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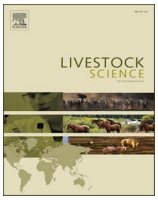

Diet, marker and fecal sampling method interactions with internal and external marker pairs when estimating dry matter intake in beef cattle

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HIGHLIGHTS

- Chromic oxide is more accurate in estimating fecal output then titanium dioxide.
- Chromic oxide paired with indigestible fibers are accurate in predicting intake.
- Grab samplings are equal or better than bulk sampling to estimate dry matter intake.
- Grab sampling can replace total fecal collection, if fecal recovery is established.

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ABSTRACT

The use of markers is currently the most used technique for estimating feed intake in production animals when direct measurement is not possible. Twelve growing Nellore bulls were used in a crossover design aiming to evaluate the accuracy of internal markers to estimate dry matter digestibility (DMD), the accuracy of external markers to estimate fecal output (FO), and the combination of internal and external markers to estimate dry matter intake (DMI) at different fecal sampling procedures. Animals received one of the four diets which varied in forage source (corn silage or Tifton-85 hay) and forage to concentrate ratio (F:C; 60:40 or 40:60) in a 2 × 2 factorial arrangement. The internal markers, acetyl bromide lignin (ABL), indigestible aNDFom (iNDF), indigestible ADFom (iADF) and cutin, are naturally present in the feedstuffs and the external markers, Cr_2O_3 and TiO_2, were daily fed to the animals. Three fecal sampling procedures were tested: bulk 72-h (continuous feces collection for 72 h), grab 9 × 3 (sample collection every 9 h over 3-d period), and grab 4 × 4 (4 fecal samples collected during daylight hours over 4-d period). None of the internal or external markers evaluated had complete fecal recovery (FR), being necessary to establish the FR of the markers in order to obtain correct estimates. The FR of ABL, iNDF and iADF were close to 100% when animals received a hay-based diets but close to 50% when feeding a corn silage-based diets, regardless the F:C ratio of the diet. However, ABL produced accurate DMD estimates only with both grab sampling procedures across all diets, while iNDF and iADF produced accurate DMD estimates across all sampling procedures and diets. Cutin failed to produce accurate DMD consistently. Both external markers produced accurate FO under both grab sampling procedures, except for TiO_2 when the grab 4 × 4 sampling procedure was performed for animals receiving the diet with high silage proportion. In general, the grab sampling procedures yielded accurate DMD, FO and DMI estimates, which were similar or better than bulk 72 h sampling procedure. The combination of internal and external markers to estimate DMI produced satisfactory and accurate results, particularly when Cr_2O_3 or TiO_2 was paired with iNDF or iADF under both grab sampling procedures. The grab sampling procedures can replace TFC, opening new possibilities for collectively housed animals.

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1. Introduction

Daily dry matter intake can be estimated by means of digestion markers. The use of markers is currently the most widely used technique to estimate DMI in ruminants when intake cannot be directly measured (Velasquez et al., 2018). 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 specific characteristics of each marker. The concentration of the marker in feces will ultimately determine its FR which is a crucial, if not the most important factor that any marker should possess in order to be free of recovery bias (Detmann et al., 2007).

Indigestible components as lignin, iNDF and iADF present in the feed ingredients of the diet has been widely used as an internal marker for digestibility studies (Lippke, 2002). However, the FR of the internal markers is highly variable, compromising the accuracy of the DMD prediction (Cochran et al., 1986). Indigestible NDF and ADF are the most common internal markers used for estimating DMD, with several studies reporting FR ranging 50 to 121% for iNDF and from 80 to 121% for iADF (Velasquez et al., 2018). Lignin as ADL has been criticized because of partial solubilization of lignin during hydrolysis with sulfuric acid solution. Goachet et al. (2009) used ADL to estimate digestibility coefficients in horses and observed an average FR of 88%. While in our previous study with dairy cows (Velasquez et al., 2018), we used lignin as ABL, which is a method where no lignin is lost in the process due its spectrophotometric procedure. The average of the ABL FR we observed was 95% for the grab sampling procedures, appearing as an appropriate internal marker for estimating DMD. Cutin is a waxy polymer present in the plant cuticle and due its indigestibility it could be used as an internal marker, however, to our knowledge there is a lack of studies evaluating the feasibility of cutin as a marker for ruminants and our previous study was the pioneer in testing it, where the average FR of cutin in dairy cows was 76% (Velasquez et al., 2018).

Chromic oxide was the most common external marker used in digestion studies for fecal recovery evaluation (Prigge et al., 1981), likely due its low-cost and proper accuracy in estimating FO in cattle. However, it was previously indicated that the FR of Cr_2O_3 could vary greatly among forage-fed ruminants (Delagarde et al., 2000). Additionally, Cr_2O_3 has been related to potential health risks (Myers et al., 2006), therefore, TiO_2 has been evaluated as an alternative to Cr_2O_3 (Titgemeyer et al., 2001). When comparing both external markers, Titgemeyer et al. (2001) observed that the FR for Cr_2O_3 averaged 113% and TiO_2 averaged 95%, overestimating and underestimating FO, respectively, suggesting that the usefulness of TiO_2 should be further investigated.

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 TFC is regularly used in intake studies as the standard reference, where estimates are compared and as the source of bulk samples for later analysis. 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.

Dietary characteristics as chemical composition and conservation method of the forage (silage vs. hay) can be responsible for the variability that is commonly observed in FR of markers (Huhtanen et al., 1994). One possible explanation is that markers attach to solid and liquid phases of the ruminal digesta differently, varying in passage rate from the rumen and consequently in recovery in the feces. Therefore, Kanani et al. (2014) suggested that markers should be validated across different types of forages before its application in research. However, there is a lack of studies evaluating internal and external markers with reduced fecal sampling procedures in beef cattle fed diets containing corn silage or Tifton-85 hay at different forage to concentrate proportion.

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 to 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 this experiment was to evaluate the accuracy of internal markers, ABL, cutin, iNDF and iADF for estimating DMD; to test the utilization of external markers, Cr_2O_3 and TiO_2 for estimating FO; examine the combination of internal and external markers for estimating DMI and to compare 3 different sampling designs.

2. 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. All experimental procedures were in agreement with the *Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 1999), with all animal procedures approved by the University of São Paulo Animal Bioethics Committee (protocol number: CEUA 24,420,603,114). No animals were harmed or fell ill during the experiment.

2.1. Animals, diets and experimental design

Twelve Nellore bulls (414 ± 14 kg BW and 18 months of age) were randomly allocated to 12 individual pens (6 m × 4 m) 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 randomly assigned to each of the four diets varying in forage source and F:C ratio: I) 60% corn silage + 40% concentrate (Silage 60:40); II) 40% corn silage + 60% concentrate (Silage 40:60); III) 60% Tifton-85 hay + 40% concentrate (Hay 60:40); and IV) 40% Tifton-85 hay + 60% concentrate (Hay 40:60). Diets were distributed in a 2 × 2 factorial arrangement of treatments with an incomplete cross-over design, where in the first period animals received diets I, II, III and IV; and in the second period animals received diets IV, III, II and I, respectively.

Diets were formulated according to NRC (2000) to have the same F:C ratios for each forage source and projected daily weight gain averaging from 0.8 to 1.4 kg/day. The composition of the concentrates and the experimental diets are presented in Table 1. Cottonseed meal was used as source of true protein due its high content of cutin. Increasing the cutin concentration of the diets was desirable in order to facilitate analysis and decrease variability of results. Urea was added to the diets in order to meet the rumen degradable protein requirements. Chemical composition of the forages and concentrates are shown in Table 2 and of the experimental diets are shown in Table 3.

Diets were offered as total mixed ration (TMR), ad-libitum, divided into 2 daily servings (0700 and 1600) to represent the natural diurnal feeding pattern of cattle. Daily allowances of the TMR, plus 10% of calculated daily fresh matter intake was fed to guarantee orts. Feed ingredients, TMR and orts subsamples were taken on Mondays, Wednesdays and Fridays for DM determination, drying in a forced draft oven (Solab Cientifica, Piracicaba, SP, Brazil) at 55 °C for 3 d Orts were collected daily so that ad libitum intake could be determined as the difference between the DM offered and refused.

The experiment was conducted for 38 d, considering a day as the 24 h period from 0800 to 0800 h. Each of the two experimental periods consisted of 19 d divided into three phases: d 1 to 5 were allocated to adaptation of the animals to the diet; 10 d (d 6 to 15) of marker excretion

Table 1
Composition of concentrates and experimental diets.

	Concentrates			
Ingredient,% of DM	I	II	III	IV
Dry ground corn	62.2	71.2	79.6	81.3
Cottonseed meal	31.1	23.2	14.9	13.3
Urea	0.96	0.66	0.25	0.50
Mineral Mixture[1]	4.78	3.31	4.98	3.32
Limestone	0.96	1.66	0.25	1.66
	Diets			
Ingredient,% of DM	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] The trace mineral mixture contained as percentage: 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.

Table 2
Chemical composition (g/kg of DM ± SD) of forages and concentrates ($n = 2$).

	Forage		Concentrates			
	Silage	Hay	I	II	III	IV
Dry matter (g/kg)	325 ± 0.58	872 ± 1.42	867 ± 0.18	865 ± 2.87	869 ± 1.62	863 ± 3.04
Mineral matter	54.9 ± 1.75	48.3 ± 1.74	54.8 ± 1.66	48.2 ± 1.66	53.3 ± 0.08	43.2 ± 3.32
aNDFom[1]	634 ± 8.23	772 ± 12.1	287 ± 2.31	263 ± 14.1	210 ± 7.05	287 ± 12.9
ADFom[2]	390 ± 3.49	444 ± 12.1	137 ± 2.41	105 ± 5.07	81.1 ± 6.5	86.3 ± 8.06
Cell wall	734 ± 8.08	776 ± 3.93	786 ± 0.92	795 ± 22.2	816 ± 2.31	823 ± 2.8
ABL[3]	153 ± 18.5	133 ± 33.5	35.0 ± 5.37	31.0 ± 10.2	23.3 ± 1.0	33.6 ± 5.38
Cutin	15.9 ± 4.47	38.4 ± 4.73	31.8 ± 3.60	29.6 ± 9.14	15.4 ± 5.11	10.9 ± 0.95
iNDF[4]	229 ± 7.91	344 ± 6.49	109 ± 8.41	83.8 ± 9.53	66.9 ± 5.88	54.5 ± 4.09
iADF[5]	147 ± 4.72	209 ± 6.39	70.7 ± 7.98	53.5 ± 5.82	40.8 ± 3.09	33.8 ± 2.67
Crude Protein	84.5 ± 0.96	87.4 ± 3.09	204 ± 3.08	171 ± 2.03	143 ± 2.89	152 ± 4.18
Ether extract	55.7 ± 1.03	72.3 ± 1.35	95.7 ± 3.55	48.3 ± 2.47	76.8 ± 2.84	59.6 ± 2.91

[1] aNDFom, neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash.
[2] ADFom, acid detergent fiber expressed exclusive of residual ash.
[3] ABL, acetyl bromide lignin;.
[4] iNDF, indigestible aNDFom;.
[5] iADF, indigestible ADFom.

stabilization and the final 4 d (d 16 to 19) were for sample collection. The animals were adapted to the experimental conditions during 14 d prior to the start of the experiment.

2.2. 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 8 combinations (1 internal marker and 1 external marker) derived from these 6 markers were used to estimate voluntary DMI. Estimates of DMD, FO, and DMI were compared against their reciprocal total-tract apparent digestibility (TTAD), real fecal output (RFO), and real dry matter intake (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, either 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. Bulls received 6 g of Cr_2O_3 and 10 g of TiO_2 daily. This dose was divided into 2 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 (0800 and 1600 h). The animals were dosed from d 5 to 18 of the experimental period and had been receiving molasses with concentrate before each meal during the 14 d before the start of the experiment.

Table 3
Chemical composition (g/kg of DM ± SD) of the experimental diets ($n = 2$).

Forage	Corn silage		Tifton-85 hay	
F:C ratio	60:40	40:60	60:40	40:60
Dry matter (g/kg)	542 ± 034	649 ± 1.93	870 ± 1.43	867 ± 2.39
Mineral matter	54.9 ± 1.61	50.9 ± 1.62	50.3 ± 1.08	45.2 ± 2.69
aNDFom[1]	495 ± 5.12	411 ± 11.6	547 ± 4.47	481 ± 7.65
ADFom[2]	289 ± 2.94	219 ± 3.33	299 ± 9.68	229 ± 1.45
Cell wall	755 ± 4.82	770 ± 16.5	792 ± 2.97	804 ± 0.81
ABL[3]	106 ± 13.3	79.9 ± 5.24	89.4 ± 19.7	73.5 ± 12.4
Cutin	22.3 ± 2.56	24.1 ± 3.73	29.2 ± 4.23	21.9 ± 2.46
iNDF[4]	181 ± 5.06	142 ± 2.91	233 ± 4.13	170 ± 4.86
iADF[5]	117 ± 5.42	91.0 ± 3.17	142 ± 3.13	104 ± 3.01
Crude Protein	132 ± 0.8	137 ± 0.84	110 ± 2.43	126 ± 2.88
Ether extract	71.7 ± 1.43	51.2 ± 1.9	74.1 ± 1.95	64.7 ± 2.14

[1] aNDFom, neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash.
[2] ADFom, acid detergent fiber expressed exclusive of residual ash.
[3] ABL, acetyl bromide lignin.
[4] iNDF, indigestible aNDFom.
[5] iADF, indigestible ADFom.

2.3. Feces

On d 16 at 0600 h stalls were thoroughly cleaned and at 0800 h the 72 h TFC period started. Two-person teams constantly watched the animals, in 8 h shifts, and collected all feces immediately after excretion to avoid contamination or partial loss of material. Collected feces were placed into individual (one per bull) blue plastic containers (100 L; previously weighed empty) that were kept sealed and away from direct sunlight. At 0730 h of d 17 to 19, the containers were weighed and the daily RFO was 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 h continuous sampling design.

Two grab sampling designs were evaluated during this study, Grab 9 × 3 (3 samples per day collected over 3-d period with samples being taken every 9 h, where on d 16 samples were collected at 0800, 1700 and 0200 h; on d 17 collected at 1100, 2000 and 0500 h; and on d 18 collected at 1400, 2300 and 0800) and Grab 4 × 4 (4 samples per day collected during daylight hours over 4-d period, where samples were collected on d 16–19 at 0800, 1100, 1400 and 1700 h). The grab samples were collected at the same time as bulk sampling. At the end of each day, grab sample weights were added to the TFC weight. The grab samples were then composited daily and analyzed by sampling method.

2.4. Chemical analysis and measurements

Feces, feed and ort samples were composed individually per animal and frozen at −20 °C. Later, 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. A 2 mm screen was used to avoid small particle loss during analyses that might contain internal markers. The determination of aNDFom and ADFom was performed as proposed by Van Soest et al. (1991) using 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 procedure (Mertens, 2002).

Cell wall was analysed according to the procedure proposed by Fukushima et al. (1991) and later used as substrate for acetyl bromide lignin (ABL) determination in feed, orts and feces samples, according to Fukushima et al. (1991). Lignin absorbance was determined with a Libra S80 spectrophotometer (BIOCHROM®; United Kingdom) at a wavelength of 280 nm. Indigestible NDF and iADF were obtained by rumen incubation in a cannulated cow for 288 h (Krizan and Huhtanen, 2013). Cutin content in feed, TMR, orts and feces samples were determined in non-woven textile (NWT) fiber bags as described by Velasquez et al. (2018).

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 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 and Muller (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, Campinas, São Paulo, Brazil) 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 regression equation obtained was: $y = 0.002x + 0.06$ ($r^2 = 0.995$).

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 regression equation obtained was: $y = 0.057x + 0.04$ ($r^2 = 0.995$).

2.5. Calculations

Real DMI was calculated as daily feed offer minus daily orts (Eq. (1)) and RFO was calculated as the average of 3 d (d 16–18) of TFC (24 h collection and daily weighing of amount excreted). Total-tract apparent digestibility was calculated as RDMI minus RFO (Eq. (2)):

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

$$\text{TTAD}\ (\text{g}/\text{kg DM}) = \frac{\left[\text{RDMI}\left(\frac{\text{g}}{\text{d}}\right) - \text{RFO}\left(\frac{\text{g DM}}{\text{d}}\right)\right] \text{ x } 1,000}{\text{RDMI}} \quad (2)$$

Eqs. (3) and (4) were used to estimate marker excretion and recovery rate. These equations derived from TFC. The overall recovery rate of markers was calculated from the total amount of marker given and the amount recovered during the fecal sampling period (d 15 - 19). Daily recovery rate of markers was calculated from d 16 to 19:

$$\text{marker excretion}\left(\frac{\text{g DM}}{\text{d}}\right) = [\text{fecal marker}]\left(\frac{\text{g DM}}{\text{g}}\right) \text{x RFO}\left(\frac{\text{g DM}}{\text{d}}\right) \quad (3)$$

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

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

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

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

Eq. (7) was used to estimate total voluntary intake from FO and DMD estimates obtained by Eqs. (5) and (6). Total DMI was estimated for both bulk and grab samples:

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

2.6. Statistical analysis

Results were analyzed with SAS 9.3 (SAS Institute Inc., Cary, NC), after verifying normality of residuals and homogeneity of variance. Values for TTAD, RFO and RDMI were analyzed using PROC-MIXED, according to the model:

$$Y_{ijkl} = \mu + F_i + C_j + FC_{ij} + A_k + P_l + \varepsilon_{ijkl}$$

where Y_{ijkl} = is the TTAD ($n = 24$), RFO ($n = 24$) or RDMI ($n = 24$) 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_k = is the random effect of animal; P_l 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, according to the model:

$$Y_{ijklm} = \mu + F_i + C_j + T_k + FC_{ij} + FT_{ik} + CT_{jk} + FCT_{ijk} + A_l + P_m + \varepsilon_{ijklm}$$

where Y_{ijklm} = is the DMD ($n = 24$), FO ($n = 24$) or DMI ($n = 24$) 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, where treatments were distributed in a split-plot design, with markers being completely randomized as the main plot and sampling procedure as sub-plots; 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_l = is the random effect of animal; P_m is the random effect of period and ε_{ijklm} is the experimental error supposed to be NIID (normal independent and identically distributed).

In virtue of the significance of the three-way 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)). Differences were considered significant at $P \leq 0.05$ and tendency to significance at $0.05 < P \leq 0.10$.

3. Results

The means of TTAD, RFO and RDMI are shown in Table 4 and were used specifically to estimate FR, FO, DMD and DMI.

3.1. Fecal recovery of markers

The FR of markers observed in the proposed bulk and grab sampling designs was generally poor. However, in order to evaluate the accuracy of markers, the FR observed in the TFC was used (Table 5). The effect of animal was tested in the model and no difference was observed, thus we recommend the TFC from at least one animal per treatment to determine FR. Hay based-diets showed a greater FR for ABL ($P < 0.001$) compared to silage based-diets. There was a tendency of interaction between forage source and F:C ratio for iNDF ($P = 0.079$) and iADF ($P = 0.057$), where the FR was greater for hay based-diets compared to silage based-diets. The FR of cutin was affected ($P < 0.001$) by forage source and F:C ratio, where hay based-diets and 60:40 diets showed greater FR than silage based diets and 40:60 diets, respectively. For Cr_2O_3, there was an interaction ($P = 0.035$) between forage source and F:C ratio, where the FR observed for the corn silage 40:60 diet was lower than the FR observed for the corn silage 60:40, hay 60:40 and hay 40:60 diets, with no difference among the last ones. The FR of TiO_2 was affected only by F:C ratio ($P = 0.001$), where animals fed 40:60 diets showed greater FR compared to animals fed 60:40 diets.

3.2. Prediction of DMD based on fecal concentration of internal markers

The mean DMD estimates are shown in Table 6. No effect ($P = 0.435$) was observed for sampling procedure on DMD estimates. Effects for marker ($P = 0.021$), diet ($P < 0.001$) and the interaction among diet, marker and method ($P < 0.001$) were observed. All markers produced accurate DMD estimates when compared with TTAD. Estimates derived from both grab (9 × 3 and 4 × 4) fecal sampling procedures were consistently accurate, except when using cutin as internal marker. Indigestible fibers (iNDF and iADF) consistently produced accurate estimates independently of the fecal sampling procedure.

3.3. Prediction of FO based on fecal concentration of external markers

The mean FO estimates are shown in Table 7. An effect ($P < 0.001$) for diet was observed. No effect for marker ($P = 0.232$) and a tendency ($P = 0.062$) were observed on FO estimates. There was a interaction among diet, sampling procedure and marker ($P = 0.017$). Both external markers produced accurate FO estimates when compared to RFO. The grab 9 × 3 sampling procedure was consistently accurate for estimating FO. Besides with TiO_2 on the silage 60:40 diet, the grab 4 × 4 sampling procedure estimated FO accurately. The bulk 72 h sampling procedure failed to produce accurate estimates of FO with TiO_2 on the hay 40:60 diet, with Cr_2O_3 on the hay 60:40 diet and had a tendency ($P = 0.074$) on the silage 60:40 diet.

3.4. Prediction of DMI based on internal and external marker pairs

The mean DMI estimates are shown in Table 8. Effects for forage source, F:C ratio and treatment (marker pair × sampling procedure) were observed ($P < 0.001$). There was interactions between forage source x F:C ratio ($P = 0.037$), forage source x treatment ($P < 0.002$) and F:C ratio x treatment ($P < 0.001$). There was a tendency ($P = 0.078$) for the three-way interaction between forage source, F:C ratio and treatment. Less DMI estimates from Cr_2O_3 marker pairs were different from their reciprocal RDMI values than the TiO_2 derived marker pairs. Among the internal markers, the indigestible fibers produced accurate estimates (not different to the reciprocal RDMI value) more constantly than ABL and cutin. Between the sampling procedures, the grab 9 × 3 and grab 4 × 4 derived DMI estimates were more often accurate than the bulk 72 h sampling procedure. None of the marker pairs or sampling procedures produced accurate DMI estimates for all diets.

4. Discussion

4.1. Fecal recovery of markers

Lignin was measured 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. The FR of ABL ranged from 51 to 98%, where the FR was close to 100% when animals received a hay-based diets but close to 50% when feeding a corn silage-based diets, regardless the F:C ratio of the diet. These results suggest that ABL is a suitable marker for young bulls fed hay-based diets but not when feeding a corn silage-based diet. However, in our previous study cows received a corn silage-based diet (55:45 F:C ration) and we observed a proper FR for ABL, ranging from 95 to 111%, suggesting that ABL is an appropriate internal marker for estimating DMD in dairy cattle (Velasquez et al., 2018). Lignin as ABL is not commonly used as an internal marker likely due to the inconsistences observed when lignin was

Table 4
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; g/kg DM), real fecal output (RFO; g DM/day), real dry matter intake (RDMI; g DM/day) obtained by total feces collection (72 h) in young bulls fed TMR diets ($n = 6$).

Forage (F)	Corn silage		Tifton-85 hay		SEM	*P*-value		
F:C[1] (C)	60:40	40:60	60:40	40:60		F	C	*F* × *C*
TTAD	708	700	613	642	22.3	<0.001	0.327	0.174
RFO	2716	3561	2941	2912	126	0.023	<0.001	0.046
RDMI	9328	11,921	7598	8126	797	<0.001	<0.001	0.035

[1]F:C, forage to concentrate ratio.

Table 5
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 ($n = 6$).

Forage (F)	Corn silage		Tifton-85 hay		SEM	*P*-value		
F:C[1] (C)	60:40	40:60	60:40	40:60		F	C	*F* × *C*
FR								
ABL	0.51	0.52	0.98	0.91	0.12	<0.001	0.104	0.520
iNDF	0.73$^{(b)}$	0.72$^{(b)}$	0.97$^{(a)}$	1.06$^{(a)}$	0.09	<0.001	0.026	0.079
iADF	0.81$^{(b)}$	0.80$^{(b)}$	1.06$^{(a)}$	1.17$^{(a)}$	0.09	<0.001	0.019	0.057
Cutin	1.16	0.89	1.45	1.38	0.11	<0.001	<0.001	0.537
Cr_2O_3	0.80^{a}	0.71^{b}	0.80^{a}	0.83^{a}	0.02	0.008	0.101	0.035
TiO_2	1.31	1.57	1.30	1.40	0.05	0.109	0.001	0.370

[1]F:C, forage to concentrate ratio.
[a–d]Within a line, mean values with common lower case superscript are not significantly different by Fisher's LSD ($P < 0.05$) or tendency to significance if in parentheses ($0.05 < P \leq 0.10$).

Table 6
Total tract apparent digestibility (TTAD; g/kg DM) (mean ± SE) and dry matter digestibility (DMD; g/kg DM) (mean ± SE) estimates derived from internal markers (ABL, cutin, iNDF and iADF) on two grab (4 × 4 and 9 × 3) and one bulk (72 h) fecal sampling procedures ($n = 6$).

Forage	Corn silage		Tifton-85 hay	
F:C ratio[1]	60:40	40:60	60:40	40:60
ABL[2]				
4 × 4	702 ± 9.2	706 ± 9.4	599 ± 21.3	659 ± 8.7
9 × 3	704 ± 8.6	734 ± 9.7	641 ± 14.6	677 ± 6.3
72 h	691 ± 15.6	608* ± 17.3	550* ± 25.4	518* ± 37.4
Cutin				
4 × 4	622* ± 29.3	676 ± 26.1	613 ± 20.0	662 ± 18.9
9 × 3	674 ± 26.7	660 ± 28.1	623* ± 52.8	543 ± 54.7
72 h	744 ± 11.1	697 ± 19.7	566* ± 38.3	606 ± 48.3
iNDF[3]				
4 × 4	695 ± 9.3	694 ± 5.8	589 ± 7.4	616 ± 4.6
9 × 3	721 ± 5.2	686 ± 8.3	595 ± 13.7	621 ± 7.3
72 h	719 ± 6.2	703 ± 11.8	638 ± 4.0	641 ± 8.2
iADF[4]				
4 × 4	688 ± 11.6	670 ± 6.3	578 ± 9.4	621 ± 6.4
9 × 3	722 ± 6.2	687 ± 9.4	608 ± 7.9	619 ± 8.8
72 h	718 ± 6.5	703 ± 12.8	638 ± 4.9	644 ± 8.5
TTAD	708 ± 11.5	700 ± 8.8	613 ± 8.8	642 ± 14.4

[1]F:C, forage to concentrate ratio.
[2]ABL, acetyl bromide lignin.
[3]iNDF, indigestible aNDFom.
[4]iADF, indigestible ADFom.
*Within columns are significantly different from TTAD for diet according to contrast analysis ($P < 0.05$).
Grab 4 × 4 = 4 samples per day collected during daylight hours over 4-d period, where samples were collected on d 16–19 at 0800, 1100, 1400 and 1700 h; Grab 9 × 3 = 3 samples per day collected over 3-d period with samples being taken every 9 h, where on d 16 samples were collected at 0800, 1700 and 0200 h; on d 17 collected at 1100, 2000 and 0500 h; and on d 18 collected at 1400, 2300 and 0800; Bulk (72 h) = total feces collection over 72 h on d 16–19.

Table 7
Real fecal output (RFO; g DM/day) (mean ± SE) and fecal output (FO; g DM/day) (mean ± SE) estimates derived from external markers on two grab (4 × 4 and 9 × 3) and one bulk (72 h) fecal sampling procedures ($n = 6$)[1].

Forage	Corn silage		Tifton-85 hay	
F:C ratio[2]	60:40	40:60	60:40	40:60
Cr_2O_3				
4 × 4	2712 ± 183	3761 ± 318	3008 ± 178	2791 ± 121
9 × 3	2618 ± 124	3733 ± 207	2749 ± 104	3092 ± 174
72 h	3107^{T} ± 79	3802 ± 210	3416* ± 217	2928 ± 88.2
TiO_2				
4 × 4	3157* ± 164	3339 ± 123	3180 ± 171	3199 ± 145
9 × 3	2522 ± 140	3586 ± 241	2946 ± 219	3614 ± 327
72 h	3060 ± 137	3909 ± 253	3508* ± 366	2788 ± 265
RFO	2716 ± 115	3561 ± 120	2941 ± 102	2912 ± 150

[1]Bulls received 6 g of Cr_2O_3 and 10 g of TiO_2 daily.
[2]F:C, forage to concentrate ratio.
*Within columns are significantly different from RFO for diet according to contrast analysis ($P < 0.05$).
TWithin columns means tendency of difference from RFO for diet according to contrast analysis ($0.05 < P \leq 0.10$).
Grab 4 × 4 = 4 samples per day collected during daylight hours over 4-d period, where samples were collected on d 16–19 at 0800, 1100, 1400 and 1700 h; Grab 9 × 3 = 3 samples per day collected over 3-d period with samples being taken every 9 h, where on d 16 samples were collected at 0800, 1700 and 0200 h; on d 17 collected at 1100, 2000 and 0500 h; and on d 18 collected at 1400, 2300 and 0800; Bulk (72 h) = total feces collection over 72 h on d 16–19.

used as ADL (Goachet et al., 2009), thus further research is necessary to increase the knowledge on how ABL can be applied to ruminant nutrition studies and how appropriate this internal marker for estimating DMD in beef cattle under different conditions.

Fecal recovery for iNDF and iADF was close to 100% when animals received a hay-based diets, regardless the F:C ratio of the diet. However, when corn silage-based diets were fed the FR varied from 72 to 81%. A possible explanation for the difference regarding the forage source is probably related to the DMI, where animals fed corn silage-based diets showed greater DMI compared to hay-based diets. Greater intake leads to faster passage rate likely modifying the composition of the feces throughout the day, as digestible compounds will remain in the rumen longer in attempt to be digested and indigestible compounds will disappear by passage (Owens and Hanson, 1992). Lower DMI of hay-based diets likely contributed to a more homogenous feces composition throughout the day, due to the slower passage rate. A more homogenous feces might result in a better representation of the material when the sampling is performed. The chances of mistaking the sample collection is greater if the material is heterogeneous.

Besides our previous study (Velasquez et al., 2018), no reports were found on the use of cutin as an internal marker for ruminant digestion studies and therefore our data is novel to this field of research. When using cutin as an internal marker in dairy cows the average FR was 76%, however, in the present study where beef cattle were used, the average FR was 122%. A possible explanation for incomplete FR of internal markers is that when fiber cell wall is treated with cellulases, part of the components that is ester-linked by ferulic or ρ-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). In order to evaluate the viability of cutin as an internal marker, more studies are necessary.

The FR for TiO_2 varied from 130 to 157%. Titgemeyer et al. (2001) used steers in two different studies, first feeding forage-based diets and

Table 8
Real dry matter intake (RDMI; g DM/day) (mean ± SE) and dry matter intake (DMI; g DM/day) (mean ± SE) estimates derived from 1 internal + 1 external marker pairs on two grab (4 × 4 and 9 × 3) and one bulk (72 h) fecal sampling procedures ($n = 6$)[1].

Forage	Corn silage		Tifton-85 hay	
F:C ratio[2]	60:40	40:60	60:40	40:60
Cr_2O_3+ABL[3]				
4 × 4	9206 ± 610	12,210 ± 1114	7644 ± 413	8216 ± 364
9 × 3	8890 ± 384	14,763* ± 1298	7862 ± 433	9558* ± 488
72 h	10,187 ± 501	9894* ± 559	7662 ± 322	6595* ± 444
Cr_2O_3+Cutin				
4 × 4	7749* ± 602	12,308 ± 926	8350 ± 771	8779 ± 669
9 × 3	8785 ± 652	12,005 ± 1020	8415 ± 857	8627 ± 1256
72 h	12,595* ± 595	13,331 ± 987	9347* ± 1102	9146 ± 1109
Cr_2O_3+iNDF[4]				
4 × 4	8831 ± 450	11,672 ± 995	7295 ± 399	7254 ± 301
9 × 3	9356 ± 335	11,881 ± 559	6865 ± 293	8113 ± 381
72 h	10,797* ± 522	12,441 ± 803	9436* ± 597	8221 ± 288
Cr_2O_3+iADF[5]				
4 × 4	8677 ± 443	11,892 ± 1000	7131 ± 400	7377 ± 317
9 × 3	9402 ± 357	11,950 ± 555	7030 ± 269	8113 ± 409
72 h	10,806 ± 579	12,452 ± 821	9443* ± 597	8303 ± 302
TiO_2+ABL[3]				
4 × 4	10,817* ± 730	11,578 ± 590	8326 ± 682	9490 ± 520
9 × 3	8595 ± 490	13,753* ± 1023	8166 ± 440	11,176* ± 969
72 h	10,512 ± 805	10,771 ± 847	7563 ± 465	6071* ± 550
TiO_2+Cutin				
4 × 4	9118 ± 706	11,451 ± 977	8636 ± 621	9903* ± 652
9 × 3	8511 ± 730	10,830 ± 507	8878 ± 1781	9358 ± 1982
72 h	12,271* ± 721	14,647* ± 1258	9948* ± 1606	9440 ± 1447
TiO_2+iNDF[4]				
4 × 4	10,560 ± 675	11,009 ± 500	7784 ± 465	8383 ± 438
9 × 3	9131 ± 553	11,334 ± 595	7347 ± 562	9464 ± 800
72 h	11,212* ± 701	13,815* ± 774	9618* ± 962	7760 ± 713
TiO_2+iADF[5]				
4 × 4	10,404 ± 688	11,264 ± 535	7622 ± 470	8533 ± 462
9 × 3	9179 ± 565	11,407 ± 607	7514 ± 546	9454 ± 812
72 h	11,158* ± 671	13,901* ± 807	9608* ± 950	7828 ± 711
RDMI	9328 ± 199	11,921 ± 310	7598 ± 201	8126 ± 266

[1]Bulls received 6 g of Cr_2O_3 and 10 g of TiO_2 daily.
[2]F:C, forage to concentrate ratio.
[3]ABL, acetyl bromide lignin.
[4]iNDF, indigestible aNDFom.
[5]iADF, indigestible ADFom.
*Within columns are significantly different from RDMI for diet according to contrast analysis ($P < 0.05$).
Grab 4 × 4 = 4 samples per day collected during daylight hours over 4-d period, where samples were collected on d 16–19 at 0800, 1100, 1400 and 1700 h; Grab 9 × 3 = 3 samples per day collected over 3-d period with samples being taken every 9 h, where on d 16 samples were collected at 0800, 1700 and 0200 h; on d 17 collected at 1100, 2000 and 0500 h; and on d 18 collected at 1400, 2300 and 0800; Bulk (72 h) = total feces collection over 72 h on d 16–19.

second feeding grain-based diets, observed that the FR for TiO_2 averaged 93% and 95%, respectively. The results of the present study showed that FR of TiO_2 was generally higher than 100% and varied between diets with different F:C ratio, where animals fed 40:60 diets showed greater FR than animals fed 60:40 diets. In our previous study with dairy cattle we also observed a FR greater than 100%, where the FR ranged from 183 to 199% for TiO_2 (Velasquez et al., 2018). According to Glindemann et al. (2009), FR greater than 100% was associated to consumption of soil containing TiO_2, increasing its fecal concentration. Although, in our current study animals were kept in concrete floor pens and diets were well preserved and handled, so the risk of soil contamination was minimum.

Titgemeyer et al. (2001) analyzed TiO_2 using the colorimetric procedure proposed by Short et al. (1996), with an adaptation where 10 mL of 30% H2O2 was used instead of 20 mL recommended by the original procedure. In our previous and present study, TiO_2 was quantified by a spectrophotometry procedure according to Myers et al. (2004) and the accuracy of the analysis was validated by a regression equation proposed by Glindemann et al. (2009). Despite the differences regarding the methodology of marker quantification, (Owens and Hanson, 1992) suggested that the variation in marker recovery must be taken into consideration other sources of variation, as animal differences and environmental effects. The FR for Cr_2O_3 ranged from 71 to 83%, indicating that Cr_2O_3 was not completely recovered in the feces when administered orally to growing beef cattle fed corn silage and Tifton-85 hay diets with different F:C ratios. As observed in our previous study using dairy cattle (Velasquez et al., 2018) and others with beef cattle (Benvennuti et al., 2014; Sampaio et al., 2011a, 2011b; Paixão et al., 2007) Cr_2O_3 was almost completely recovered and considered as an appropriate external marker for estimating FO. The consistency of our results suggest that for this specific conditions, where growing Nellore bulls were fed corn silage or Tifton-85 hay based-diets with varying F:C ratios, the FR of the internal and external markers evaluated were generally poorly recovered. Therefore, it is necessary to perform TFC on at least one animal per treatment to establish the FR by using the RFO in the equation to estimate the markers excretion.

4.2. Prediction of DMD based on fecal concentration of internal markers

In general, once FR was established 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 underestimated and differed from TTAD when the bulk 72 h 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 procedures of lignin analysis. The inaccuracy in estimating DMD using ABL and cutin is associated to their low concentration in the feed and feces samples. According to Velasquez et al. (2018), 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. Nevertheless, our findings suggest that both iNDF and iADF are suitable internal markers for estimating DMD under both bulk and grab sampling procedures proposed in this study.

4.3. Prediction of FO based on fecal concentration of external markers

In the present study, once FR was established both external markers were able to produce accurate FO estimates when compared to the RFO values. However, only under the 72 h bulk sampling procedure on the hay 60:40 diet both Cr_2O_3 and TiO_2 did not estimate FO accurately. A tendency 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 forage source and F:C ratios that was observed for FO. Besides the 72 h bulk sampling, both grab procedures produced accurate FO when Cr_2O_3 was used as external marker. The same was true for TiO_2, except when the grab 4 × 4 sampling procedure was performed for animals receiving the silage 60:40 diet. In general, the FO estimates produced by Cr_2O_3 and TiO_2 were accurate for most of the sampling procedures, where FO did not differ from the RFO.

According to Myers et al. (2006), the use of TiO_2 as external marker has increased due the potential human health threats caused by inhalation when manipulating Cr_2O_3. Inconsistent responses are often observed, where FO estimates are underestimated (Glindemann et al., 2009) or overestimated compared to RFO (Titgemeyer et al., 2001). The variability of the results appear to be caused by differences in FR which is affected by the FO estimation (Velasquez et al., 2018). In the current study, all but two, of the TiO_2 derived FO estimates were not significantly different from RFO. Once again, this shows the importance of TFC to have the real FR in order to correct the fecal concentration values.

Grab sampling procedures produced accurate FO estimates with both external markers and therefore TFC is not strictly necessary. Although, we recommend the TFC on at least one animal per treatment to establish the FR of markers. Interestingly, the bulk 72 h sampling procedure was the less accurate among the procedures being studied. This procedure requires collecting all feces in a 24 h 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 the small quantities collected are usually completely used to make up the composite sample.

4.4. 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 FO 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 perhaps is explained by the same difficulty of obtaining representative samples from bulk samples.

Titgemeyer et al. (2001) using TiO_2 and Benvennuti et al. (2014) using Cr_2O_3 to estimate DMD and FO, and then DMI, observed lower intake compared to RDMI. These authors stated that when FR is different than 100% will result in under or over estimation of FO, consequently, under or over estimating DMI. The lower the FR, the higher FO estimates that will be obtained and vice versa. A precise estimation of DMD (or TTAD), if FO is underestimated then DMI will also be lower than RDMI. Similar would occur when DMD is underestimated at a given FO (or RFO). In the current study, FR was 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.

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 the external markers paired 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. Due to the more consistent DMI prediction results showed by the combination between both external markers and the internal markers iNDF and iADF under grab sampling procedures, the effect of the markers combinations was averaged across diets and compared. The results suggest that TiO_2 paired with iNDF or iADF resulted in a slightly better DMI prediction than when the same internal markers were paired with Cr_2O_3. The average DMI prediction using TiO_2 was only 1.7% higher than the average RDMI, while when Cr_2O_3 was used paired with iNDF or iADF, the average DMI prediction was 3.6% lower than the average RDMI observed. There was no difference in using iNDF or iADF as the internal marker.

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 4 × 4 daylight procedure can be adapted to various management practices as 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 under grab sampling procedures is an available tool to predict DMI in ruminant nutrition studies.

5. Conclusion

The FR of all internal and external markers evaluated in growing Nellore bulls fed corn silage or Tifton-85 hay based-diets with varying F:C ratios was poorly recovered, thus it is necessary to adjust the marker excretion using the RFO. Therefore we recommend to perform a TFC from at least one animal per treatment in order to adjust FR. Once FR is established, the internal markers iNDF and iADF produce accurate DMD estimates; and both external markers produce properly FO estimates under both grab sampling procedures. As a result, the DMI is accurately predict by the combination of internal and external markers, specifically when Cr_2O_3 or TiO_2 is paired with iNDF or iADF under grab 4 × 4 or grab 9 × 3 sampling procedures. In this case, both grab sampling procedures can replace TFC.

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CRediT authorship contribution statement

Alejandro V. Velásquez: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Cassiele A. Oliveira:** Investigation. **Cristian M.M.R. Martins:** Formal analysis. **Julio C.C. Balieiro:** Formal analysis. **Luis F.P. Silva:** Resources. **Romualdo S. Fukushima:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Dannylo O. Sousa:** Conceptualization, Validation, Investigation, Data curation, Writing – review & editing, Project administration.

Declarations of Competing Interest

None.

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