e	318.0 ^e	404.1 ^d	822.1 ^b	879.7	883.5	880.5	879.9	877.4
MM ²	39.3 ^c	80.8 ^b	104.2 ^a	122.2 ^a	103.9 ^{ab}	11.0 ^a	80.6 ^b	145.0 ^b	146.0 ^{ab}	175.4 ^a	166.3 ^a	118.4 ^c
NDF ³	288.9 ^f	473.1 ^e	646.1 ^b	545.6 ^d	595.0 ^c	556.8 ^d	715.7 ^a	191.5 ^{bc}	176.4 ^c	216.0 ^a	178.9 ^c	201.6 ^{ab}
ADF ⁴	165.0 ^f	342.7 ^e	410.7 ^b	350.3 ^e	376.5 ^d	385.9 ^c	480.0 ^a	75.4 ^{ab}	68.3 ^{bc}	77.3 ^{ab}	66.5 ^c	79.1 ^a
CW ⁵	-	715.0 ^c	761.7 ^b	649.8 ^d	724.2 ^c	747.9 ^b	856.0 ^a	689.1	674.7	691.2	695.7	678.1
iNDF ⁶	-	319.8 ^b	307.4 ^b	258.1 ^c	284.0 ^{bc}	306.5 ^b	406.8 ^a	58.8	65.5	79.0	47.2	63.3
iADF ⁷	-	238.3 ^b	195.2 ^c	178.9 ^c	176.4 ^c	209.5 ^{bc}	288.5 ^a	21.7	36.2	28.1	22.4	25.1
ABL ⁸	-	51.4 ^d	87.2 ^b	65.0 ^c	82.0 ^b	84.7 ^b	115.7 ^a	2.3	2.2	4.7	2.3	1.8
CP ⁹	102.0 ^{cd}	197.6 ^a	146.1 ^b	152.2 ^b	146.1 ^b	124.5 ^{bc}	92.4 ^d	440.0	440.6	412.1	414.2	437.2
Ee ¹⁰	84.0 ^a	39.7 ^c	46.4 ^b	26.8 ^d	22.9 ^d	22.8 ^d	43.3 ^{bc}	19.8 ^b	27.0 ^a	10.4 ^c	25.8 ^a	17.7 ^b

¹ dry matter; ² mineral matter; ³ neutral detergent soluble fiber; ⁴ acid detergent soluble fiber; ⁵ cell wall; ⁶ indigestible neutral detergent soluble fiber; ⁷ indigestible acid detergent soluble fiber; ⁸ acetyl bromide lignin; ⁹ crude protein (N% $\times$ 6.25); ¹⁰ ether extract; ^{a, b, c, d, e, f} means within the same line, followed by different superscripts, are different according to Fisher's LSD at 5% declared significance.

Table 3. Chemical composition (g/kg of DM) of experimental diets

	Diets					
	D1 Alfalfa	D2 MaxQ	D3 Oats	D4 SS	D5 V. Plot	D6 WG
DM ¹	775.7 ^a	517.0 ^c	442.6 ^e	414.5 ^f	470.0 ^d	743.8 ^b
MM ²	104.8 ^a	100.2 ^a	116.8 ^a	98.3 ^{ab}	102.3 ^a	85.0 ^b
NDF ³	399.8 ^d	464.4 ^b	444.9 ^c	464.0 ^b	469.8 ^b	528.9 ^a
ADF ⁴	223.9 ^d	260.0 ^c	258.4 ^c	260.8 ^c	294.4 ^b	316.7 ^a
CW ⁵	677.2 ^c	721.9 ^b	671.5 ^c	702.3 ^b	723.7 ^b	780.4 ^a
iNDF ⁶	197.4 ^c	260.1 ^b	245.2 ^{bc}	209.0 ^c	256.3 ^b	371.0 ^a
iADF ⁷	139.6 ^{bc}	163.6 ^{bc}	157.8 ^{bc}	129.6 ^c	175.2 ^b	229.6 ^a
ABL ⁸	40.0 ^c	56.8 ^b	50.5 ^d	56.4 ^b	61.9 ^b	74.8 ^a
CP ⁹	226.1 ^a	227.8 ^a	234.2 ^a	213.2 ^{ab}	186.0 ^{bc}	175.2 ^c
Ee ¹⁰	37.2 ^b	46.2 ^a	45.5 ^a	45.9 ^a	43.3 ^a	31.8 ^c

¹ dry matter; ² mineral matter; ³ neutral detergent soluble fiber; ⁴ acid detergent soluble fiber; ⁵ cell wall; ⁶ indigestible neutral detergent soluble fiber; ⁷ indigestible acid detergent soluble fiber; ⁸ acetyl bromide lignin; ⁹ crude protein (N% x 6.25); ¹⁰ ether extract; a, b, c, d, e, f means within the same line, followed by different superscripts, are different according to Fisher's LSD at 5% declared significance.

Fecal recovery of markers

A highly significant effect for marker ($P < .0001$) was observed. An effect for diet ($P = 0.002$) was also observed, but no effect for the marker x diet interaction ($P = 0.114$) was detected. The lowest FR was observed for ABL and it differed from unity. The FR of TiO_2 was complete and did not differ from unity. Indigestible NDF and ADF produced similar incomplete FR, around 78% of what was consumed for the 24-hour period. All the internal markers had higher FR on the hay diets and the recoveries were not different from unity (data not shown).

Table 4. Twenty-four hour fecal recovery (FR) of internal markers and TiO_2 in steers fed diets with different forage sources

With different forage sources								
	Diet							
	Alfalfa	MaxQ II	Oats	SS ⁴	V. Plot ⁵	WG mix ⁶	SD	P value
FR	1.10a	0.81b	0.79c	0.78c	0.85bc	0.97ab	0.066	0.002
	Marker							
	ABL ¹	iNDF ²	iADF ³		TiO ₂		SD	P value
FR	0.73b	0.77b	0.79b		1.15a		0.062	<.0001

¹ acetyl bromide lignin; ² indigestible neutral detergent soluble fiber; ³ indigestible acid detergent soluble fiber; ⁴ sorghum Sudan haylage; ⁵ variety plot haylage; ⁶ Wheatgrass hay; Within a line, mean values with common upper case superscript are not significantly different by Fisher's LSD ($P < 0.05$).

Prediction of DMD based on fecal concentration of internal markers

The mean DMD estimates are shown in Table 5. Significant effects for forage, marker and their interaction were observed ($P = 0.001$, $P < .0001$ and $P < .0001$, respectively) on DMD. Estimates of DMD from the Grow Safe® intake data and TiO_2 estimates for FO (GS+ TiO_2) were compared against DMD estimates from the internal markers. Values for DMD varied among markers and

forages. Estimates from GS+TiO₂ were significantly higher than estimates from internal markers, in all forages except for Alfalfa haylage. For all forages, DMD from GS+TiO₂ was in accordance with what was biologically expected, thus DMD was higher for haylage containing diets than hay based diets.

Table 5. Mean DMD estimates $\pm$ SE (g/kg DM) for ABL, iNDF, iADF and Grow Safe® intake data with TiO₂ estimates of FO

Forage	Marker			
	ABL ¹	iNDF ²	iADF ³	GS+TiO ₂
Alfalfa	586.9 ^{bc} $\pm$ 14.3	657.5 ^{Aa} $\pm$ 5.8	626.0 ^{ABab} $\pm$ 11.6	533.8 ^{Cc} $\pm$ 41.6
MaxQ II	497.7 ^b $\pm$ 18.9	525.8 ^{Cb} $\pm$ 23.9	544.5 ^{BCb} $\pm$ 22.5	665.6 ^{Ba} $\pm$ 16.0
Oats	496.0 ^c $\pm$ 20.4	553.5 ^{BCbc} $\pm$ 15.4	589.1 ^{ABb} $\pm$ 14.8	761.4 ^{Aa} $\pm$ 44.6
SS ⁴	539.2 ^c $\pm$ 12.1	629.7 ^{ABb} $\pm$ 19.8	643.1 ^{Ab} $\pm$ 17.7	745.6 ^{ABa} $\pm$ 21.2
V. Plot ⁵	527.1 ^c $\pm$ 27.7	608.6 ^{ABCb} $\pm$ 35.3	610.8 ^{ABb} $\pm$ 35.0	748.4 ^{ABa} $\pm$ 22.6
WG mix ⁶	509.9 ^{ab} $\pm$ 33.9	396.8 ^{Db} $\pm$ 33.1	454.9 ^{Cb} $\pm$ 33.7	544.0 ^{Ca} $\pm$ 34.9

¹ acetyl bromide lignin; ² indigestible neutral detergent soluble fiber; ³ indigestible acid detergent soluble fiber; ⁴ sorghum Sudan haylage; ⁵ variety plot haylage; ⁶ Wheatgrass hay; Within a line, mean values with common upper case superscript are not significantly different by Fisher's LSD (P<0.05). Within a column, mean values with common lower case superscript are not significantly different by Fisher's LSD (P<0.05).

Prediction of FO based on fecal concentration of TiO₂

The mean FO estimates are shown in Table 6. As with DMD, significant effects for forage, marker and their interaction were observed (P=0.003, P<.0001 and P<.0001, respectively) on FO. Estimates for FO, produced by TiO₂ analysis, were compared against estimates derived from internal marker DMD and Grow Safe® intake data. Values for FO varied among markers and forages. Estimates from ABL were highest among all markers especially for the more digestible haylages. Estimates from TiO₂ were more in accordance to what was expected for the RDMI values obtained from Grow Safe® technology.

Table 6. Mean FO (g DM/day) estimates for TiO₂ and Grow Safe® intake data with DMD estimates from internal markers

Forage	Marker			
	ABL ¹ +GS	iNDF ² +GS	iADF ³ +GS	TiO ₂
Alfalfa	2791.9 ^{Cab} ± 286.5	2301.9 ^{Cbc} ± 202.0	1959.3 ^C ± 196.4	3152.0 ^a ± 443.3
MaxQ II	5673.7 ^{Ba} ± 553.0	5447.3 ^{ABa} ± 704.8	2707.5 ^C ± 356.0	3797.1 ^b ± 438.4
Oats	7385.1 ^{Aa} ± 417.4	6593.6 ^{Aa} ± 494.6	2682.5 ^C ± 191.1	3609.9 ^b ± 810.8
SS ⁴	5595.9 ^{Ba} ± 868.6	4686.8 ^{Bb} ± 964.5	2114.3 ^d ± 352.8	2971.7 ^c ± 352.8
V. Plot ⁵	5793.8 ^{Ba} ± 373.5	5062.2 ^{ABa} ± 447.0	2364.9 ^b ± 214.9	3252.5 ^b ± 414.4
WG mix ⁶	3532.9 ^{Cab} ± 503.7	4368.3 ^{Ba} ± 497.1	2945.5 ^b ± 396.1	3708.8 ^{ab} ± 448.6

¹ acetyl bromide lignin; ² indigestible neutral detergent soluble fiber; ³ indigestible acid detergent soluble fiber; ⁴ sorghum Sudan haylage; ⁵ variety plot haylage; ⁶ Wheatgrass hay; Within a line, mean values with common upper case superscript are not significantly different by Fisher's LSD (P<0.05). Within a column, mean values with common lower case superscript are not significantly different by Fisher's LSD (P<0.05).

Prediction of DMI based on internal markers and TiO₂ pairs

The mean DMI estimates are shown in Table 7. No significant effect (P=0.211) for forage on DMI was observed in this study. Highly significant effects for marker and forage*marker interaction (P<.0001) were observed. Differences in RDMI (Grow Safe® data) were observed among forages, especially when comparing the haylage containing against the hay containing diets. None of the 3 marker pairs produced accurate estimates of DMI when compared to RDMI. Estimates from ABL+TiO₂ and iADF+TiO₂ were not different among forages but differed significantly from RDMI in the MaxQ II (P=0.020, P=0.052), Oats (P<.0001, P=0.0002), sorghum Sudan (P=0.0004, P=0.013) and variety plot (P=0.010, P=0.004)) diets, respectively. When DMI values were not significantly different from RDMI, high SD values indicated low precision of the estimates which were up to 13 percentage points different from real intake values (GS).

Table 7. Mean RDMI (g DM/day) from Grow Safe® data and DMI (g DM/day) estimates from internal markers and TiO₂ pairs

Forage	Marker			
	ABL ¹ +TiO ₂	iNDF ² + TiO ₂	iADF ³ +TiO ₂	GS
Alfalfa hay	7668.6 ^{ab} ± 1127.0	9194.1 ^{Aa} ± 1295.1	8384.4 ^{ab} ± 1090.7	6704.5 ^{Cb} ± 545.6
MaxQ II	7572.6 ^b ± 909.4	7901.5 ^{ABb} ± 644.7	8208.3 ^b ± 622.0	11276.0 ^{Ba} ± 1063.0
Oats	7393.0 ^b ± 1889.3	7889.5 ^{ABb} ± 1627.5	8642.9 ^b ± 1844.2	14679.0 ^{Aa} ± 689.2
Sorg. Sudan ⁴	6511.7 ^c ± 823.2	7910.6 ^{ABbc} ± 587.2	8228.2 ^b ± 665.7	12185.0 ^{ABa} ± 1894.4
V. Plot ⁵	5567.8 ^b ± 840.3	6495.0 ^{ABb} ± 839.4	6268.8 ^b ± 918.5	10579.0 ^{Ba} ± 838.2
WG mix ⁶	7077.8 ± 1393.2	5413.1 ^B ± 918.2	5871.4 ± 1106.4	6306.2 ^C ± 541.0

¹ acetyl bromide lignin; ² indigestible neutral detergent soluble fiber; ³ indigestible acid detergent soluble fiber; ⁴ sorghum Sudan haylage; ⁵ variety plot haylage; ⁶ Wheatgrass hay; Within a line, mean values with common upper case superscript are not significantly different by Fisher's LSD (P<0.05). Within a column, mean values with common lower case superscript are not significantly different by Fisher's LSD (P<0.05).

Discussion

Fecal recovery of markers

Fecal recovery varied among markers and forages, ranging from 0.5 - 1.1 and from 0.6 – 1.3 for ABL and iNDF, respectively. Lee and Hristov (2013) reported FR for iNDF of 0.89 for multiparous Holstein cows fed corn silage based diets. No reports were found on the use of ABL as a marker for estimating DMD in ruminants, therefore our data is novel to this field of research. Liquid and solid phase stratification of ruminal contents directly influence how particles interact and bind to digesta around it. This interaction will in turn, determine the retention time (T_R) and passage rate (Q_P) of the marker/particle and ultimately affect what fraction of what is ingested will be excreted in feces over a 24-hour period. Mean FR of ABL, iNDF and iADF were all lower than and different than unity. Njaa (1961) reported that mean FR less than one could be attributed to inaccuracy of the analysis. These 3 procedures are all gravimetric and since the residue left after acid digestion or rumen incubation is small, errors are more likely to happen.

Several authors (KOTB & LUCKEY, 1972; FAHEY & JUNG, 1983) have long described what the properties of the ideal marker should be. Among these properties, one of the most important ones is, being totally recovered in a 24-hour period. Many studies have been conducted over the last 3 decades using TiO_2 and the FR observed for it has been around 100%. Based on these findings the routinary use of TiO_2 in ruminal digestion trails has been suggested. Our findings support the use of TiO_2 as an external marker to estimate FO in ruminants, since we observed mean FR of 1.1 for the 6 experimental diets. These findings are in accordance to those of Brandt and Allam, (1987); Hafez *et al.*, (1988) and Titgemeyer *et al.*, (2001), who observed mean FR for TiO_2 of 0.96-0.99 in sheep and 0.96-1.02, 0.90-0.95 in dairy cattle, respectively. Glindemann *et al.*, (2009) found FR higher than 1 and attributed this to possible ingestion of soil which contained TiO_2 and therefore would increase the fecal concentration. Our diets were composed of corn silage, concentrate and the treatment forage which were all well preserved and packaged, so the risk of soil contamination was low. Nonetheless, soil residues could account in part for the slightly elevated FR observed. Also, FR in this study was calculated using the FO estimates from TiO_2 (since total feces collection was not performed) and therefore the values will carry the bias that the FO estimates may have had. As shown in Table 6, FO estimates from TiO_2 were in accordance to what was biologically expected and did not differ among diets.

Lignin, determined as acid detergent lignin (ADL) has been used as an internal marker for digestibility studies and is considered indigestible because there are no microbial or mammalian enzymes that can degrade it (VAN SOEST, 1994). The ADL method has received criticism over the years because it can measure cutin and Maillard's compounds as being lignin. Additionally, this method has also been criticized because of lignin solubilization or destruction during hydrolysis with 72% H₂SO₄ solution. According to Lowry *et al.*, (1994) this loss of lignin during analysis can be upwards of 50% in some tropical grasses. Because of this lignin had not been extensively used as an internal marker and the few published reports, account for under estimation of DMD. Goachet *et al.*, (2009) used ADL to estimate digestibility coefficients in horses and observed FR of 0.90 and 0.86 for 3 and 4 days of fecal sampling, respectively. This study measured lignin as ABL, a procedure in which no lignin loss occurs during sample preparation and that is less subject to gravimetric errors because of its spectrophotometric nature. It is necessary to point out that ABL, as a spectroscopic method, possesses the sensitivity required for detecting soluble lignin components that would be lost by conventional gravimetric analysis (Fahey and Jung, 1983). Mean ABL FR was 0.73, significantly different ($P<.0001$) than TiO₂ FR and unity ($P=0.001$). These results suggest that fecal recovery of lignin is incomplete when measured as ABL and that it would not be an appropriate internal marker for estimating DMD in cattle. A possible explanation for incomplete FR is that when vegetable cell walls are treated with cellulases, the components that ester-link ferulic or p-coumaric acids to a hemicellulosic side chain of xylose and arabinose are released (MUELLER-HARVEY *et al.*, 1986). These soluble hemicellulosic complexes do not appear to be digestible; they undergo precipitation on reaching gastric acidity in the lower tract and are recoverable in feces (NEILSON and RICHARDS, 1978). Although the studies did not consider the possibility that rumen bacteria might adapt, and metabolism of monomeric units has been reported (FUKUSHIMA *et al.*, 1991).

Indigestible neutral detergent and acid detergent fibers are two of the most extensively used internal markers today. Mean FR ranging from 0.50 to 1.21 (BARROS *et al.*, 2009; KOZLOSKY *et al.*, 2009; RODRIGUES *et al.*, 2010; SAMPAIO *et al.*, 2011a and LEE & HRISTOV, 2013) and from 0.80 to 1.21 (OLIVEIRA Jr. *et al.*, 2004; RODRIGUES *et al.*, 2010; SAMPAIO *et al.*, 2011a) for iNDF and iADF, respectively, have been reported. Oliveira *et al.*, (2003) worked with horses and observed FR of 1.08 to 1.34 and up to 1.54 for iNDF and iADF, respectively. In this study, mean FR was 0.53 to 1.25 for iNDF and 0.58 to 1.15 for iADF. These results were lower than expected and do not agree with previous author's findings. The disparity of results for

different experiments is, at least in part, due to differences in incubation time of the samples for obtaining the fibrous fractions. Our study followed the procedure proposed by Krizsan and Huhtanen (2013) who compared various incubation times (144h, 240h and 288h) and reported that at least 12 days of incubation were necessary to isolate the completely indigestible fiber pool in feeds fed to dairy cows. The results in this study suggest that iNDF and iADF would not be adequate internal markers for ruminant digestion studies, because low (<1) FR would lead to overestimation of DMD. Even so, the opposite was observed with low FR and probable underestimation of DMD by iNDF and iADF in all diets. Estimates of DMD from these markers were significantly different from GS+TiO₂ estimates and the values lacked biological coherence for the different types of forages being studied.

Prediction of DMD based on fecal concentration of internal markers

Estimated values for DMD from GS+TiO₂ were significantly different than estimates produced by either of the three internal markers. The ABL procedure consistently produced the lowest DMD estimates and this was caused, at least partially, because fecal recovery was not complete. Another possible explanation for the low DMD estimates would be the low concentration of lignin in both diet and feces samples. Lower concentrations make chemical analysis less reliable because of increased difficulty to detect differences in such small amounts of residue that are left behind for weighing after the gravimetric procedures are performed. The ABL procedure is of spectrophotometric nature and therefore should not be subject to this type of issues.

Mean DMD estimates produced by iNDF and iADF fecal concentrations were also lower than expected. Estimates were not different between the two indigestible fractions when compared within the same diet, evidencing the close relationship between both fibrous fractions. Using animals on pasture, Detmann *et al.*, (2001) reported more accurate DMD estimates when using iNDF compared to iADF. Berchielli *et al.*, (2005) reported conflicting results when using these indigestible fractions, in response to the incubation procedure as was mentioned before when discussing FR. With heifers fed fresh sugar-cane iNDF produced better DMD estimates than iADF but, when corn silage was fed to dairy cows, iADF estimates were more accurate than those of iNDF (FERREIRA *et al.*, 2008). Our findings suggest that both iNDF and iADF are not suitable internal markers for estimating DMD as the estimates they produced bare no biological meaning.

Prediction of FO based on fecal concentration of TiO_2

During the last decade or so TiO_2 has received increasing attention by researchers in response to the potential health threats that have been attributed to Cr_2O_3 (MYERS *et al.*, 2006). Studies have reported that FO estimates are over-estimated, not significantly different from RFO (TITGEMEYER *et al.*, 2001) or under-estimated (GLINDEMANN *et al.*, 2009). The variety of results appear to be caused by differences in FR which directly affects the FO estimates. When used as an external marker for FO, TiO_2 could produce satisfactory estimates that hold biological meaning. Although FR of TiO_2 was 10 percentage points higher than unity, the FO estimates were coherent considering RDMI obtained from the Grow Safe® System in this study.

Values for FO did not differ between the 6 experimental diets, even though DMD of diets was different. What this implies is that total digestibility of diets may have been similar but the rate of digestion of each diet varied. Haylage containing diets, apart from having top quality forages, had gone through a sort of “pre-digestion” during the ensiling process. The acid fermentation that kills harmful microorganisms (MO's) and allows for feed to be conserved by this process, also breaks down part of the cell wall of plant cells. This break down allows for easier and faster access of ruminal MO's to the nutrients and digestible portion of the feed, thus increasing DMD and DMI when consumed by ruminants. The Alfalfa and Wheatgrass hays did not go through the “pre-digestion” process and therefore had lower digestion rates when compared to haylages. The lower digestion rate resulted higher rumen retention time and ultimately lower DMI for the diets containing these two forage sources. The FO estimates from TiO_2 reflected this phenomenon and thus deserve attention. From our observations, TiO_2 seems to be a suitable external marker for estimating FO in ruminants that are housed collectively or when total feces collection is not possible.

Prediction of DMI based on internal markers and TiO_2 pairs

Estimates of DMI varied widely among marker pairs and diets. Values of RDMI obtained by the Grow Safe® software were different among the haylage diets and between hay containing and haylage containing diets. Only on the hay diets (Alfalfa and WG mix) were the marker pairs able to accurately estimate DMI. Even then, estimates differed from RDMI $\pm$ 15 percentage points. Such numerical differences, although not significant from a statistical point of view, discourages the use of the double marker technique because the practical consequences of such error can be expensive in both time and resources.

When TiO_2 was paired with internal markers, accurate DMI estimates were only obtained under 4x4 GRAB sampling for cutin and under 72h BULK sampling for ABL, iADF and iNDF. Once again, although estimates were “accurate”, the mean difference was 4027 g/day or 17 percentage units less than RDMI. This difference would not be acceptable because decisions made based on them would carry error with them.

A previous study with TiO_2 (TITGEMEYER *et al.*, 2001) observed that DMI estimates were lower than or equal to RDMI for this marker. In this study, the authors used the marker to estimate both DMD and FO, and from these values also estimate DMI. They reported as the main cause for the inaccuracy of DMI estimates was that FR was different from 100% which resulted in under or over estimation of FO. The higher the FR the lower FO estimates that will be obtained and *vice-versa*. Given an accurate DMD estimate (or real TTAD from total feces collection), if FO is underestimated then DMI will also be lower than RDMI. The same thing happens when DMD is underestimated at a given or accurate FO (real FO from total feces collection). The degree of recovery of these markers has been variable (BERCHIELLE *et al.*, 2005 and RODRIGUES *et al.*, 2006) and this happens because these fractions do not constitute uniform chemical units that are constant throughout feeds. The consequence of this is non-uniformity of markers in feces. Additionally, no standard method has been defined to determine them (LIPPKE, 2002). Possibly, marker dosing schedule will also affect fecal concentration of markers and more information should be collected on this issue. Also, as mentioned before, the uniformization of fecal samples during collection and later during analysis has great impact on fecal marker concentration.

The results of our study were in accordance with the observations of the above-mentioned authors. Most of the TiO_2 pairs derived estimates of DMI differed from RDMI and this can be explained by incomplete FR of internal markers. When TiO_2 derived FO estimates were paired with underestimated DMD estimates from the internal markers, the resulting DMI estimates carried over the bias from low DMD. Even though FR was a little higher than 1, this was not enough to compensate the incomplete FR of internal markers.

Conclusions

In steers fed diets with different forage sources FR of TiO_2 was close to 1 and this marker produced adequate FO estimates. Internal markers, ABL, iNDF and iADF had FR lower than unity which compromised the accuracy of DMD estimates derived from these markers. The FR was affected by the forage source that composed the diet, possibly due to soluble cell wall

fractions of the different forages. Although DMI estimates were not affected by the diet, the low DMD estimates from internal markers resulted in DMI estimates different from RDMI. Because of these observations, we can recommend the use of TiO_2 for estimating FO with concomitant TFC on at least 1 animal per treatment in order to determine FR. Based on our results we cannot recommend the use of ABL, iNDF or iADF for estimating DMD. Although none of the marker pairs produced accurate DMI estimates, these substances deserve further attention for research in ruminant nutrition studies.

Chapter 3.

Diet, marker and fecal sampling method interactions with internal and external marker pairs when estimating dry matter intake in ruminants.

Introduction

The use of dietary markers to study feed utilization was proposed as far back as 1874 (KOTB and LUCKEY, 1972). Daily dry matter intake (DMI) can be estimated by means of digestion markers. The most frequently used method separately estimates fecal output (FO) and dry matter digestibility (DMD) (PENNING, 2004).

The use of markers is currently the most widely used technique to estimate DMI in ruminants. Markers are indigestible substances that are not secreted by the animal, have passage rates similar to the feed, can be recovered completely after ingestion and allow for practical and precise chemical analysis (FAHEY and JUNG, 1983), among others characteristics. According to Merchen (1993), none of the substances that are used as markers in ruminant digestion studies meet all of the requirements but, some are adequate enough to produce important data for the advance of research.

The quality of forage consumed by animals affects the passage rate (Q_p) (POPPI *et al.*, 1981a,b) and thus the retention time (T_r) (DIAS *et al.*, 2011) of digesta in the GIT, therefore influencing the rate of change of marker concentration in feces. The concentration of the marker in feces will ultimately determine its fecal recovery (FR) which is a crucial characteristic, if not the most, that any marker should possess in order to be free of recovery bias (DETMANN *et al.*,

2007). According to Langlands *et al.*, (1963), FR appears to be an inherent characteristic of each marker and how it interacts with feed particles.

The marker technique to estimate intake uses an external marker to estimate fecal output (FO) and an internal marker (naturally occurring in feedstuffs) to estimate dry matter digestibility (DMD). Intake is then calculated by dividing FO by the indigestibility (1-DMD) of the feed. The main criticisms to the technique are the difficulty and labor of feeding the external markers twice a day, for several days; the need for excessive interaction between animals and people which may negatively affect intake and the intensive labor and high cost of feces sample collection.

Total feces collection (TFC) is regularly used in intake studies as the standard to which estimates are compared to and as the source of BULK samples for later analysis. The recommended number of days for TFC ranges from 7-10 days (SCHNEIDER & FLATT, 1975) to 3-5 days (FERREIRA *et al.*, 2008), with authors reporting no significant differences for digestibility estimates. Aiming to reduce the number of days for TFC, different fecal sampling designs have been proposed (LANGLANDS *et al.*, 1963), which accounts for the inconsistencies of obtaining the feces samples. In this approach, GRAB samples collected at specific times during the day, either after spontaneous excretion or collected directly from the rectum, are composited to make up the daily samples. Sampaio *et al.*, (2011b) compared 9 different fecal sampling designs and reported that four daily GRAB samples distributed along the whole day or just during the diurnal period will produce accurate estimates.

From the above, it can be hypothesized that the combination of internal and external markers, with fecal sampling designs that produce composite GRAB samples over 3-4 days of collection period, should result in accurate estimates for DMD, FO and DMI in bulls consuming corn silage or Tifton 85 hay based diets, with varying forage to concentrate ratios.

The objective of the experiment was to test the accuracy of internal markers, acetyl bromide lignin (ABL), cutin, indigestible neutral detergent fiber (iNDF) and indigestible acid detergent fiber (iADF) for estimating DMD; to test the utilization of external markers, chromic oxide (Cr_2O_3) and titanium dioxide (TiO_2) for estimating FO; examine the combination of internal and external markers for estimating DMI and to compare 3 different sampling designs against TFC.

Material and Methods

The experiment was conducted at the Beef Cattle Research Laboratory of the Animal Nutrition and Production Department at the University of São Paulo. The experiment was approved by

the Ethics Committee for Animal Use (CEUA) of the University of São Paulo (Approval number: CEUA 24420603114). No animals were harmed or fell ill during the experiment.

Animals, diets and experimental design

Twelve commercial Nelore bulls (avg. age: 18 months; avg. BW: 413.7 kg) were randomly allocated to 12 individual pens (6m x 4m) with concrete flooring. The mouth, tongue and teeth of all bulls were examined prior to starting the experiment in order to guarantee absence of wounds or abnormalities that could compromise feed intake. Each pen had a feed bunk and individual waterer. Three pens were assigned at random to each of the 4 experimental diets.

Diets were offered as total mixed ration (TMR), *ad-libitum*, divided into 2 daily servings (07:00 and 16:00) to represent the natural diurnal feeding pattern of cattle. Daily allowances of the TMR, plus 10% of calculated daily natural matter intake was fed to guaranteeorts. Feed subsamples were taken on Mondays, Wednesdays and Fridays for dry matter (DM) determination (drying in a forced draft oven (SOLAB Cientifica®) at 55°C for 3 days). Orts were collected daily so that *ad libitum* intake could be determined as the difference between the DM offered and refused.

Diets were composed of either corn silage (inoculated with *L. buchneri*) or Tifton-83 grass hay and their corresponding concentrate (Table 1). Diets were formulated to meet maintenance requirements and projected daily weight gain (DWG) of 0.8 – 1.4 kg/day. Urea was added to the diets in order to meet the rumen degradable protein (RDP) requirements and soybean meal was completely replaced by cottonseed meal. This substitution was made due to the high contents of cutin (from added cotton hulls) in this by-product. Increasing the cutin concentration of the diets was desirable in order to facilitate analysis and decrease variability of results. Chemical composition of forages, concentrates and TMR are shown in Table 2.

The experiment ran for 38 days (day regarded as the 24 h period from 8 am to 8 am). The experimental period consisted of 19 days divided into three phases: d1-d5 were allocated to adaptation of the animals to the diet; ten days (d5-d15) of marker excretion stabilization and the final 4 days (d16-19) were for sample collection. The animals were adapted to the experimental conditions during 14 days prior to the experimental period. Treatments followed a 2x2 factorial arrangement, being F1 the forage source (corn silage vs. hay) and F2 the forage to concentrate ratio (F:C) (60:40 vs. 40:60). The 12 bulls were randomly assigned to one of two blocks (forage source) and the blocks distributed into an incomplete cross-over design.

Table 1. Composition of concentrates and experimental diets

Ingredient, DM (%)	Concentrates			
	I	II	III	IV
Dry ground corn	26.0	43.0	32.0	49.0
Cottonseed meal ¹	13.0	14.0	6.0	8.0
Urea ²	0.4	0.4	0.1	0.3
Mineral+Vitamin mix ³	2.0	2.0	2.0	2.0
Lime ⁴	0.4	1.0	0.1	1.0

Ingredient, DM (%)	Diets			
	I (Silage 60:40)	II (Silage 40:60)	III (Hay 60:40)	IV (Hay 40:60)
Corn silage	60	40	-	-
Tifton-85 hay	-	-	60	40
Concentrate				
I	40	-	-	-
II	-	60	-	-
III	-	-	40	-
IV	-	-	-	60

¹ 28% CP; ² Reforce N® (Petrobras (N_{minimum} = 460g/kg)); ³ Minerthal Corte MD® (18.8-23.1% Ca, 0.2% Co, 0.07% Cu, 7.4% S, >0.02% F, 2.4% P, 0.04% I, 3% Mg, 0.15% Mn, 0.01% Se, 6% Na, 0.2% Zn and 0.2% monensin sodium salt); ⁴ FortCal®.

Markers

Four internal markers (ABL, cutin, iNDF and iADF) were used to estimate DMD and two external markers (Cr₂O₃ and TiO₂) were used to estimate FO. The eight combinations (1 internal marker and 1 external marker) derived from these six markers were used to estimate voluntary DMI. Estimates of DMD, FO and DMI were compared against their reciprocal TTAD, RFO and RDMI values, respectively.

Internal markers are naturally occurring indigestible fractions of feedstuffs. External markers, in this case Cr₂O₃ and TiO₂, need to be fed to the animals exogenously, be it orally or via ruminal cannula. For the experiment, capsules containing 0.75 g of Cr₂O₃ or 1 g of TiO₂ were prepared at a local pharmacy. Bulls received 6 g of Cr₂O₃ and 10 g of TiO₂ daily. This dose was divided into two equal parts and the animals were fed the capsules (mixed with a small amount of concentrate and molasses) before receiving the rest of the meal (08:00 and 16:00). The animals were dosed from d5 to d18 of each experimental period and had been receiving molasses with concentrate before each meal during the 14 days prior to the start of the experiment.

Feces

On d15, all stalls were thoroughly cleaned (pressure hosed). On d16 at 06:00 stalls were cleaned of what was excreted overnight and at 08:00 the 72 h TFC period started. Two people teams constantly watched the animals, in 8 hour shifts, and collected all feces immediately after excretion to avoid contamination or partial loss of material. Collected feces were placed into individual (1 per bull) plastic containers (previously weighed empty) which were kept sealed and away from direct sunlight. At 07:30 of d17 to d19 the containers were weighed and the daily RFO recorded for each animal. Fecal matter in each container was homogenized (mixed thoroughly with a ladle) and an approximately 500 g sample was taken for each animal daily, during the sampling period. These BULK samples constituted the 72-hour continuous sampling design.

Two GRAB sampling designs were evaluated during this study. A commonly used design in marker studies is the 3-day sampling period with samples being taken every 9 hours. This way, at the end of the three days, the 24 hours of a day will be represented by the various samples. Sampaio *et al.*, (2011b), compared 9 different sampling design protocols and reported that 4 samples collected during daylight hours were enough to obtain a representative sample of the daily excretion profile. Table 2 illustrates the fecal sampling design protocols used in this study.

Table 2. Sampling design protocols for BULK and GRAB feces samples

Design	Sampling	Duration – Sampling Times
A	BULK 72h	72 hours (d16-d18) - Continuous
B	GRAB 9x3	3 days - d16 = 8:00, 17:00, 2:00; d17 = 11:00, 20:00, 5:00; d18 = 14:00, 23:00, 8:00
C	GRAB 4x4 Daylight	4 days (d16-d19) - 8:00, 11:00, 14:00, 17:00

Chemical analysis and measurements

For quantification of voluntary intake, feed supplied, feces and orts were collected. Composite samples were elaborated per animal and frozen at -20°C. Later, feces, feed and ort samples were thawed at room temperature, oven-dried at 55°C and processed in a MA-048 knife mill

(MARCONI®, São Paulo, Brazil), with a 2 mm mesh screen. Preference was given to the 2 mm screen because, according to Froidmont *et al.*, (2008), fine grinding can cause loss of small particles (containing lignin and cutin) which would have a significant impact on our expected results.

Feed and TMR samples were analyzed for dry-matter (DM) and mineral matter (MM) by incineration in a muffle furnace (MARCONI®, São Paulo, Brazil). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by using the traditional method procedure (VAN SOEST, ROBERTSON & LEWIS, 1991) where filter bags replaced the glass-sintered crucibles, with addition of heat-stable amylase and without sodium sulfite (MERTENS, 2002), in a TE-149 Fiber Analyzer (TECNAL®, São Paulo, Brazil). Sodium sulfite was not used because it has been reported to cause loss of lignin particles during the detergent procedure (MERTENS, 2002).

Cell wall (CW) preparation was obtained according to the procedure proposed by Fukushima and Hatfield (2001) and later used as substrate for acetyl bromide lignin (ABL) determination in feed, Orts and feces samples, according to Fukushima and Kerley (2011). Lignin absorbance was determined with a Libra S80 spectrophotometer (BIOCHROM®; United Kingdom) at a wavelength of 280 nm. Soluble fiber was calculated as the difference between CW and NDF contents, with pectin being one of its components. Indigestible neutral detergent fiber and indigestible acid detergent fiber (iNDF and iADF, respectively) were obtained by weighing silage and feces (0.5 g), and concentrate (1.0 g) samples into F57 bags (ANKOM Technology Corp.®, Fairport, NY). Samples were incubated inside the rumen of a cannulated cow for 288 hours (CASALI *et al.*, 2008; KRIZAN and HUHTANEN, 2013), washed with abundant water and treated according to Van Soest and Robertson (1985) for determination of these indigestible fractions.

Cutin content in feed, TMR and feces samples were determined in non-woven textile (NWT) fiber bags according to the procedure described by Fukushima (personal communication). Briefly, dry matter samples were sequentially treated with neutral detergent and acid detergent solutions to obtain an acid detergent residue (ADR). Fiber bags containing ADR were treated with potassium permanganate solution (KMnO_4) in order to oxidize lignin and afterwards treated with 72% sulfuric acid solution (H_2SO_4) to hydrolyze cellulose, leaving behind cutin and mineral residue. Bags were dried in a forced draft oven at 105°C for 12 hours and weighed. Finally, the residue containing bags were incinerated at 450°C in a muffle furnace and cutin content was calculated as:

$$\text{Cutin\%} = ((\text{WaH} - (\text{Wal} - \text{Wc}) \times 100) \div \text{SiW})$$

where: WaH = weight after sulfuric acid hydrolysis, Wal = weight after incineration, Wc = weight of crucible and SiW = sample initial weight.

Crude protein (N% x 6.25) was determined in feed and TMR samples by the micro-kjeldahl procedure in a TE 036/1 Nitrogen Distiller (TECNAL®, São Paulo, Brazil). Ether extract (Ee) content of feeds and TMR was determined by petroleum ether extraction using a XT10 Extractor (ANKOM Technology Corp.®, Fairport, NY).

The quantification of Cr₂O₃ in feces samples was performed by visible light spectrophotometry (550 nm) according to the procedure proposed by Kimura *et al.*, (1957) with the modifications proposed by Graner (1972). Standard samples containing 0, 2, 4, 6, 8 and 10 mg of chromium per g DM were analyzed in triplicate, totalizing 18 standard samples. Pure Cr₂O₃ (99% trace metals basis; Solumix®) was employed to produce the standards. The concentration of Cr₂O₃ was analyzed and compared with the added amount of Cr₂O₃. The regression equation obtained was: $y = 0.002x + 0.06$ ($r^2 = 0.995$).

The quantification of TiO₂ in feces samples was performed by visible light spectrophotometry (410 nm) according to Myers *et al.*, (2004). To validate the accuracy of the TiO₂ analysis, a regression equation was created using the method proposed by Glindemann *et al.*, (2009). Standard samples containing 0, 1, 2, 3, 4, 5 and 6 mg of titanium per g DM were analyzed in triplicate, totalizing 21 standard samples. Pure TiO₂ (99.3% trace metals basis; Dinâmica® #1093) was employed to produce the standards. The concentration of TiO₂ was analyzed and compared with the added amount of TiO₂. The regression equation obtained was: $y = 0.057x + 0.04$ ($r^2 = 0.995$).

Calculations

Real DMI (RDMI) was calculated as daily feed offer minus daily orts (Equation 1) and Real FO (RFO) was obtained by 3 days (d16-d18) of TFC (24-hour collection and daily weighing of amount excreted). Total-tract apparent digestibility (TTAD) was calculated as RDMI minus RFO (Equation 2):

(1)

$$\text{RDMI(g/day)} = \text{daily feed offered(g/day)} - \text{orts collected(g/day)}$$

(2)

$$\text{TTAD (g/kg DM)} = \frac{\left[\text{RDMI} \left(\frac{\text{g}}{\text{day}} \right) - \text{RFO} \left(\frac{\text{g}}{\text{day}} \right) \right] \times 1000}{\text{RDMI}}$$

Equations (3) and (4) were used to estimate marker excretion and recovery rate. These equations derived from both BULK and GRAB samples. The overall recovery rate of markers was calculated from the total amount of marker given and the amount recovered during the fecal sampling period (d15-d19). Daily recovery rate of markers was calculated from d16 to d19:

(3)

$$\text{marker excretion} \left(\frac{\text{g}}{\text{day}} \right) = [\text{fecal marker}] \left(\frac{\text{g}}{\text{g}} \right) \times \text{RFO} \left(\frac{\text{g}}{\text{day}} \right)$$

(4)

$$\text{recovery rate of the marker} = \frac{\text{marker excreted} \left(\frac{\text{g}}{\text{day}} \right)}{\text{marker ingested} \left(\frac{\text{g}}{\text{day}} \right)}$$

Equation (5) was used to estimate FO using external markers and Equation (6) was used to estimate DMD using internal markers:

(5)

$$\text{FO estimated by external marker} \left(\frac{\text{g DM}}{\text{day}} \right) = \frac{\text{daily marker dose} \left(\frac{\text{g}}{\text{day}} \right)}{[\text{fecal marker}] \left(\frac{\text{g}}{\text{g DM}} \right)}$$

(6)

$$\text{DMD estimated by internal marker} \left(\frac{\text{g}}{\text{kg DM}} \right) = 100 - \left(100 \times \left(\frac{[\text{marker in feed}] \left(\frac{\text{g}}{\text{g DM}} \right)}{[\text{fecal marker}] \left(\frac{\text{g}}{\text{g DM}} \right)} \right) \right)$$

Equation (7) was used to estimate total voluntary intake from FO and DMD estimates obtained by equations (5) and (6). Total DMI was estimated for both BULK and GRAB samples:

(7)

$$\text{DMI estimated by markers} \left(\frac{\text{g}}{\text{day}} \right) = \frac{\text{FO} \left(\text{kg} \frac{\text{DM}}{\text{day}} \right)}{1 - \text{diet DMD}}$$

Statistical Analysis

Results were analyzed with SAS® 9.3 (SAS®, 2011), after verifying normality of residuals and homogeneity of variance. Normally distributed values for TTAD, RFO and RDMI, data was analyzed using PROC-MIXED, at 0.05 significance level, according to the model:

$$Y_{ijkl} = \mu + F_i + C_j + FC_{ij} + A_{ijk} + P_{ijkl} + \varepsilon_{ijkl},$$

where Y_{ijkl} = is the TTAD, RFO or RDMI value for animal k, on forage i, with F:C ratio j, in period l; μ = is the general constant; F_i = is the fixed effect of forage source; C_j = is the fixed effect of forage to concentrate ratio; FC_{ij} = is the interaction between forage source i and F:C ratio j (fixed); A_{ijk} = is the random effect of animal; P_{ijkl} is the random effect of period and ε_{ijkl} is the sampling error supposed to be NIID (normal independent and identically distributed).

Values for TTAD, RFO and RDMI were compared against DMD, FO and DMI estimates using PROC MIXED, at 0.05 significance level, according to the model:

$$Y_{ijklmn} = \mu + F_i + C_j + T_k + FC_{ij} + FT_{ik} + CT_{jk} + FCT_{ijk} + A_{ijkl} + P_{m(ij)} + \varepsilon_{ijklmn},$$

where Y_{ijklmn} = is the DMD, FO or DMI estimated in animal l, on treatment (marker versus sampling procedure) k; μ = is the general constant; F_i = is the fixed effect of forage; C_j = is the fixed effect of forage to concentrate ratio; T_k = is the fixed effect of treatments, being, for DMD: 4 internal markers combined with 3 sampling procedures plus one treatment considered the “real value” derived from TFC; for FO: 2 external markers combined with 3 sampling procedures plus one treatment considered the “real value” derived from TFC; for DMI: 8 marker pairs (1 external and 1 internal) combined with 3 sampling procedures plus one treatment considered the “real value” derived from daily weighing of all feed and orts; FC_{ij} = is the interaction between forage source i and F:C ratio j (fixed); FT_{ik} = is the interaction between forage source i and treatment k; CT_{jk} = is the interaction between forage to concentrate ratio j and treatment k; FCT_{ijk} = is the interaction between forage source i, forage to concentrate ratio j and treatment k; A_{ijkl} = is the random effect of animal; $P_{m(ij)}$ is the random effect of period and ε_{ijklmn} is the experimental error supposed to be NIID (normal independent and identically distributed).

In virtue of the significance of the triple interactions, slicing of these interactions was performed considering the treatments within each FC (forage source and forage:concentrate ratio combination). In this case, for DMD: contrast analysis was used to compare the TTAD (“real values”) versus sampling method estimates, within each internal marker; for FO: contrast analysis was used to compare RFO versus sampling method estimates, within each external marker and for DMI: contrast analysis was used to compare RDMI values versus sampling method estimates, within each marker pair (1 internal (DMD) plus 1 external (FO)).

Results

Chemical composition of feeds and diets is shown in Table 3. Mean RDMI (average $\pm$ SD) was 10.3 ± 1.2 kg DM/animal/day, equivalent to $2.4 \pm 0.1\%$ of BW for the silage 60:40 F:C (S64) diet, 10.9 ± 2.0 kg DM/animal/day, equivalent to $3.1 \pm 0.3\%$ of BW for the silage 40:60 F:C (S46) diet, 8.0 ± 1.3 kg DM/animal/day, equivalent to $1.7 \pm 0.1\%$ of BW for the hay 60:40 (H64) diet and 7.7 ± 0.7 kg DM/animal/day, equivalent to $1.8 \pm 0.2\%$ of BW for the hay 40:60 (H46) diet. Mean RFO as a % of BW (average $\pm$ SD) was 0.7 ± 0.05 , 0.9 ± 0.02 , 0.7 ± 0.05 and 0.6 ± 0.07 for the S64, S46, H64 and H46 diets, respectively. The chemical composition of the forages, concentrates and TMR diets were in accordance to what was formulated for the experiment (Tables 3 and 4). The main difference between the diets was the NDF concentration which ranged from 41-55%. The highest level of NDF from the experimental diets should not have been enough to limit DMI when pressure receptors are stimulated by rumen fill. Even so, a highly significant effect ($P < .0001$) for forage source on TTAD and RDMI was observed (Table 5).

Table 3. Chemical composition (g/kg of DM) of forages and concentrates

		Forage		Concentrates			
		Silage	Hay	I	II	III	IV
Dry matter (g/kg DM)		325.2 ^c	872.2 ^a	867.5 ^{ab}	865.3 ^{ab}	869.4 ^{ab}	863.1 ^b
Mineral matter		54.9 ^a	48.3 ^b	54.8 ^a	48.2 ^b	53.3 ^{ab}	43.2 ^b
NDF ¹		633.8 ^b	772.5 ^a	287.5 ^c	262.6 ^c	209.7 ^c	286.6 ^c
ADF ²		390.0 ^b	444.0 ^a	137.1 ^c	105.4 ^d	81.1 ^e	86.3 ^d
Cell wall		733.8 ^c	776.0 ^b	786.2 ^b	794.8 ^{ab}	816.0 ^a	822.8 ^a
ABL ³		153.4 ^a	133.4 ^a	35.0 ^b	31.0 ^b	23.3 ^b	33.6 ^b
Cutin		15.9 ^b	38.4 ^a	31.8 ^a	29.6 ^{ab}	15.4 ^b	10.9 ^b
iNDF ⁴		228.8 ^b	344.3 ^a	108.6 ^c	83.8 ^d	66.9 ^e	54.5 ^e
iADF ⁵		147.3 ^b	209.5 ^a	70.7 ^c	53.5 ^d	40.8 ^e	33.8 ^e
Crude Protein		84.5 ^e	87.4 ^e	204.0 ^a	171.3 ^b	143.0 ^d	151.6 ^c
Ether extract		55.7	72.3	95.7	48.3	76.8	59.6

¹ neutral detergent fiber; ² acid detergent fiber; ³ acetyl bromide lignin; ⁴ indigestible neutral detergent fiber; ⁵ indigestible acid detergent fiber. Within a line, mean values with common lower case superscript are not significantly different by Fisher's LSD ($P < 0.05$).

Table 4. Chemical composition (g/kg of DM), of the experimental diets

Forage F:C ratio	Corn silage		Tifton-85 hay	
	60:40	40:60	60:40	40:60
Dry matter (g/kg)	542.1 ^c	649.3 ^b	870.1 ^a	866.8 ^a
Mineral matter	54.9 ^a	50.9 ^b	50.3 ^b	45.2 ^c
NDF ¹	495.3 ^{ab}	411.1 ^b	547.3 ^a	481.0 ^{ab}
ADF ²	288.8 ^a	219.2 ^b	298.8 ^a	229.4 ^b
Cell wall	754.8 ^c	770.4 ^{bc}	792.0 ^{ab}	804.1 ^a
ABL ³	106.0	79.9	89.4	73.5
Cutin	22.3	24.1	29.2	21.9
iNDF ⁴	180.7 ^b	141.8 ^d	233.3 ^a	170.4 ^c
iADF ⁵	116.7 ^b	91.0 ^d	142.0 ^a	104.1 ^c
Crude Protein	132.3 ^a	136.6 ^a	109.6 ^c	125.9 ^b
Ether extract	71.7	51.2	74.1	64.7

¹ neutral detergent fiber; ² acid detergent fiber; ³ acetyl bromide lignin; ⁴ indigestible neutral detergent fiber; ⁵ indigestible acid detergent fiber. Within a line, mean values with common lower case superscript are not significantly different by Fisher's LSD (P<0.05).

Table 5. Effect of forage source (F, silage vs. hay) with two forage:concentrate ratios (C, 60:40 vs. 40:60) on total tract apparent digestibility (TTAD), real fecal output (RFO), real dry matter intake (RDMI) obtained by total feces collection (72 hours) in young bulls fed TMR diets

Forage (F) F:C ¹ (C)	Corn silage		Tifton-85 hay		SD _F ²	SD _C ³	P-value		
	60:40	40:60	60:40	40:60			F	C	FxC
TTAD	708.3	700.5	612.8	641.7	54.6	14.0	<.0001	0.327	0.174
RFO	2715.7	3561.1	2940.7	2912.3	308.9	308.9	0.023	<.0001	0.046
RDMI	9328.5	11920.8	7597.8	8126.1	1953.5	1103.3	<.0001	<.0001	0.035

¹ F:C, forage:concentrate ratio; ² standard deviation of forage source; ³ standard deviation of F:C.

Fecal recovery of markers

Mean fecal recovery of markers varied among them and are shown in Table 6. Significant effects were observed for both forage source and F:C ratio on both indigestible fibers (iNDF and iADF) and cutin. For iNDF and iADF, FR was incomplete (P=0.028 and P=0.024, respectively) on silage based diets and complete (P=0.625 and P=0.176, respectively) for the hay based diets. The FR of cutin was unexpectedly high, around 120%, not in accordance to what would be biologically expected. Both Cr₂O₃ and ABL were affected by forage source but not by F:C ratio. For Cr₂O₃, a significant effect (P=0.035) was observed for the interaction between forage source and F:C ratio, resulting in incomplete FR (P=0.019) for the corn silage 40:60 diet and "complete" FR (P=0.110, P=0.102, P=0.154) for the corn silage 60:40, hay 60:40 and hay 40:60 diets, respectively. For ABL, FR was only complete (P=0.889 and P=0.476) on the hay diets and

around 50% for the corn silage diets ($P<.0001$). Contrarily, TiO_2 was unaffected by forage source but suffered an effect by F:C ratio, which led to, as with cutin, to FR higher than 1, around 140%, not consistent to what would be expected.

Table 6. Effect of forage source (F, silage vs. hay) with two forage:concentrate ratios (C, 60:40 vs. 40:60) on fecal recovery (FR) of external markers chromic oxide (Cr_2O_3), titanium dioxide (TiO_2) and of internal markers acetyl bromide lignin (ABL), indigestible neutral detergent fiber (iNDF), indigestible acid detergent fiber (iADF) and cutin in young bulls fed TMR diets

Forage (F)	Corn silage		Tifton-85 hay		SD _F ²	SD _C ³	P-value			
	F:C ¹ (C)	60:40	40:60	60:40			40:60	F	C	FxC
FR										
Cr ₂ O ₃		0.80a	0.71b	0.80a	0.83a	0.04	0.04	0.008	0.101	0.035
TiO ₂		1.31	1.57	1.30	1.40	0.06	0.13	0.109	0.001	0.370
ABL		0.51	0.52	0.98	0.91	0.30	0.03	<.0001	0.104	0.520
iNDF		0.73	0.72	0.97	1.06	0.21	0.04	<.0001	0.026	0.079
iADF		0.81	0.80	1.06	1.17	0.22	0.04	<.0001	0.019	0.057
Cutin		1.16	0.89	1.45	1.38	0.28	0.12	<.0001	<.0001	0.537

¹ F:C, forage:concentrate ratio; ² standard deviation of forage source; ³ standard deviation of F:C. Within a line, mean values with common lower case superscript are not significantly different by Fisher's LSD ($P<0.05$).

Prediction of DMD based on fecal concentration of internal markers

The mean DMD estimates are shown in Table 7. No effect ($P=0.435$) was observed for sampling procedure on DMD estimates. Significant effects for marker ($P=0.021$), diet ($P<.0001$) and the interaction diet x marker x method ($P<.0001$) were observed. All markers produced accurate DMD estimates when compared with TTAD. Estimates derived from the GRAB 9x3 fecal sampling procedure were consistently accurate, independently of the internal marker used. Indigestible fibers (iNDF and iADF) consistently produced accurate estimates independently of the fecal sampling procedure.

Table 7. Total tract apparent digestibility (TTAD) (mean $\pm$ SE) (g/kg DM) and dry matter digestibility (DMD) (mean $\pm$ SE) (g/kg DM) estimates derived from internal markers on two GRAB (4x4 and 9x3) and one BULK (72h) fecal sampling procedures

Marker	Forage				
	Corn silage		F:C ratio	Tifton-85 hay	
	60:40	40:60		60:40	40:60
Sampling ¹					
ABL ²					
4x4	702.0 $\pm$ 9.2	705.7 $\pm$ 9.4		599.4 $\pm$ 21.3	658.9 $\pm$ 8.7
9x3	703.9 $\pm$ 8.6	734.1 $\pm$ 9.7		641.0 $\pm$ 14.6	677.3 $\pm$ 6.3
72h	691.1 $\pm$ 15.6	608.5* $\pm$ 17.3		550.1* $\pm$ 25.4	518.5* $\pm$ 37.4
Cutin					
4x4	622.2* $\pm$ 29.3	676.4 $\pm$ 26.1		612.9 $\pm$ 20.0	662.0 $\pm$ 18.9
9x3	673.7 $\pm$ 26.7	659.7 $\pm$ 28.1		623.2* $\pm$ 52.8	543.2 $\pm$ 54.7
72h	744.2 $\pm$ 11.1	696.8 $\pm$ 19.7		565.9* $\pm$ 38.3	606.2 $\pm$ 48.3
iNDF ³					
4x4	695.0 $\pm$ 9.3	693.9 $\pm$ 5.8		588.7 $\pm$ 7.4	615.6 $\pm$ 4.6
9x3	721.4 $\pm$ 5.2	685.9 $\pm$ 8.3		595.0 $\pm$ 13.7	620.7 $\pm$ 7.3
72h	719.3 $\pm$ 6.2	703.4 $\pm$ 11.8		637.9 $\pm$ 4.0	641.1 $\pm$ 8.2
iADF ⁴					
4x4	688.3 $\pm$ 11.6	670.0 $\pm$ 6.3		578.4 $\pm$ 9.4	621.4 $\pm$ 6.4
9x3	722.2 $\pm$ 6.2	687.2 $\pm$ 9.4		607.7 $\pm$ 7.9	619.2 $\pm$ 8.8
72h	718.4 $\pm$ 6.5	703.1 $\pm$ 12.8		638.1 $\pm$ 4.9	644.3 $\pm$ 8.5
TTAD	708.3 $\pm$ 11.5	700.5 $\pm$ 8.8		612.8 $\pm$ 8.8	641.7 $\pm$ 14.4

¹ sampling procedure; ² acetyl bromide lignin; ³ indigestible neutral detergent fiber; ⁴ indigestible acid detergent fiber. Within columns, means followed by *, are significantly different from TTAD for diet according to contrast analysis (P<0.05).

Prediction of FO based on fecal concentration of external markers

The mean FO estimates are shown in Table 8. A highly significant effect (P<.0001) for diet was observed. No significant effect for marker (P=0.232) and a tendency (P=0.062) were observed on FO estimates. For the interaction diet x sampling procedure x marker, a significant effect (P=0.017) was observed. Both external markers produced accurate FO estimates when compared to RFO. The GRAB 9X3 sampling procedure was, as with DMD estimates, consistently accurate for estimating FO. The BULK 72h sampling procedure failed to produced accurate estimates with TiO₂ on the hay 40:60 diet, with Cr₂O₃ on the H64 diet and had a tendency (p=0.074) on the silage 60:40 diet.

Table 8. Real fecal output (RFO) (mean $\pm$ SE) (g DM/day) and fecal output (FO) (mean $\pm$ SE) (g DM/day) estimates derived from external markers on two GRAB (4x4 and 9x3) and one BULK (72h) fecal sampling procedures

Marker	Forage				
	Corn silage		F:C ratio	Tifton-85 hay	
S. PROC ¹	60:40	40:60		60:40	40:60
Cr ₂ O ₃					
4x4	2712.4 $\pm$ 183.0	3761.0 $\pm$ 318.1		3008.0 $\pm$ 178.4	2790.6 $\pm$ 120.6
9x3	2618.4 $\pm$ 124.2	3732.8 $\pm$ 207.4		2749.3 $\pm$ 103.7	3091.9 $\pm$ 174.0
72h	3107.0 ^T $\pm$ 178.9	3801.3 $\pm$ 210.2		3416.4* $\pm$ 216.6	2927.7 $\pm$ 88.2
TiO ₂					
4x4	3157.3* $\pm$ 163.7	3338.6 $\pm$ 122.8		3180.4 $\pm$ 171.1	3199.6 $\pm$ 145.5
9x3	2522.5 $\pm$ 140.0	3586.3 $\pm$ 240.8		2946.0 $\pm$ 218.6	3613.7 $\pm$ 326.9
72h	3059.9 $\pm$ 136.9	3909.5 $\pm$ 253.0		3508.0 $\pm$ 365.7	2787.9* $\pm$ 265.0
RFO	2715.7 $\pm$ 114.8	3561.1 $\pm$ 120.5		2940.7 $\pm$ 101.7	2912.3 $\pm$ 150.3

¹ sampling procedure. Within columns, means followed by *, are significantly different from RFO for diet according to contrast analysis (P<0.05).

Prediction of DMI based on internal and external marker pairs

The mean DMI estimates are shown in Table 9. Highly significant effects for forage source (P<.0001), F:C ratio (P<.0001) and treatment (marker pair x sampling procedure) (P<.0001) were observed. Interactions between forage source x F:C ratio, forage source x treatment and F:C ratio x treatment were also significant with P=0.037, P<.002 and P<.0001, respectively. For the triple interaction forage source x F:C ratio x treatment, a tendency (P=0.078) was observed. Less DMI estimates from Cr₂O₃ marker pairs were significantly different from their reciprocal RDMI values than the TiO₂ derived marker pairs. Among the internal markers, the indigestible fibers produced accurate estimates (not significantly different to the reciprocal RDMI value) more constantly than ABL and cutin. Between the sampling procedures, the GRAB 9x3 and GRAB 4x4 derived DMI estimates were more often accurate than the BULK 72h sampling procedure. None of the marker pairs or sampling procedures produced accurate DMI estimates for all diets.

Table 9. Real dry matter intake (RDMI) (mean $\pm$ SE) (g DM/day) and dry matter intake (DMI) (mean $\pm$ SE) (g DM/day) estimates derived from 1 internal + 1 external marker pairs on two GRAB (4x4 and 9x3) and one BULK (72h) fecal sampling procedures

Marker Sampling ¹	Forage				
	Corn silage		F:C ratio	Tifton-85 hay	
	60:40	40:60		60:40	40:60
Cr ₂ O ₃ +ABL ²					
4x4	9205.8 $\pm$ 610.2	12210.0 $\pm$ 1113.7		7644.0 $\pm$ 413.3	8216.4 $\pm$ 364.1
9x3	8890.2 $\pm$ 383.8	14763.0* $\pm$ 1298.2		7862.1 $\pm$ 433.2	9558.0* $\pm$ 488.3
72h	10187.0 $\pm$ 500.9	9893.8* $\pm$ 559.1		7661.9 $\pm$ 322.5	6595.1* $\pm$ 444.3
Cr ₂ O ₃ +Cutin					
4x4	7748.9* $\pm$ 601.8	12308.0 $\pm$ 926.4		8349.8 $\pm$ 771.5	8779.4 $\pm$ 669.1
9x3	8784.9 $\pm$ 652.5	12005.0 $\pm$ 1019.7		8415.5 $\pm$ 857.3	8627.5 $\pm$ 1255.7
72h	12595.0* $\pm$ 595.0	13331.0 $\pm$ 987.0		9347.5* $\pm$ 1102.4	9145.7 $\pm$ 1109.0
Cr ₂ O ₃ +iNDF ³					
4x4	883.6 $\pm$ 450.2	11672.0 $\pm$ 994.9		7294.6 $\pm$ 399.5	7253.8 $\pm$ 300.7
9x3	9355.8 $\pm$ 335.0	11881.0 $\pm$ 559.5		6865.4 $\pm$ 292.9	8113.4 $\pm$ 380.8
72h	10797.0* $\pm$ 521.7	12441.0 $\pm$ 803.4		9436.4* $\pm$ 597.1	8221.2 $\pm$ 287.9
Cr ₂ O ₃ +iADF ⁴					
4x4	8676.7 $\pm$ 443.3	11892.0 $\pm$ 1000.1		7131.4 $\pm$ 400.4	7376.9 $\pm$ 317.4
9x3	9402.0 $\pm$ 357.3	11950.0 $\pm$ 555.0		7030.1 $\pm$ 268.9	8112.8 $\pm$ 409.0
72h	10806.0 $\pm$ 579.4	12452.0 $\pm$ 820.6		9443.3* $\pm$ 596.9	8303.1 $\pm$ 301.9
TiO ₂ +ABL ²					
4x4	10817.0* $\pm$ 730.5	11578.0 $\pm$ 590.4		8326.0 $\pm$ 681.6	9489.9 $\pm$ 519.9
9x3	8595.1 $\pm$ 490.2	13753.0* $\pm$ 1023.5		8166.5 $\pm$ 439.6	11176.0* $\pm$ 969.1
72h	10512.0 $\pm$ 804.8	10771.0 $\pm$ 847.5		7563.3 $\pm$ 465.2	6070.7* $\pm$ 550.5
TiO ₂ +Cutin					
4x4	9117.7 $\pm$ 706.3	11451.0 $\pm$ 977.0		8635.7 $\pm$ 620.7	9903.0* $\pm$ 652.1
9x3	8511.4 $\pm$ 730.2	10830.0 $\pm$ 507.4		8878.5 $\pm$ 1781.3	9358.1 $\pm$ 1981.9
72h	12271.0* $\pm$ 720.6	14647.0* $\pm$ 1258.0		9948.4* $\pm$ 1606.1	9439.8 $\pm$ 1446.6
TiO ₂ +iNDF ³					
4x4	10560.0 $\pm$ 675.0	11009.0 $\pm$ 500.5		7783.8 $\pm$ 465.0	8382.9 $\pm$ 437.8
9x3	9131.0 $\pm$ 552.6	11334.0 $\pm$ 595.3		7347.5 $\pm$ 561.9	9464.3 $\pm$ 800.2
72h	11212.0* $\pm$ 701.0	13815.0* $\pm$ 773.7		9617.7* $\pm$ 962.2	7759.6 $\pm$ 712.8
TiO ₂ +iADF ⁴					
4x4	10404.0 $\pm$ 687.6	11264.0 $\pm$ 535.0		7622.5 $\pm$ 470.5	8533.4 $\pm$ 462.5
9x3	9179.2 $\pm$ 564.6	11407.0 $\pm$ 607.5		7514.5 $\pm$ 546.0	9454.4 $\pm$ 812.5
72h	11158.0* $\pm$ 671.4	13901.0* $\pm$ 807.4		9607.9* $\pm$ 950.1	7828.5 $\pm$ 710.8
RDMI	9328.5 $\pm$ 198.7	11921.0 $\pm$ 310.2		7597.8 $\pm$ 201.3	8126.1 $\pm$ 265.7

¹ sampling procedure; ² acetyl bromide lignin; ³ indigestible neutral detergent fiber; ⁴ indigestible acid detergent fiber. Within columns, means followed by *, are significantly different from TTAD for diet according to contrast analysis (P<0.05).

Discussion

Fecal recovery of markers

Fecal recovery varied widely among markers and ranged from 0.71-0.83 for Cr_2O_3 to 1.30-1.57 for TiO_2 . No reports were found on the use of cutin as a marker in ruminant digestion studies and therefore our data is novel to this field of research. Siqueira *et al.*, (2009) used cutin as DMD marker in horses consuming four different diets and observed FR around 1.51. The FR in horses was very close to what was observed in this study. The interaction between the marker molecule and digesta around it, will determine the retention time (T_R) and passage rate (Q_P) of the marker and ultimately affect what fraction of what is ingested will be excreted in feces over a 24-hour period. Njaa (1961) reported that mean FR less than one could be attributed to inaccuracy of the analysis. A possible explanation for incomplete FR of internal markers is that when vegetable cell walls are treated with cellulases, the components that ester-link ferulic or p-coumaric acids to a hemicellulosic side chain of xylose and arabinose are released (MUELLER-HARVEY *et al.*, 1986). These soluble hemicellulosic complexes do not appear to be digestible; they undergo precipitation on reaching gastric acidity in the lower tract and are recoverable in feces (NEILSON and RICHARDS, 1978). Although the studies did not consider the possibility that rumen bacteria might adapt, and metabolism of monomeric units has been reported (FUKUSHIMA *et al.*, 1991).

Several authors (KOTB and LUCKEY, 1972; FAHEY and JUNG, 1983) have long described the properties of an ideal marker. Among them, one of the most important is to be totally recovered in a 24-hour period. Many studies have been conducted over the last 3 decades using TiO_2 and the FR observed for it has been around 100%. Based on these findings the routinary use of TiO_2 in ruminal digestion trails has been suggested. Our findings do not support the established concept that FR of TiO_2 is 100%. The results of this study show that FR is higher than 100% and varies between diets with different F:C ratio. These findings were contrary to those of Brandt and Allam, (1987); Hafez *et al.*, (1988) and Titgemeyer *et al.*, (2001), who observed mean FR for TiO_2 of 0.96-0.99 in sheep and 0.96-1.02, 0.90-0.95 in dairy cattle, respectively. Glindemann *et al.*, (2009) found FR higher than 1 and attributed this to possible ingestion of soil which contained TiO_2 and therefore would increase the fecal concentration. Our diets were composed of corn silage, hay and concentrates that were well preserved and packaged correctly, so the risk of soil contamination is low.

Mean FR of Cr_2O_3 was lower than 100% in this study. Benvennuti *et al.*, (2014) worked with Brahman steers fed leafy or steamy grass hay and observed FR of 0.92 to 1.03. Since Cr_2O_3

has been widely used as a marker in digestions studies, various FR have been reported for it: in horses FR ranged from 0.712 to 1.031 (OLIVEIRA *et al.*, 2003; SIQUEIRA *et al.*, 2009; LANZETTA *et al.*, 2009), in sheep from 0.80 to 0.92 (RODRIGUES *et al.*, 2010), in dairy cattle from 0.87 to 0.89 (DELAGARDE *et al.*, 2010) and in steers from 0.85 to 1.05 (SAMPAIO *et al.*, 2011a,b). Paixão *et al.*, (2007) fed Cr_2O_3 to steers and observed total fecal recovery of the administered dose. Our results are not in accordance to their findings and suggest that Cr_2O_3 is not completely recovered in feces when administered orally to steers fed corn silage and Tifton-85 hay diets with different F:C ratios. For Cr_2O_3 to be used as a marker, it is necessary to perform TCF on at least some of the animals to establish the FR.

This study measured lignin as ABL, a procedure in which no lignin loss occurs during sample preparation and that is less subject to gravimetric errors because of its spectrophotometric nature. Mean ABL FR ranged from 0.51 to 0.98 and was significantly different among forage sources. These results suggest that fecal recovery of lignin is not stable therefore ABL would not be a suitable marker. Further research is necessary to show how ABL can be applied to ruminant nutrition studies and how appropriate an internal marker for estimating DMD it can be.

Indigestible neutral detergent and acid detergent fibers are two of the most extensively used internal markers today. Mean FR ranging from 0.50 to 1.21 (BARROS *et al.*, 2009; KOZLOSKY *et al.*, 2009; RODRIGUES *et al.*, 2010; SAMPAIO *et al.*, 2011a and LEE and HRISTOV, 2013) and from 0.80 to 1.21 (OLIVEIRA Jr. *et al.*, 2004; RODRIGUES *et al.*, 2010; SAMPAIO *et al.*, 2011a) for iNDF and iADF, respectively. Oliveira *et al.*, (2003) worked with horses and observed FR of 1.08 to 1.34 and up to 1.54 for iNDF and iADF, respectively. In this study, mean FR for iNDF and iADF were 0.72 to 1.06 and 0.80 to 1.17, respectively. For these two markers, both forage source and F:C ratio had significant effect on FR. More so, the forage source *versus* F:C ratio interaction had a tendency ($P=0.071$ and $P=0.057$, for iNDF and iADF, respectively) on both markers. These results do not agree with previous findings of other authors that observed or assumed complete FR for these internal markers. The disparity of results for different experiments is, at least in part, due to differences in incubation time of the samples for obtaining the fibrous fractions. Our study followed the procedure proposed by Krizsan and Huhtanen (2013) who compared various incubation times (144h, 240h and 288h) and reported that at least 12 days (288h) of incubation were necessary to isolate the completely indigestible fiber pool in feeds fed to dairy cows. The results in this study suggest that iNDF and iADF can be used as internal markers for ruminant digestion studies, but FR must be determined by TFC, prior to or during the experimental period.

Prediction of DMD based on fecal concentration of internal markers

All internal markers were able to produce accurate DMD estimates when compared to TTAD. The indigestible fibers, iNDF and iADF, produced accurate estimates for all diets independently of the fecal sampling procedure. However, values for DMD were under-estimated and differed significantly from TTAD when the BULK 72h sampling procedure and ABL or cutin were used in some diets. When fecal concentration was measured in GRAB samples, both markers produced accurate DMD estimates in comparison to TTAD. Moreover, ABL was accurate with both GRAB sampling procedures in all diets. These two molecules (lignin and cutin) are measured together by some lignin analysis procedures. A possible explanation for the varying, inaccurate DMD estimates produced by these two markers would be their low concentration in both the feed and feces samples. Lower concentrations make chemical analysis less reliable because of increased difficulty to detect differences in such small amounts of residue that are left behind for weighing after the procedures are performed. In this study, lignin was measured as ABL and thus gravimetric related issues would not be a problem. The cutin procedure that is being proposed is gravimetric and subject to these limitations. After researching literature, nor ABL or cutin (by the procedure being proposed) had ever been used in ruminant digestion studies and therefore more research is necessary and worthwhile before discarding them as possible internal markers.

Mean DMD estimates produced by iNDF and iADF fecal concentrations did not differ from TTAD under any of the sampling procedures or diets. Detmann *et al.*, (2001) reported more accurate DMD estimates when using iNDF compared to iADF. Berchielli *et al.*, (2005) reported conflicting results when using these indigestible fractions, in response to the incubation procedure as was mentioned before when discussing FR. With heifers fed fresh sugar-cane iNDF produced better DMD estimates than iADF but, when corn silage was fed to dairy cows, iADF estimates were more accurate than those of iNDF (FERREIRA *et al.*, 2008). Our findings suggest that both iNDF and iADF are suitable internal markers for estimating DMD under both BULK and GRAB sampling procedures.

Prediction of FO based on fecal concentration of external markers

Both Cr_2O_3 and TiO_2 were able to produce accurate FO estimates when compared to RFO values in this study. Only under the 72h BULK sampling procedure, on the hay 60:40 diet was the FO estimate not accurate. A tendency ($P=0.074$) was also observed on the silage 60:40 diet when Cr_2O_3 was used to estimate FO. These differences could be explained by the significant interaction between diet x forage source x F:C ratio that was observed for FO. Also, FR of Cr_2O_3

was incomplete which would lead to overestimation of FO. Estimates for FO derived from fecal concentration of TiO_2 were inaccurate for the hay 40:60 diet on BULK 72h sampling and for the silage 60:40 diet on the GRAB 4x4 sampling procedure. Still, most of the FO estimates produced by TiO_2 were accurate even though the FR was 150% which should have resulted in underestimation. When comparing Cr_2O_3 and TiO_2 in steers, Titgemeyer *et al.*, (2001) observed that both markers significantly underestimated FO. Delagarde *et al.*, (2010) reported accurate FO estimates, derived both from BULK and GRAB sampling procedures, positively correlated to RFO (R^2 from 0.86 to 0.96) when using Cr_2O_3 as a marker in dairy cows. A more recent study that fed Cr_2O_3 to steers, reported that FO estimates from GRAB samples significantly higher than RFO (BENVENUTTI *et al.*, 2014). Our results suggest that Cr_2O_3 and TiO_2 can be used as a marker for estimating FO in cattle. Our results show that GRAB sampling procedures produce accurate FO estimates with these two external markers and therefore TFC is not strictly necessary. Although, it is recommended that FR of markers is determined by trial TFC prior to or concomitant with the study. Interestingly, the BULK 72h sampling procedure was the less accurate among the procedures being studied. This procedure requires collecting all feces in a 24-hour period and then homogenizing the material to take a representative sample. The process is not simple and therefore the concentration of markers in feces may vary, altering the results. Error associated with homogenization of samples, will have greater impact on the BULK sampling procedure because of the volume collected prior to sampling. The GRAB sampling procedures on the other hand, do not have this problem because small quantities (150-500 g max.) are usually completely used to make up the composite sample.

During the last decade or so TiO_2 has received increasing attention by researchers in response to the potential health threats that have been attributed to Cr_2O_3 (MYERS *et al.*, 2006). Studies have reported that FO estimates are overestimated, not significantly different from RFO (TITGEMEYER *et al.*, 2001) or underestimated (GLINDEMANN *et al.*, 2009). The variety of results appear to be caused by differences in FR which directly affects the FO estimates. In our study, all but two, of the TiO_2 derived FO estimates were not significantly different from RFO. This was unexpected due to the high (140%) FR observed for this marker. Once again, this shows the importance of TFC to have the “real” FR and correct fecal concentration values.

Prediction of DMI based on internal and external marker pairs

Estimates of DMI varied among marker pairs, sampling procedures and diets. All marker pairs produced accurate DMI estimates, when compared to RDMI, but not under all fecal sampling procedures. The Cr_2O_3 +iNDF and Cr_2O_3 +iADF marker pairs were the most accurate among

pairs which is consequent with the accurate DMD and FI estimates that the markers produced individually. When iNDF and iADF were paired with TiO_2 , DMD estimates were accurate on the GRAB sampling procedures but not for the BULK sampling procedure. This is similar to what was observed for FO estimates from TiO_2 and maybe be explained by the same difficulty of obtaining representative samples from BULK samples.

Previous studies with TiO_2 (TITGEMEYER *et al.*, 2001) and Cr_2O_3 (RODRIGUES *et al.*, 2006 and BENVENUTTI *et al.*, 2014) observed that DMI estimates were lower than or equal to RDMI for these markers, respectively. In their studies, the authors used each marker to estimate both DMD and FO, and, from these values also estimate DMI. They reported as the main cause for the inaccuracy of DMI estimates the FR different than 100% which resulted in under or over estimation of FO. The higher the FR the lower FO estimates that will be obtained and *vice-versa*. Given an accurate DMD estimate (or TTAD), if FO is underestimated then DMI will also be lower than RDMI. The same thing happens when DMD is underestimated at a given FO (or RFO). In our study, we observed FR different than 100% for all markers calculated from RFO. Once fecal marker concentration was corrected for FR, accurate DMD and FO estimates were obtained from internal and external markers, respectively. Accurate DMD and FO estimates led to accurate DMI estimates for internal + external marker pairs. This highlights the importance of performing FR trials prior to DMI experiments or having a representative group of animals to determine FR simultaneously as a pasture based or collective housing experiment is conducted.

Conclusions

In young Nelore bulls fed corn silage or Tifton-85 hay based diets with varying F:C ratios, the average FR of internal markers ABL, iNDF, iADF and cutin was different from 100%. Lower for ABL, iNDF and iADF, and higher for cutin. After correction, iNDF and iADF produced accurate DMD estimates under GRAB and BULK fecal sampling procedures. As long as RFO is determined, these two markers are adequate to be used in ruminal digestion studies. External markers Cr_2O_3 and TiO_2 had FR lower and higher than 100%, respectively. As with internal markers, the correction of fecal concentration by FR resulted in accurate FO estimates. These two markers are also adequate for use in ruminant digestion studies, with preference being given to Cr_2O_3 , according to our results. Finally, the combination of internal and external markers to estimate DMI produced satisfactory and accurate results and can definitely be recommended for use in ruminant digestion studies. Preference is given to Cr_2O_3 based pairs with iNDF and iADF. Internal markers ABL and cutin deserve more attention and further research in order to better understand how they can be useful in ruminant nutrition studies.

GRAB sampling procedures yielded accurate DMD, FO and DMI estimates were as good as or better than BULK sampling procedures. They are also much less invasive and labor demanding, which make them an excellent alternative to the classical BULK sampling procedure from TFC. The GRAB 4x4 daylight procedure can be adapted to various management practices like milking and/or feeding schedules. GRAB sampling designs deserve more research in order to propose new and better ones or to further validate the existing ones. The double marker procedure is an available tool for ruminant nutrition studies that may be adopted by researchers, to use in ruminant digestion trials.

Regardless of the fecal sampling method and the composition of the diet, it is necessary to establish the FR of the markers to be used in each experiment, in order to obtain correct estimates. Consistently throughout the experiments, FR was lower than and higher than unity for internal and external markers, respectively. Corrected FO estimates were accurate for Cr₂O₃ on various diets under GRAB sampling. The same was not true for TiO₂, which even after FR correction was applied failed to produce accurate FO estimates consistently. The method of dosing the external markers has to allow the animals to display normal feeding behavior and not affect performance. GRAB sampling procedures can replace TFC (once FR is established) which will open new possibilities for pasture based or collectively housed animals. Accurately knowing how much an animal eats under a given situation is a useful tool.

Final Remarks

None of the markers used in this study had stable FR after the 10 days of adaptation. Consistently throughout the experiments, FR was lower than and higher than unity for internal and external markers, respectively. Variation in fecal marker concentration was identified as the major issue in regard to the accuracy of marker estimates. Regardless of the fecal sampling method and the composition of the diet, it is necessary to establish the FR of the markers to be used in each experiment, in order to obtain correct estimates. A short preliminary study can be performed using one animal *per* experimental diet in order to determine FR. Once FR is established, experiments can use a greater number of animals, housed in group or on pasture and use different fecal GRAB sampling procedures because TFC is not necessary. Within each laboratory, this information should be kept and organized, in order to create a FR log for the markers used. This will allow comparison between diets and possible establishment of a standard FR for markers in each laboratory conditions.

In spite of incomplete FR, corrected FO estimates were accurate for Cr_2O_3 on various diets under GRAB sampling. The same was not true for TiO_2 , which even after FR correction was applied failed to produce accurate FO estimates consistently. Various authors have reported complete FR for TiO_2 under diverse conditions. Our observations from two experiments where TFC was performed do not support these statements. When FR of TiO_2 is calculated from FO estimates (like on experiment 2) in some cases FR does not differ from unity, but when it is calculated from RFO obtained by TFC, FR is always higher than unity. Since it is legal to include TiO_2 in commercial diets and it is well known to the scientific community, more research with TiO_2 is important. Nonetheless, our observations do not support its use as an external marker for estimating FO in ruminants. On the other hand, Cr_2O_3 can be used to estimate FO and concomitantly with iADF to estimate DMI, as long as FR for both markers is determined by TFC trial.

For internal markers, ABL, cutin, iNDF, and iADF the FR situation was similar to external markers. None of the markers had complete FR consistently throughout the 3 experiments. Cutin and ABL had lower than unity FR on all diets and failed to produce accurate DMD estimates which resulted in inaccurate DMI estimates when either one was paired with Cr_2O_3 or TiO_2 . Of the indigestible fibers, iNDF and iADF, the later was more accurate in estimating DMI when paired with Cr_2O_3 . Still, iNDF produced accurate DMD estimates on different diets, confirming its potential as an internal marker for ruminant studies.

From results of experiment 2 it is possible to infer that twice a day fecal GRAB sampling is not enough to obtain a representative daily sample. These results varied greatly and DMI estimates for all marker pairs were significantly different to RDMI measured by the Grow Safe® System. Also from this experiment, we observed that the need to pass all animals through the chute, twice a day for dosing external markers and fecal sampling, has a strong influence in feeding behavior and therefore in animal performance. Although the method for dosing the animals worked correctly, it should not be recommended because of the above observations. Less invasive procedures, like mixing the external markers in the supplements should be given more attention and be subject of study, since external marker dosing is probably the second major issue for this type of studies. More research is necessary in this area and the development of slow release capsules (formerly available) would greatly contribute to the task.

Limitations

Our study used 6 of the most commonly used markers today under different conditions. Still, there are several other substances available that have been reported to work properly for estimating DMI in ruminants. Of these substances, alkanes have been receiving major attention and the published reports consistently support their use for ruminant digestion studies. Because of budgetary issues, our study did not compare alkanes and conventional markers so no conclusions can be drawn in regards to alkanes.

Take home messages

Marker technology for ruminant studies is available and widely documented. Although results vary, data are useful enough to provide important information about DMD, FO and DMI. It is necessary to determine real (TFC) FR for each marker that will be used. The method of dosing the external markers has to allow the animals to display normal feeding behavior and not affect performance. GRAB sampling procedures can replace TFC (once FR is established) which will open new possibilities for pasture based or collectively housed animals. Obtaining accurate DMI estimates will allow for better supplement formulation, more cost efficient feeding programs, other ways of monitoring ruminal health and ultimately a better understanding of the ruminant GIT. In a world where resources are becoming limited and where environmental concerns gain political strength every day, it is important to improve economic efficiency of animal protein production. Accurately knowing how much an animal eats under a given situation is a useful tool for this purpose.

References

ASSOCIATION OF AMERICAN FEED CONTROL OFFICIALS (AAFCO). Official Publication, Atlanta, GA, 1996.

ASTIGARRAGA, L. Técnicas para la medición del consumo de rumiantes en pastoreo. In: **Simpósio sobre avaliação de pastagens com animais**, Maringá. Anais... p.1-23. Maringá: UEM, 1997.

BARBOSA, A. M. (2005). **Período de coleta de urina e de fezes para avaliação da excreção de creatinina, produção microbiana e digestibilidade aparente dos nutrientes em Nelore** (Doctoral dissertation, Universidade Federal de Viçosa).

BARROS, E. D., FONTES, C. D. A., DETMANN, E., VIEIRA, R. A. M., HENRIQUES, L. T.; FERNANDES, A. M. Vícios na estimação da excreção fecal utilizando indicadores internos e óxido crômico em ensaios de digestão com ruminantes. **Revista Brasileira de Zootecnia**, v. 38, no. 10, p. 2015-2020, 2009.

BENVENUTI, M.A.; COATES, D. B.; BINDELLE, J.; POPPI, D. P.; GORDON, I. J. Can faecal markers detect a short term reduction in forage intake by cattle? **Animal Feed Science and Technology**, v. 194, p. 44–57, 2014.

BERCHIELLI, T.T.; ANDRADE, P.; FURLAN, C.L. Avaliação dos indicadores internos em ensaios de digestibilidade. **Revista Brasileira de Zootecnia**, v.29, n.3, p.830-833, 2000.

BERCHIELLI, T.T.; OLIVEIRA, S.G.; GARCIA, A.V. Aplicação de técnicas para estudos de ingestão, composição da dieta e digestibilidade. **Archives of Veterinary Science**, v.10, p.29-40, 2005.

BERCHIELLI, T.T.; PIRES, A.V.; OLIVEIRA, S.G. **Nutrição de Ruminantes**. 2ª edição. Jaboticabal : Funep, 2011. 616p.

BOWMAN, J. G. P.; SOWELL, B. F. Delivery method and supplement consumption by grazing ruminants: a review. **Journal of Animal Science**, v.75, p. 543-550, 1997.

BRANDT, M.; ALLAM, S. M. Analytik von Titandioxid im Darminhalt und Kot nach Kjeldahlaufschluß. **Archives of Animal Nutrition**, v. 37, p. 453–454, 1987. .

BURNS, J. C.; POND, K. R.; FISHER, P. S. (1994). Measurement of forage intake. In: **Forage Quality, Evaluation and Utilization** (Ed. G. C. FAHEY JR), PP. 494-532. Madison, WI.

BUXTON, D. R.; RUSSELL, J. R. Lignin constituents and cell-wall digestibility of grass and legume stems. **Crop Science**, v. 28, n. 3, p. 553-558, May-Jun 1988.

CASALI, A.O.; DETMANN, E.; VALADARES FILHO, S.C.; PEREIRA, J.C.; HENRIQUES, L.T.; FREITAS, S.G.; PAULINO, M.F. (2008). Influência do tempo de incubação e do tamanho de partículas sobre os teores de compostos indigestíveis em alimentos e fezes bovinas obtidos por procedimentos *in situ*. **Revista Brasileira de Zootecnia**, v.37, n.2, p.335-342.

CORBET, J. L. Measurement of forage intake of grazing animals. In: WHEELER, J. L.; MOCHRIE, R. D. (Eds.). **Forage Evaluation: Concepts and Techniques**. Victoria: CSIRO, 1980. P. 287-298.

CRAMPTON, E. W. Weaknesses in feedstuff analysis. **Feedstuffs**, 1940, vol. 12, p. 16.

COSTA, L.T.; SILVA, F.F.; VELOSO, C.M.; PIRES, A.J.V.; ROCHA NETO, A.L.; MENDES, F.B.L.; RODRIGUES, E.S.O.; SILVA, V.L. Análise econômica da adição de níveis crescentes de concentrado em dietas para vacas leiteiras mestiças alimentadas com cana-de-açúcar. **Revista Brasileira de Zootecnia**, v.11, n.5, p.1155-1162, 2011.

DECRUYENAERE, V.; BULDGEN, A.; STILMANT, D. Factors affecting intake by grazing ruminants and related quantification methods: a review. **Biotechnology, Agronomy and Society and Environment**, v.13, n. 4, p. 559-573, 2009.

DELAGARDE, R.; PEYRAUD, J. L.; DELABY, L. Influence of herbage allowance on intake and behavior of dairy cows grazing perennial ryegrass swards in autumn. In: Rook, A. J., Penning, P. D.(Eds.), *Grazing Management ,Occasional Symposium No. 34. The British Grassland Society, UK*, pp. 101–102, 2000.

DEMMENT, M. W.; VAN SOEST, P. J. (1985). A nutritional explanation for body-size patterns of ruminant and non-ruminant herbivores. **American Naturalist**, v.125, p.641-672.

DETMANN, E.; PAULINO, M. F.; ZERVOUDAKIS, J. T.; VALADARES FILHO, S. D. C.; EUCLYDES, R. F.; LANA, R. D. P.; QUEIROZ, D. D. (2001). Cromo e indicadores internos na determinação do consumo de novilhos mestiços, suplementados, a pasto. **Revista Brasileira de Zootecnia**, vol. 30, no 5, p, 1600-1609.

DETMANN, E.; SOUZA, A.L.; GARCIA, R.; VALADARES FILHO, S.C.; CABRAL, L.S.; ZERVOUDAKIS, J.T. (2007). Avaliação do vício de "tempo longo" de indicadores internos em ensaio de digestão com ruminantes. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, 59(1), 182-188.

DETMANN, E.; ZERVOUDAKIS, J.T.; CABRAL, L.S.; JUNIOR, V. R. R.; VALADARES FILHO, S. C.; QUEIROZ, A. C.; PONCIANO, N. J.; MAGNO, F. A. Validação de equações preditivas da fração indigestível da fibra em detergente neutro em gramíneas tropicais. **Revista Brasileira de Zootecnia**, v.33, n.6, p.1866-1875, 2004.

DIAS, R. S.; PATINO, H. O.; LOPEZ, S.; PRATES, E.; SWANSON, K. C.; FRANCE, J. Relationship between chewing behavior, digestibility, and digesta kinetics in steers fed oat hay at restricted and *ad libitum* intakes. **Journal of Animal Science**, v. 89, p. 1873-1880, 2014.

EDIRISINGHE, A.; CLARK, D.; WAUGH, D. (2012) Spatio-temporal modelling of biomass of intensively grazed perennial dairy pastures using multispectral remote sensing. **International Journal of Applied Earth Observation and Geoinformation**, v.16, p. 5-16.

ELLIS, W.C.; MATIS, J.H.; HILL, T.M. et al. Methodology for estimating digestion and passage kinetics of forage. In: FAHEY JR., G.C., MOSER, L.E., MERTENS, D.R., et al. (Eds.) **National Conference on Forage Quality, Evaluation, and Utilization**. Madison: Soil Science Society of America. 1994. p.682-756.

FAHEY, G. C.; JUNG, H. G. Lignin as a marker in digestion studies: a review. **Journal of Animal Science**, v.57, n.1, p. 220-225, 1983.

FAHEY Jr, G.C.; McLAREN, G. A.; WILLIAMS, J. E. (1979). Lignin digestibility by lambs fed both low quality and high quality roughages. **Journal of Animal Science**, 48:941.

FERREIRA M. A.; VALADARES FILHO, S. C.; MARCONDES, M. I. et al. Consumo Individual de Matéria Seca Estimado em Bovinos de Corte e Leite Alimentados em Grupos. 1. Avaliação de Indicadores. **Revista Brasileira de Zootecnia**, 2008.

FORBES, J. M. A personal view of how ruminant animals control their intake and Choice of food: minimal total discomfort. **Nutrition Research Reviews**, v.20, p. 132-146, 2007.

FORBES, J. M. Conceitos sobre o consumo voluntário e seleção de dieta com referência especial aos animais domésticos. In: VIEIRA, S. L. et al., (1a Ed.). Consumo e preferência alimentar dos animais domesticos. Londrina: Phytobiotics Brasil, 2010. p. 315.

FRIEND, T. H.; POLAN, C. E. Social rank, feeding behavior and free stall utilization by dairy cattle. *Journal of Animal Science*, v.57, n. 4, p. 1214-1224, 1974.

FROIDMONT, E.; BONNET, M.; OGER, R.; DECRUYENAERE, V.; ROMNÉE, J.; BECKERS, Y.; BARTIAUX-THILL, N. Influence of the grinding level and extrusion on the nutritional value of lupin seed (*Lupinus albus*) for cattle in the context of the Dutch protein evaluation system. **Animal Feed Science and Technology**, 2008, no.142, p. 59-73.

FUKUSHIMA, R. S.; DEHORITY, B. A.; LOERCH, S. C. Modification of colorimetric analysis for lignin and its use in studying the inhibitory effects of lignin on forage digestion by ruminal microorganisms. **Journal of Animal Science**, v. 69, no. 1, p. 295-304, 1991.

FUKUSHIMA, R. S.; HATFIELD, R. D. Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. **Journal of Agricultural and Food Chemistry**, v. 49, n. 7, p. 3133-3139, Jul. 2001. ISSN 0021-8561.

FUKUSHIMA, R. S.; KERLEY, M. S. Use of lignin extracted from different plant sources as standards in the spectrophotometric acetyl bromide lignin method. **Journal of Agricultural and Food Chemistry**, v. 59, n. 8, p. 3505-3509, Apr. 27 2011.

FUKUSHIMA, R. S.; KERLEY, M. S.; RAMOS, M. H.; PORTER, J. H.; KALLENBACH, R. L. Comparison of acetyl bromide lignin with acid detergent lignin and Klason lignin and correlation with in vitro forage digestibility. **Journal of Feed Science and Technology**, v.201, p. 25-37, 2015.

GARDNER, A.L. **Técnicas de pesquisa em pastagens e aplicabilidade de resultados em sistemas de produção**. Brasília: IICA/Embrapa CNPGL, 1986. 197p. (Série Publicações Miscelâneas, 634).

GLINDEMANN, T.; TAS, B.M.; WANG, C.; ALVERS, S.; SUSENBETH, A. Evaluation of titanium dioxide as an inert marker for estimating faecal excretion in grazing sheep. **Animal Feed Science and Technology**, v.152, p.186-197, 2009.

GOACHET, A. G.; PHILIPPEAU, C.; VARLOUD, M.; JULIAND, V. Adaptations to standard approaches for measuring total tract apparent digestibility and gastro-intestinal retention time in horses in training. **Animal Feed Science and Technology**, v. 152, n. 1-2, p. 141-151, 2009.

GRANER, C.A.F. **Determinação do cromo pelo método colorimétrico da σ -difênilcarbazida**. 1972. 112f. Dissertação (Mestrado em Zootecnia) – Faculdade de Ciências Médicas e Biológicas/Universidade Estadual Paulista, Botucatu.

GRANT, R. J.; ALBRIGHT, J. L. Feeding behavior and management factors during the transition period in dairy cattle. **Journal of Animal Science**, v.73, n. 9, p. 543-550, 1995.

HAFEZ, S.; JUNGE, W.; KALM, E. Schätzung der verdaulichkeit mit einer indicator-methode bei milchkuhen im vergleich zum hohenheimer-futterwert-test. **Arch. Tierernähr**, v. 38, p. 929-945, 1988.

HORN, F. P.; MILLER, G. E. (1979). Bovine boots: a new research tool. **Oklahoma Agricultural Research Station Miscellaneous Publication**, MP-104, p. 44-46.

JANK, L.; VALLE, C. D.; RESENDE, R. M. S.; MCGILLOWAY, D. A. (2005). Grass and forage plant improvement in the tropics and sub-tropics. **Grassland: a global resource**. Wageningen: Wageningen Academic Publishers, 69-81.

KIMURA, F.T.; MÜLLER, V.L. Improved determination of chromic oxide in cal feed and feces. **Journal of Agricultural and Food Chemistry**, v.5, n.2, p.216, 1957.

KONDO, T.; MIZUNO, K.; KATO, T. Some characteristics of forage plant lignin. **Jarq-Japan Agricultural Research Quarterly**, v. 21, n. 1, Jul. 1987. ISSN 0021-3551.

KOTB, A. R.; LUCKEY, T. D. (1972). Markers in nutrition. **Nutrition Abstracts and Reviews**, v.42, p. 813-845.

KOZLOSKI, G. V.; MESQUITA, F. R.; ALVES T. P.; CASTAGNINO, D. S.; STEFANELLO, C. M.; SANCHEZ, L. M. D. Avaliação do uso de frações indigestíveis do alimento como indicadores internos de digestibilidade em ovinos. **Revista Brasileira de Zootecnia**, v.38, n.9, p.1819-1823, 2009.

KRIZAN, S. J.; HUHTANEN, P. (2013). Effect of diet composition and incubation time on feed indigestible neutral detergent fiber concentration in dairy cows. **Journal of Dairy Science**, vol. 96, no. 3, p. 1715-1726.

LANGLANDS, J. P.; CORBETT, J. L.; McDONALD, I.; REID, G. W. (1963). Estimation of the faeces output of grazing animals from the concentration of chromium sesquioxide in a sample of faeces. **British Journal of Nutrition**, 17, pp 211-218.

LANZETTA, V.A.S.; REZENDE, A.S.C. et al. Validation of Lipe™ as method to evaluate the apparent digestibility of nutrients in equines. **Revista Brasileira de Zootecnia**, v.38, n.1, p.69-74, 2009.

LE DU, Y. L. P.; PENNING, P. D. (1982). Animal based techniques for estimating herbage intake. In: LEAVER, J. D. (Ed.), **Herbage Intake Handbook**. British Grassland Society, Reading, UK, pp. 37-75.

LEE, C.; HRISTOV, A. N. Short communication: Evaluation of acid-insoluble ash and indigestible neutral detergent fiber as total-tract digestibility markers in dairy cows fed corn-silage based diets. **Journal of Dairy Science**, v. 96, n. 8, p. 5295-5299, 2013.

LIPPKE, H. Estimation of forage intake by ruminants on pasture. **Crop Science**, v. 42, n. 3, p. 869-872, 2002.

LOWRY, J. B.; CONLAN, L. L.; SCHLINK, A. C.; McSWEENEY, C. S. Acid detergent dispersible lignin in tropical grasses. **Journal of the Science of Food and Agriculture**, v. 65, n. 1, p. 41-49, May 1994.

LUGINBUHL, J. M.; POND, K. R.; BURNS, J. C. Changes in ruminal and fecal particle weight distribution of steers fed coastal Bermuda grass hay at four levels. **Journal of Animal Science**, v.68, p. 2864–2873, 1990.

MERCHEN, N. R. Digestion, absorption and excretion in ruminants. In: CHURCH, D. C. (Ed). **The ruminant animal digestive physiology and nutrition**. 4.ed. Carvallis: O&B Books. 1993, p. 172-201.

MERTENS, D. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. **Journal of AOAC International**, v. 85, n. 6, p. 1217-1240, 2002.

MUELLER-HARVEY, I; HARTLEY, R. D. Linkage of p-coumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. **Carbohydrate Research**, v. 148, n. 1, p. 71-85, 1986.

MYERS, W. D.; LUDDEN, P. A.; NAYIGIHUGU, V.; HESS, B. W. Thecnical Note: a procedure for the preparation and quantitative analysis of samples for titanium dioxide. **Journal of Animal Science**, v. 82, n. 1, p.179-183, 2004.

MYERS, W. D.; LUDDEN, P. A.; NAYIGIHUGU, V.; HESS, B. W. Excretion patterns of titanium dioxide and chromic oxide in duodenal digesta and feces of ewews. **Small Ruminant Research**, v. 63, n. 1-2, p. 135-141, 2006.

NATIONAL RESEARCH COUNCIL - NRC. **Nutritional requirements of dairy cattle**. 6.ed. Washington, D.C.: National Academy Press, 1989. 157p.

NEILSON, M. J.; GEOFFREY, N. R. The fate of the soluble lignin-carbohydrate complex produced in the bovine rumen. **Journal of the Science of Food and Agriculture**, v. 29, no. 6, p. 513-519, 1978.

NJAA, L. R. Determination of protein digestibility with titanium dioxide as indicator substance. **Acta Agriculturae Scandinavica**, v. 11, n. 3-4, p. 227-241, 1961.

OLIVEIRA, CAA.; ALMEIDA, F. Q.; VALADARES FILHO, S. C.; VIEIRA, A. A.; ALMEIDA, M. I. V.; CORASSA, A.; LOPES, B. A.; MACEDO, R. Estimativa da digestibilidade aparente de nutrientes em dietas para equinos com uso de oxido crômico e indicadores internos. **Revista Brasileira de Zootecnia**, v. 32, no. 6, p. 1681-1689, 2003.

OLIVEIRA JÚNIOR, R. C.; PIRES, A. V.; FERNANDES, J. J. R.; SUSIN, I.; SANTOS, F. A. P.; NASCIMENTO FILHO, V. F.; ARAUJO, F. C. Avaliação de marcadores para estimar a digestibilidade dos nutrientes em novilhos nelore alimentados com dietas contendo alto teor de concentrado e fontes nitrogenadas. **Revista Brasileira de Zootecnia**, v. 3, p. 749-758, 2004.

OWENS, F. N.; HANSON, C. F. External and internal markers for appraising site and extent of digestion in ruminants. **Journal of Dairy Science**, 1992, vol. 75, no 9, p. 2605-2617.

PAIXÃO, M. L.; FILHO, S. D. C. V.; LEÃO, M. I.; CECON, P. R.; MARCONDES, M. I.; SILVA, P. A.; PINA, M. G. D. S. (2007). Variação diária na excreção de indicadores interno (FDAi) e externo. **Revista Brasileira de Zootecnia**, vol. 36, no 3, p. 739-747.

PENNING, P.D. Animal-based techniques for estimating herbage intake. In: PENNING, P.D. (Ed.). **Herbage Intake Handbook**. 2ed. Reading, UK: The British Grassland Society, 2ed. p.53-94. 2004.

PENNING, P.D., JONHSON, R.H. 1983. The use of internal markers to estimate herbage digestibility and intake. 2. Indigestible acid insoluble fiber. **Journal of Agricultural Science**, v.100:1, p. 133-138.

PIAGGIO, L.M.; PRATES, E.R.; PIRES, F.F. et al. Avaliação das cinzas insolúveis em ácido, fibra em detergente ácido indigestível e lignina em detergente ácido indigestível como indicadores internos da digestibilidade. **Revista Brasileira de Zootecnia**, v.20, n.2, p.306-312, 1991.

POPPI, D. P. (1996). Predictions of food intake in ruminants from analyses of food composition. **Australian Journal of Agriculture Research**, v.47, p. 489–504.

POPPI, D.P.; HUGHES, T.P.; L'HUILLIER, P.J. Intake of pasture by grazing ruminants. In: NICOL, A.M. (Ed.) **Livestock feeding at pasture**. Hamilton: New Zealand Society of Animal Production, 1987. p.55-64. (Occasional publication, 10).

POPPI, D. P.; MINSON, D. J.; TERNOUTH, J. H. Studies of cattle and sheep eating leaf and stem fractions of grasses. 1. The voluntary intake, digestibility and retention time in the reticulo-rumen. **Crop and Pasture Science**, v. 32, n. 1, p. 99-108, 1981a.

POPPI, D. P.; MINSON, D. J.; TERNOUTH, J. H. Studies of cattle and sheep eating leaf and stem fractions of grasses. 3. The retention time in the rumen of large feed particles. **Crop and Pasture Science**, v. 32, n. 1, p. 123-137, 1981b.

PORTER, A.; SINGLETON, A. G. (1971). The degradation of lignin and quantitative aspects of ruminant digestion. **British Journal of Nutrition**, 25:3.

PRIGGE, E. C.; VARGA, G. A.; VICINI, J. L. et al. Comparison of ytterbium chloride and sesquioxide as fecal indicators. **Journal of Animal Science**, v.53, p. 1629-1633, 1981.

RODRIGUES, P. H. M.; GOMES, R. C.; SIQUEIRA, R. F. et al. Acurácia, precisão e robustez das estimativas da digestibilidade aparente da matéria seca determinada com o uso de indicadores em ovinos. **Revista Brasileira de Zootecnia**. V.39, p. 1118-1126, 2010.

SAMPAIO, C.B.; DETMANN, E.; VALENTE, T.N.P.; SOUZA, M. A.; VALADARES FILHO, S. C.; PAULINO, M. F. Evaluation of fecal recovering and long term bias of internal and external markers in a digestion assay with cattle. **Revista Brasileira de Zootecnia**, v.40, p.174-182, (2011a).

SAMPAIO, C. B.; DETMANN, E.; VALENTE, T. N. P.; COSTA, V. A. C.; VALADARES FILHO, S. C.; QUEIROZ, A. C. Fecal excretion patterns and short term bias of internal and external markers in a digestion assay with cattle. **Revista Brasileira de Zootecnia**, 40(3), 657-665, (2011b).

SCHNEIDER, B. H.; FLATT, W. P. (1975). The evaluation of feeds through digestibility experiments. Athens: **The University of Georgia Press**. 1975. 422p.

SILVA, L. D. F.; EZEQUIEL, J. M. B.; AZEVEDO, P. S. CATTELAN, J. W. ; RIBEIRO, E. L. A.; ROCHA, M. A.; CASTRO, V. S.; MENDES, A. R. Uso da cutina na estimativa das digestões total e parcial de alguns componentes de rações contendo diferentes fontes de nitrogênio, em bovinos. **Revista Brasileira de Zootecnia**. v. 35, n 2, p.600-606, 2006.

SILVA, R.R.; MAGALHÃES, A.F.; CARVALHO, G.G.P.; SILVA, F. F.; PRADO, I. N.; FRANCO, I.L.; VELOSO, C.M.; CHAVES, M.A. Comportamento ingestivo de novilhas mestiças de holandes suplementadas em pastejo de *brachiaria decumbes*. Aspectos metodológicos. **Revista Electrónica de Veterinaria**, v.5, n.10, p.1-7, 2004.

SIQUEIRA, R. F.; GOMES, R. C.; RODRIGUES, P. H.; FUKUSHIMA, R. S.; GOBESSO, A. A. Uso da cutina na estimativa da digestibilidade aparente de dietas para equinos; Use of cutin for determining apparent digestibility of diets for horses. **Arquivos Brasileiro de Medicina Veterinaria e Zootecnia**, vol. 61, no 6, p. 1373-1381, (2009).

SMITH, H. J.; TAWHEEL, H. Z.; TAM, B.M. et al. Comparison of techniques for estimating herbage intake of grazing dairy cows. **Journal of Dairy Science**, v.88, n. 5, p. 1827-1836, 2005.

STATISTICAL ANALYSIS SYSTEM INSTITUTE - SAS. **Statistical analysis user's guide**. Version 8.2. Cary, 2001. 1686p.

THONNEY, M. L.; DUHAIME, D. J.; MOE, P. W. et al. Acid insoluble ash and permanganate lignin as indicators to determine digestibility of cattle rations. **Journal of Animal Science**, v.49, n. 4, p. 112-146. 1979.

TITGEMEYER, E. C. Design and interpretation of nutrient digestion studies. **Journal of Animal Science**, 1997, vol. 75, no 8, p. 2235-2247.

TITGEMEYER, E. C.; ARMENDARIZ, C. K.; BINDEL, D. J. et al. Evaluation of titanium dioxide as a digestibility marker for cattle. **Journal of Animal Science**, v. 79, n. 4, p. 1059-1063, 2001.

VALADARES FILHO, S.C.; PAULINO, P.V.R.; DETMANN, E. et al. **Exigências nutricionais de zebuínos e tabelas de composição de alimentos BR-CORTE**. Viçosa:UFV, DZO, 1ªed., 2010, (142p.)

VALADARES FILHO, S.C.; PAULINO, P.V.R.; DETMANN, E. et al. Exigências nutricionais de zebuínos no Brasil: I. Energia. In: VALADARES FILHO, S.C.; PAULINO, P.V.R.; MAGALHÃES, K.A. **Exigências nutricionais de zebuínos e tabelas de composição de alimentos BR-Corte**. Viçosa: UFV, 2006. 142p.

VAN SOEST, P.J. Nutritional Ecology of the Ruminant. 2ed. New York: Cornell University Press, 1994. 476p.

VAN SOEST, P. J.; ROBERTSON, J. B. Analysis of forages and fibrous foods. Ithaca: Cornell University, 1985. 202p..

VANSOEST, P. J.; ROBERTSON, J. B.; LEWIS, B. A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. **Journal of Dairy Science**, v. 74, n. 10, p. 3583-3597, Oct 1991.