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

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ABSTRACT

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. The experiment used 10 multiparous Holstein cows fed a corn silage-based diet, with 55:45 forage-to-concentrate ratio, the average fecal recovery (FR) of TiO_2 was higher than FR of Cr_2O_3 , and both FR were more than unity. With internal markers, acetyl bromide lignin and cutin FR were lower than unity, and average FR for indigestible neutral detergent fiber (iNDF) and indigestible acid detergent fiber (iADF) 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 2 external markers, only Cr_2O_3 produced accurate fecal output (FO) estimates and the same happened to dry matter digestibility (DMD) when iNDF and iADF were used. Estimates for DMD and FO were affected by sampling procedure; 72-h bulk [sub-sample from total feces collection (TFC)] sampling consistently produced accurate results. The grab (sub-samples taken at specific times during the day) sampling procedures were accurate when using either of the indigestible fibers (iNDF or iADF) to estimate DMD. However, grab sampling procedures can only be recommended when concomitant TFC is performed on at least one animal per treatment to determine FR. Under these conditions, Cr_2O_3 is a suitable marker for estimating FO, and iNDF and iADF are adequate for estimating DMD. Moreover, the Cr_2O_3 +iADF marker pair produces accurate dry matter intake estimates and deserves further attention in ruminant nutrition studies. 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.

Key words: chromic oxide, intake, indigestible fiber, lignin, titanium dioxide

INTRODUCTION

Dry matter intake is a key determinant of nutrient intake and may be an index of the efficiency with which nutrients are metabolized; therefore, it is the major 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 or absorbed 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). 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 advancement 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 of this technique are the difficulty and labor of feeding the

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external markers twice a day, for several days; the need for excessive interaction between animals and people, which may 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 and as the source of bulk samples for later analysis. The recommended number of days for TFC ranges from 7 to 10 d (Schneider and Flatt, 1975) to 3 to 5 d (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 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. (2011a) compared 9 different fecal sampling designs and reported that a 4-d sampling period, in which 4 daily grab samples are taken, 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 to 4 d of collection period, should result in accurate estimates for DMD, FO, and DMI in dairy cows consuming a corn silage-based diet.

The objectives of the experiment were 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; the external markers Cr_2O_3 and TiO_2 for estimating FO; the combination of internal and external markers for estimating DMI; and to compare 3 different sampling designs against TFC.

MATERIALS 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 became ill during the experiment.

Animals, Diet, and Experimental Design

Ten multiparous Holstein cows (average $\pm$ SD; parity: 1.2 ± 0.4 lactations; DIM: 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 before starting the experiment to guarantee absence of wounds or abnormalities that could compromise feed intake. Cows were housed in a covered, concrete floor freestall, 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 (0700 and 1500 h) and milk yield was recorded during the experimental period.

All cows received a corn silage-based diet, with 55:45 forage-to-concentrate ratio. Daily allowances of the TMR, plus 10% of calculated DMI (to guarantee *orts*), were fed in 2 equal portions at 0800 and 1600 h, to represent a natural diurnal feeding pattern of cattle. Subsamples of individual feeds and TMR were taken on Mondays, Wednesdays, and Fridays for DM determination [drying in a forced-draft oven (Solab Científica, 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 concentrate was composed of coarsely ground corn grains (50.9% of total grain mix DM), soybean meal (25% of total grain mix DM), and whole roasted soybeans (8.8% of total grain mix DM), cottonseed meal (10.2% of total grain mix DM), and mineral mix (4.4% of total grain mix DM). 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 to facilitate analysis and decrease variability of results. Composition of silage, concentrate, and TMR are shown in Table 1.

The experiment ran for 19 d (day regarded as the 24-h period from 0800 to 0800 h). The experimental period consisted of 3 phases: d 1 to 5 were allocated to adaptation of the animals to the diet; 10 d (d 5 to 15) of marker excretion 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 before 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 2 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. All cows received 15 g of Cr_2O_3 and 15 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.

Feces

On d 15, all stalls were thoroughly cleaned (pressure hosed). On d 16 at 0600 h stalls were cleaned of what was excreted overnight and at 0800 h the 72-h TFC period started. Two-person teams constantly watched all the animals, in 8-h 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 (one per cow) 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. Sampaio et al. (2011a) 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-d sampling period with samples being taken every 9 h.

Table 1. Chemical composition of feeds and TMR, total-tract apparent digestibility (TTAD), real fecal output (RFO), and real dry matter intake (RDMI) of TMR

Item	Silage	Concentrate	TMR
DM (g/kg)	288.4	822.4	528.7
Ash ¹ (g/kg of DM)	43.0	56.9	49.3
CP ² (g/kg of DM)	94.0	265.9	171.4
EE ³ (g/kg of DM)	66.0	127.9	93.9
NDF ⁴ (g/kg of DM)	560.0	457.6	535.7
CW ⁵ (g/kg of DM)	736.9	763.9	749.1
SF ⁶ (g/kg of DM)	137.4	306.3	213.4
ADF (g/kg of DM)	348.1	97.7	235.5
iNDF ⁷ (g/kg of DM)	247.2	29.6	155.7
iADF ⁸ (g/kg of DM)	160.0	20.0	99.6
ABL ⁹ (g/kg of DM)	95.8	13.0	58.5
Cutin (g/kg of DM)	3.5	7.6	5.3
TTAD (g/kg of DM)	—	—	710.7
RFO (g of DM/d)	—	—	6,900.3
RDMI (g of DM/d)	—	—	24,078.1

¹Ash (500°C).

²N% $\times$ 6.25.

³EE = ether extract.

⁴Neutral detergent soluble fiber.

⁵CW = cell wall.

⁶Soluble fiber (SF) = CW – NDF.

⁷Indigestible NDF, 288 h in situ assay.

⁸Indigestible ADF, 288 h in situ assay.

⁹ABL = acetyl bromide lignin.

This way, at the end of the 3 d, the 24 h of a day will be represented by the various samples. Table 2 illustrates the fecal sampling design protocols used in this study.

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 sample weights varied between 50 and 500 g per sample. These grab samples were the composited daily and analyzed by sampling method.

Chemical Analysis and Measurements

For quantification of voluntary intake, feed supplied and feces collected between d 15 to 18, and orts collected between d 16 to 19 were measured. Samples were composited by animal and frozen at -20°C . Later, feces, feed, and ort samples (approximately 500 g) 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

Table 2. Sampling design protocols for bulk and grab feces samples

Design	Sampling	Duration and sampling times
A	Bulk 72 h	72 h (d 16–18), continuous
B	Grab 9 $\times$ 3	3 d (d 16 = 0800, 1700, 0200 h; d 17 = 1100, 2000, 0500 h; d 18 = 1400, 2300, 0800 h)
C	Grab 4 $\times$ 4 daylight	4 d (d 16–19), 0800, 1100, 1400, 1700 h

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 effect on our expected results.

Feed and TMR samples were analyzed for DM (105°C for 24 h) and ash (500°C for 8 h) by incineration in a muffle furnace (Marconi). Neutral detergent fiber and ADF were determined using the fiber bag procedure (Van Soest et al., 1991), with addition of heat-stable amylase and without sodium sulfite (Mertens, 2002), in a TE-149 Fiber Analyzer (Tecnal, São Paulo, Brazil). Briefly, 0.5-g samples were weighed into nonwoven textile fiber bags (25 µm pore size; Baglab, Pirassununga, SP, Brazil). The bags were heat-sealed and placed into the fiber analyzer (30 bags per batch, 10 mL of solution per bag) for 60 min. After this, the bags were thoroughly washed 3 times with boiling water and dried at 105°C, overnight.

Cell wall preparation was obtained according to the procedure proposed by Fukushima and Hatfield (2001) and later used as substrate for ABL determination in feed, orts, and feces samples, according to Fukushima and Kerley (2011). Lignin absorbance was determined with a Libra S80 spectrophotometer (Biochrom, Cambridge, United Kingdom) at a wavelength of 280 nm. Soluble fiber was calculated as the difference between cell wall and NDF contents, composed mainly of pectin. Indigestible neutral detergent fiber and indigestible acid detergent fiber (iNDF and iADF, respectively), with ash, were obtained by weighing silage and feces (0.5 g), orts (1.0 g), and concentrate (1.0 g) samples into F57 bags (25 µm pore size, Ankom Technology Corp., Fairport, NY). Samples were incubated inside the rumen of a cannulated cow for 288 h (Casali et al., 2008; Krizsan and Huhtanen, 2013), washed with abundant water, and treated according to Van Soest and Robertson (1985) for determination of these indigestible fractions. The ABL, iNDF, and iADF content that was measured in orts samples was then discounted from the content measured in feeds to correct the ingested amount of marker.

Cutin contents in feed, TMR, orts, and feces samples were determined in nonwoven textile fiber bags according to the procedure described by R. S. Fukushima (personal communication). Briefly, DM samples are sequentially treated with neutral detergent and acid detergent solutions to obtain an acid detergent residue. Fiber bags containing acid detergent residue were treated with potassium permanganate solution (KMnO₄) to oxidize lignin and afterward treated with 72% sulfuric acid solution (H₂SO₄) to hydrolyze cellulose, leaving behind cutin and mineral residue. Bags were dried in a forced-draft oven at 105°C for 12 h

and weighed. Finally, the residue-containing bags were incinerated at 450°C in a muffle furnace, and cutin contents are calculated as

$$\text{Cutin\%} = \{[\text{WaH} - (\text{WaI} - \text{Wc})] \times 100\} \div \text{SiW},$$

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

The cutin content that was measured in orts samples was then discounted from the content measured in feeds to correct the ingested amount of marker.

Crude protein (N% × 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 Cr₂O₃ 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 (1 g of feces from an animal that had not received Cr₂O₃) containing 0, 2, 4, 6, 8, and 10 mg of chromium per g of DM were analyzed in triplicate, totaling 18 standard samples. Pure Cr₂O₃ (99% trace metals basis; Solumix, Campinas, São Paulo, Brazil) was employed to produce the standards. The concentration of Cr₂O₃ was analyzed and compared with the added amount of Cr₂O₃. The results of the regression are shown in Figure 1.

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

Figure 1. Relationship between Cr₂O₃ added and Cr₂O₃ analyzed in feces matrix.

Figure 2. Relationship between TiO_2 added and TiO_2 analyzed in feces matrix.

was created using the method proposed by Glindemann et al. (2009). Standard samples (1 g of feces from an animal that had not received TiO_2) containing 0, 1, 2, 3, 4, 5, and 6 mg of titanium per g of DM were analyzed in triplicate, totaling 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.

Calculations

Real dry matter intake was calculated as daily feed offer minus daily orts (Equation [1]), and RFO was calculated as the average of 3 d (d 16 to 18) of TFC (24 h collection and daily weighing of amount excreted). Total-tract apparent digestibility was calculated as RDMI minus RFO (Equation [2]):

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

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

Equations [3] and [4] were used to estimate marker excretion and recovery rate. These equations were derived from both bulk and grab samples. The overall recovery rate of markers was calculated from the total amount of marker given (minus the amount of marker measured in orts) and the amount recovered during the fecal sampling period (d 15 to 19). Daily recovery rate of markers was calculated from d 16 to 19:

$$\text{marker excretion (g/d)} = [\text{fecal marker}] (\text{g/g}) \times \text{RFO (g/d)}, \quad [3]$$

$$\text{recovery rate of the marker} = \frac{\text{marker excreted (g/d)}}{\text{marker ingested (g/d)}}. \quad [4]$$

Equation [5] was used to estimate FO using external markers and Equation [6] was used to estimate DMD using internal markers:

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

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

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:

$$\text{DMI estimated by markers (g/d)} = \frac{\text{FO (kg of DM/d)}}{1 - \text{diet DMD}}. \quad [7]$$

Statistical Analysis

Results were analyzed with SAS 9.3 (SAS Institute Inc., Cary, NC), after verifying normality of residuals and homogeneity of variance. With normally distributed TTAD values and DMD estimates, a 4×4 factorial arrangement was adopted (4 markers and 4 sampling procedures) and data were analyzed using PROC MIXED, at a 0.05 significance level, according to the following model:

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

where Y_{ijk} = the DMD estimated in animal k , by marker i , under sampling procedure j ; μ = the general constant; T_i = the fixed effect of internal marker; M_j = the fixed effect of sampling procedure; TM_{ij} = the interaction between internal marker i and sampling procedure j

(fixed); $A(T)_k$ = the random effect of animal k nested to marker i ; and ε_{ijk} = the sampling error supposed to be normal and independently distributed.

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

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

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

Values of RDMI and DMI estimates were checked for normal distribution and an 8×4 factorial arrangement was adopted [8 marker pairs (one internal + one external) and 4 sampling procedures]. Data were analyzed using PROC MIXED, at a 0.05 significance level, according to the model

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

where Y_{bcd} = the DMI estimated in animal d , by marker b , under sampling procedure c ; μ = the general constant; T_b = the fixed effect of marker pair; M_c = the fixed effect of sampling procedure; TM_{bc} = the interaction between marker pair b and sampling procedure c (fixed); $A(T)_d$ = the random effect of animal d nested to marker b ; and ε_{bcd} = 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 24.1 ± 4.0 kg/animal per d, equivalent to $3.9 \pm 0.4\%$ of BW. Mean RFO (average $\pm$ SD) was 6.9 ± 1.1 kg of DM/animal per d, equivalent to $1.1 \pm 0.2\%$ of BW. The chemical composition of the feeds and TMR were in accordance with the diet formulated for the experiment. The values for TTAD (average $\pm$ SD) of 710.7 ± 41.1 g/kg of DM were higher than expected, as were those for ether

extract (average $\pm$ SD) of $9.4 \pm 0.4\%$ DM, considering the average quality of the corn silage that composed 55% of the diet.

Fecal Recovery of Markers

Mean fecal recovery (FR) of markers varied widely and are shown in Table 3. A highly significant effect for marker ($P < 0.0001$) 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. In contrast, fecal recovery of TiO_2 was unexpectedly high (around 200%) independent of the sampling procedure. Indigestible NDF and ADF produced similar FR, around 150% for the 24-h period and ABL had a FR around 100%, which was similar in all sampling procedures. The FR of Cr_2O_3 differed ($P = 0.044$) between the 4×4 and 9×3 grab sampling procedures, but neither one was different ($P = 0.344$ and $P = 0.285$, respectively) from FR of the 72-h sampling procedure.

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 ($P < 0.0001$, $P = 0.003$, and $P = 0.060$, respectively). Estimates of DMD from iADF and iNDF were not significantly different from TTAD, independent of the

Table 3. Twenty-four hour fecal recovery (FR; mean $\pm$ SE) of markers under 2 grab (4×4 and 9×3) and 1 bulk (72 h) fecal sampling procedures

Marker ¹	Method		
	4×4 grab	9×3 grab	72 h bulk
ABL	$0.98^{cd} \pm 0.09$	$0.95^d \pm 0.08$	$1.11^c \pm 0.13$
Cutin	$0.81^d \pm 0.14$	$0.67^c \pm 0.09$	$0.80^d \pm 0.11$
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_2O_3	$1.01^{B,c} \pm 0.06$	$1.23^{A,c} \pm 0.06$	$1.12^{AB,c} \pm 0.06$
TiO_2	$1.95^a \pm 0.16$	$1.99^a \pm 0.14$	$1.83^a \pm 0.14$

^{A,B}Within a row, mean values with common uppercase superscripts are not significantly different by Fisher's LSD ($P < 0.05$).

^{a-d}Within a column, mean values with common lowercase superscripts are not significantly different by Fisher's LSD ($P < 0.05$).

¹ABL = acetyl bromide lignin; iADF = indigestible ADF; iNDF = indigestible NDF.

Table 4. Mean total-tract apparent digestibility (TTAD; g/kg of DM) and dry matter digestibility (DMD; g/kg of DM) estimates derived from internal markers on 2 grab (4 × 4 and 9 × 3) and 1 bulk (72 h) fecal sampling procedures (mean ± SE)

Marker ¹	Sampling procedure			
	4 × 4 (grab)	9 × 3 (grab)	72 h (bulk)	TTAD
ABL	660.6 ^{B,ab} ± 20.8	663.5 ^{B,b} ± 17.0	698.9 ^{AB} ± 17.5	710.7 ^A ± 6.6
Cutin	621.1 ^{B,b} ± 38.3	572.5 ^{B,c} ± 39.0	690.2 ^A ± 33.9	710.7 ^A ± 6.6
iADF	703.8 ^a ± 13.7	720.9 ^a ± 11.7	728.6 ± 12.2	710.7 ± 6.6
iNDF	710.5 ^a ± 12.7	726.0 ^a ± 10.6	728.5 ± 11.7	710.7 ± 6.6

^{A,B}Within a row, mean values with common uppercase superscripts are not significantly different by Fisher's LSD ($P < 0.05$).

^{a-c}Within a column, mean values with common lowercase superscripts are not significantly different by Fisher's LSD ($P < 0.05$).

¹ABL = acetyl bromide lignin; iADF = indigestible ADF; iNDF = indigestible NDF.

sampling procedure. Cutin and ABL estimates of DMD were only accurate under the 72-h sampling procedure ($P = 0.479$ and $P = 0.654$, respectively).

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 ($P < 0.0001$, $P = 0.002$, and $P = 0.006$, respectively). Estimates for FO produced by TiO₂ were not accurate when compared with RFO. Estimates of FO from Cr₂O₃ concentrations were only accurate for the 9 × 3 and 72-h fecal sampling procedures ($P = 0.206$, $P = 0.306$, respectively).

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 ($P < 0.0001$) were observed, yet no effect ($P = 0.447$) for their interaction was detected. Most DMI estimates by TiO₂ differed substantially from RFO under all sampling regimens. When TiO₂ was paired with iNDF and

iADF, under the 72-h 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 4 × 4 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 were not detected as different from RDMI independently of the sampling procedure used.

DISCUSSION

Fecal Recovery of Markers

Fecal recovery varied widely among markers and ranged from 0.67 to 0.81 g/g of DM for cutin to 1.83 to 1.99 g/g of DM for TiO₂. No reports were found on the use of cutin as a marker in ruminant digestion studies and therefore our data are novel to this field of research. Siqueira et al. (2009) used cutin as a DMD marker in horses consuming 4 different diets and observed FR around 1.51 g/g of 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.

Table 5. Mean real fecal output (RFO; g of DM/d) and fecal output (FO; g of DM/d) estimates derived from external markers on 2 grab (4 × 4 and 9 × 3) and 1 bulk (72 h) fecal sampling procedures (mean ± SE)

Marker	Sampling procedure			
	4 × 4 grab	9 × 3 grab	72 h bulk	RFO
Cr ₂ O ₃	7,971.0 ^{A,a} ± 286.2	6,455.1 ^{C,a} ± 208.3	7,260.1 ^{B,a} ± 293.3	6,900.3 ^{BC} ± 204.9
TiO ₂	5,015.3 ^{B,b} ± 223.3	4,823.8 ^{B,b} ± 202.2	5,403.6 ^{B,b} ± 307.5	6,900.3 ^A ± 204.9

^{A-C}Within a row, mean values with common uppercase superscripts are not significantly different by Fisher's LSD ($P < 0.05$).

^{a,b}Within a column, mean values with common lowercase superscripts are not significantly different by Fisher's LSD ($P < 0.05$).

Liquid and solid phase stratification of contents in the rumen compared with 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-h period. Njaa (1961) reported that mean FR less than one could be attributed to inaccuracy of the analysis. Because cutin makes up such a small fraction of DM, gravimetric errors tend to be more likely to happen.

Several authors (Kotb and Luckey, 1972; Fahey and 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-h 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 routine 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 because we observed FR that ranged from 1.83 to 1.99 g/g of 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 to 0.99 in sheep, and 0.96 to 1.02 and 0.90 to 0.95 in dairy cattle, respectively. Glindemann et al. (2009) found FR higher than 1 and attributed this to possible ingestion of soil that contained TiO_2 and therefore would increase the fecal concentration. Our diet was composed of corn silage and concentrate, and the animals were kept in stalls with concrete flooring, so the risk of soil contamination was low.

Mean FR of Cr_2O_3 varied from 1.01 to 1.23 g/g of DM in this study. Benvenuti et al. (2014) worked with Brahman steers fed leafy or stemmy grass hay and observed FR of 0.92 to 1.03 g/g of DM. Because 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 of DM (Oliveira et al., 2003; Siqueira et al., 2009; Lanzetta et al., 2009), in sheep from 0.80 to 0.92 g/g of DM (Rodrigues et al., 2010), in dairy cattle from 0.87 to 0.89 g/g of DM (Delagarde et al., 2000), and in steers from 0.85 to 1.05 g/g of DM (Sampaio et al., 2011b). 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 ADL, has been used as an internal marker for digestibility studies and is considered indigestible because no microbial or mammalian enzymes 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 upward 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 underestimation 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 of DM for 3 and 4 d 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

Table 6. Mean real dry matter intake (RDMI; g of DM/d) and DMI (g of DM/d) estimates derived from 1 internal + 1 external marker pairs on 2 grab (4×4 and 9×3) and 1 bulk (72 h) fecal sampling procedures (mean $\pm$ SE)

Marker ¹	Sampling procedure			
	4×4 grab	9×3 grab	72 h bulk	RDMI
Cr_2O_3				
ABL	26,381 ^A $\pm$ 1,816	21,521 ^B $\pm$ 1,881	26,586 ^A $\pm$ 1,724	24,078 ^{AB} $\pm$ 633
Cutin	28,559 ^A $\pm$ 4,497	19,039 ^B $\pm$ 1,978	25,345 ^A $\pm$ 3,839	24,078 ^A $\pm$ 633
iADF	28,259 $\pm$ 1,394	24,435 $\pm$ 1,423	28,116 $\pm$ 1,634	24,078 $\pm$ 633
iNDF	28,712 ^A $\pm$ 1,340	24,626 ^{AB} $\pm$ 1,335	28,046 ^{AB} $\pm$ 1,621	24,078 ^B $\pm$ 633
TiO_2				
ABL	16,656 ^B $\pm$ 1,231	15,707 ^B $\pm$ 1,126	19,726 ^{AB} $\pm$ 1,398	24,078 ^A $\pm$ 633
Cutin	19,661 ^{AB} $\pm$ 765	13,799 ^C $\pm$ 1,461	17,245 ^{BC} $\pm$ 1,951	24,078 ^A $\pm$ 633
iADF	17,793 ^B $\pm$ 985	17,967 ^B $\pm$ 896	20,411 ^{AB} $\pm$ 1,111	24,078 ^A $\pm$ 633
iNDF	18,158 ^B $\pm$ 1,008	18,170 ^B $\pm$ 853	20,406 ^{AB} $\pm$ 1,126	24,078 ^A $\pm$ 633

^{A-C}Within a row, mean values with common uppercase superscripts are not significantly different by Fisher's LSD ($P < 0.05$).

¹ABL = acetyl bromide lignin; iADF = indigestible ADF; iNDF = indigestible NDF.

to gravimetric errors because of its spectrophotometric nature. Mean ABL FR ranged from 0.95 to 1.11 g/g of 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.

Indigestible neutral detergent and acid detergent fibers are 2 of the most extensively used internal markers today. Mean FR ranging from 0.50 to 1.21 (Barros et al., 2009; Kozloski et al., 2009; Rodrigues et al., 2010; Sampaio et al., 2011b; Lee and Hristov, 2013) and from 0.80 to 1.21 g/g of DM (Oliveira Júnior et al., 2004; Rodrigues et al., 2010; Sampaio et al., 2011b) for iNDF and iADF, respectively. Oliveira et al. (2003) worked with horses and observed FR of 1.08 to 1.34 g/g of DM and up to 1.54 g/g of DM for iNDF and iADF, respectively. In this study, mean FR for iNDF and iADF were 1.43 to 1.53 g/g of DM and 1.47 to 1.60 g/g of 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 and Huhtanen (2013) who compared various incubation times (144, 240, and 288 h) and reported that at least 12 d 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 be adequate internal markers for ruminant digestion studies because of accurate DMD estimates. Although this is true, RFO need to be determined to correct FR, which tended to be overestimated in our study.

Prediction of DMD Based on Fecal Concentration of Internal Markers

Values for DMD were underestimated 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 ADL method. A possible explanation for the low DMD estimates produced by these 2 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 markers for determination of DMD in dairy cows if grab sampling procedures were to be used. With the use of bulk sampling procedures, these 2 markers deserve further attention to better understand how they behave. From what we know after researching the literature of the subject, ABL or cutin had not 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 2 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 with 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 more accurate 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 with RFO values. Under the 72-h 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 4×4 grab sampling procedure was 100%, and therefore, overestimated FO was unexpected. No consistency for FO was observed for Cr_2O_3 estimates as values were overestimated on 4×4 grab sampling, no difference was observed when 72-h bulk sampling was used, and underestimation occurred under 9×3 grab sampling. 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 with 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 (Benvenuti et al., 2014). Our results suggest that Cr_2O_3 could be used as a marker for estimating FO, but because FR differs from 100%, TFC is needed and the best results derive from the 72-h 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 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 of the TiO_2 -derived FO estimates were significantly underestimated, under all sampling procedures, when compared with RFO. This is a reflection of the unusually high FR observed in our experiment because 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 4×4 grab and 72-h bulk sampling, as well as for Cr_2O_3 +iNDF under 9×3 grab and 72-h 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 2,456 g/d when compared with 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 4×4 grab sampling for cutin and under 72-h bulk sampling for ABL, iADF, and iNDF. Once again, although estimates were "accurate," the mean difference was 4,027 g/d 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., 2010; Benvenuti 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 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 (Berchielli et al., 2005; Rodrigues et al., 2010), 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 overestimated and DMD estimates were lower than TTAD 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 2 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; 72-h bulk consistently produced accurate results. The 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 one animal per treatment 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.

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