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ANIMAL PRODUCTION

Titanium dioxide (TiO_2) as a marker to estimate fecal output in sheep

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ABSTRACT: *Titanium dioxide (TiO_2) is an external marker used to estimate fecal output based on forage intake of grazing ruminants; however, its use as marker for grazing sheep still needs attention. In the present study the TiO_2 role to estimate fecal output of sheep grazing annual ryegrass was tested. Two essays were conducted in sequence using the same forage and animals. Six lacaune lambs were housed in metabolic cages. Lambs were administered 1 g TiO_2 in a single (at 8:30 am, essay 1), or in two daily doses (at 8:30 am and 4:30 pm, essay 2). In both essays the TiO_2 administration was followed by a 7-day period to stabilize the fecal excretion of the marker and then by a 5-day period for total collection of feces. Observed and TiO_2-based estimated fecal outputs were compared, with or without correction for TiO_2 fecal recovery rate ($kTiO_2$). The $kTiO_2$ was around 1.0 and the estimated fecal outputs were similar to the observed ones ($p>0.05$), regardless the number of daily doses. These results suggested that TiO_2, administered in single or two daily doses, can be used as an external marker in grazing sheep.*
Key words: *external marker, fecal excretion, forage intake.*

Dióxido de titânio (TiO_2) para a estimativa da produção fecal em ovinos

RESUMO: *O dióxido de titânio (TiO_2) é um marcador externo utilizado para estimativas da excreção fecal, visando medidas de consumo de forragem em ruminantes a pasto. No entanto, o uso deste marcador para ovinos em pastejo ainda pode ser melhor explorado. Neste trabalho, o TiO_2 foi testado para estimar a produção fecal de ovinos ingerindo azevém anual. Foram conduzidos dois ensaios sequenciais utilizando a mesma forragem e os mesmos animais. Os animais eram seis cordeiros da raça Lacaune, mantidos em gaiolas metabólicas. No primeiro ensaio, os cordeiros receberam diariamente 1 g TiO_2 em única dosagem (às 08 h 30), e no segundo foram oferecidas duas dosagens (08h30 e 16 h 30). Em ambos os ensaios, após sete dias para estabilização da excreção fecal do marcador foram realizados cinco dias de coleta total de fezes. A produção fecal observada foi comparada com a produção fecal estimada pelo TiO_2, com ou sem correções, para sua taxa de recuperação fecal ($kTiO_2$). A $kTiO_2$ foi próxima de 1.0 e a produção fecal estimada foi similar à observada ($P>0,05$), independente do número de dosagens. Estes resultados mostram que o TiO_2 é um indicador externo que pode ser utilizado, em única ou dupla dosagem, em experimentos com ovinos em pastejo.*
Palavras-chave: *excreção fecal, ingestão de forragem, marcador externo.*

It is important to assess forage intake of grazing ruminants, given its key effect on their performance (AZEVEDO et al., 2014). However, the currently available methodologies are neither precise nor easy to implement (PENNING, 2004). There are several types of internal (e.g. fecal proteins and dietary fibers) and external (e.g. *n*-alkanes, CrO_2, Yb and TiO_2) markers used to estimate forage intake and each one of them has its own limitations (CARVALHO et al., 2007).

Titanium dioxide (TiO_2) has been considered as a suitable, viable and cost effective external marker to assess fecal outputs of grazing animals (TITGEMEYER et al., 2001), and to replace other markers, such as n-alkanes and chromium oxide ($Cr2O_3$), known for its carcinogenic potential (SEDMAN et al., 2006). Nevertheless, the reliability of TiO_2 as a marker needs to be evaluated by comparing observed and estimated fecal outputs of housed animals submitted to different dietary protocols (COATES & PENNING, 2000).

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Two sequential essays were performed using 1 g of TiO_2, in a single (essay 1) or in two daily doses (essay 2). Six castrated Lacaune lambs, fed exclusively with annual ryegrass silage (*Lolium multiflorum* Lam.), were housed in metabolic cages (110 x 50 cm). In the former essay – single dose of TiO_2 - animals had initial average body weight (BW) and body condition score of 24.1 ± 2.3 kg and 3.0 ± 0.3, respectively. In the latter – two daily doses – animals presented 25.9 ± 1.7 kg and 3.0 ± 0.2, respectively.

The ryegrass silage dry matter (DM), crude protein (CP) and neutral detergent fiber (NDF) were 213 g/kg, 132 g/kg DM and 594 g/kg DM, respectively. Silage supply was adjusted to meet NRC (2007) requirements, starting from 0.672 kg DM/d – single – and 0.725 kg DM/d – two doses (about 2.8% BW). An additional 100 g of green matter (GM)/kg GM consumed per day was fed to the lambs to allow diet selection. The same forage was used for both treatments.

Titanium dioxide (pure TiO_2, Synth®, Brazil) was administered in cellulose capsules in two ways for 12 consecutive days: a single 1 g daily dose at 8:30 am (essay 1); two 0.5 g daily doses at 8:30 am and 4:30 pm (GMT: -03 h 00) (essay 2). The later essay started immediately after the former; ie, on the 13th day, with the same lambs. A 7-day period was allowed to stabilize fecal excretion of the marker. Between the 8th and the 12th days, feces samples were collected, identified and dried in aluminum trays using a forced-air oven (MA035, Marconi, Piracicaba, Brazil) at 60 ºC for 48 h. Dry feces were ground in a micro mill and further passed through a sieve of 0.25 mm. The collected samples from each animal were combined in a single pool per animal, which were homogenized and had the concentration of TiO_2 measured.

One day before the administration of TiO_2, each animal had its marker baseline concentration (standard samples) established based on a feces sample, so all further results could be corrected for it. This procedure is important because small concentrations of TiO_2 can be reported in the soil, thus its presence in the feces may indicate the amount of soil eaten by the animal (PENNING, 2004). Furthermore, higher concentrations of TiO_2 detected by spectrophotometry in this previous analysis would indicate contamination of the lab equipment.

Feces were collected twice a day using stainless steel trays placed under the metabolic cages. The trays were emptied and weighed daily at 8 am and 4 pm and a 200 g feces sample from each animal was collected and dried to determine its dry matter content. To avoid feces contamination by urine, lambs were fitted with canvas urine collector bags.

The following steps were used to determine the concentration of TiO_2 in the samples: 1) 0.1 g of feces, 3.5 g of potassium sulphate (K_2SO_4) and 0.4 g of copper sulphate ($CuSO_4$) were weighed in duplicates on an AY-220 scale (Shimadzu®, Tokyo, Japan), transferred into micro-Kjeldahl digestion tubes (100 mL), in which 10 mL of concentrated sulfuric acid (H_2SO_4) was added; 2) samples were placed in a digestion block at 420 °C for 2 h (or until they became translucent green); 3) after cooling for at least 30 minutes, the contents were diluted to 100 mL in a volumetric flask with distilled water, carefully to avoid any supernatant; 4) 2.5 mL of the digested and diluted samples were loaded to a spectrophotometer together with 0.1 mL of hydrogen peroxide P.A. (H_2O_2), so the color of the solution became orange; 5) the absorbance was measured at 410 nm using a UV–visible light spectrophotometer IL-592-LC (Kazuaki®, Wuxi, China).

The calibration curve was obtained by measuring the absorbance of the control sample (100 mg of TiO_2 diluted in 100 mL of distilled water), as well as of the samples with 0, 2, 6, 8 and 10 mg of TiO_2 (Table 1) in distilled water and 0.1 mL of H_2O_2 (Labsynth, Diadema, Brazil). Before the dilution in 100 mL of distilled water, the TiO_2 was transferred into macro-Kjeldahl digestion tubes (45 × 200 mm), in which 14 mL of concentrated sulfuric acid (H_2SO_4) and 3.5 g of potassium sulphate (K_2SO_4) and 0.4 g of copper sulphate ($CuSO_4$) were added and placed in a digestion block at 420 °C until they became translucent green. A standard sample (no TiO_2 added) was used to standardize the spectrophotometer at 410 nm. The TiO_2 fecal recovery rate (kTiO_2) was calculated by dividing the daily excretion of TiO_2 by its daily dose (1 g), with the former being obtained by multiplying the fecal mass (g DM/d) by the concentration of TiO_2 in the feces, corrected by its baseline concentration in the standard samples. Fecal outputs were estimated as follows:

$$FO = (TiO_{2_{ingested}} \, x \, k_{TiO_2}) / ([TiO_2]_{feces} - [TiO_2]_{standard})$$

in which FO is the fecal output (g DM/d); $TiO_{2_{ingested}}$ is the amount of TiO_2 ingested by the animal (g TiO_2/d); k_{TiO_2} is the fecal recovery rate of TiO_2 (g/g ingested); $([TiO_2]_{feces}$ is the concentration of TiO_2 in the feces (mg TiO_2/g DM); and $[TiO_2]_{standard}$ is the concentration of TiO_2 (mg TiO_2/g DM) in the standard samples collected on the day before the experiment started.

Statistical differences between estimated and observed fecal outputs were calculated using

Table 1 - Volumetric and mean absorbance values of various samples dilutions used to obtain the spectrophotometric calibration curve for the external marker titanium dioxide (TiO_2).

Cuvette	Standard Solution (mL)	Distilled Water (mL)	H_2O_2 (mL)	Absorbance (dimensionless)
1	0.000	2.500	0.1	0.000
2	0.025	2.475	0.1	0.027
3	0.050	2.450	0.1	0.057
4	0.100	2.400	0.1	0.113
5	0.150	2.350	0.1	0.215
6	0.200	2.300	0.1	0.297
7	0.250	2.250	0.1	0.380
8	0.300	2.200	0.1	0.447
9	0.350	2.150	0.1	0.515
10	0.400	2.100	0.1	0.591
11	0.450	2.050	0.1	0.665
12	0.500	2.000	0.1	0.731

one-way ANOVA followed by a t-Student pairwise comparison test ($P<0.05$) using SigmaPlot Version 12 (Systat Software, San Jose, CA). Normal distribution and equal variances assumptions were confirmed by Shapiro-Wilk normality and Barlett homoscedasticity tests, respectively.

The $kTiO_2$ was around 1.0 and no statistical difference ($P>0.05$) between estimated and observed fecal outputs was reported neither in the single dose (168 ± 90 g DM/d and 151 ± 48 g DM/d, respectively) nor in the two daily doses (112 ± 11 g DM/d and 188 ± 46 g DM/d, respectively) essays. The highest individual variances of both estimated and observed fecal outputs were reported (SD of 90 g and 48 g, respectively), as well as the smallest mean difference between the two of them (17 g/d), with the administration of a single TiO_2 daily dose. Furthermore, with the two doses, both estimated and observed fecal outputs varied less (SD of 11 g and 46 g, respectively), but the mean difference between them increased to 76 g/d.

It was expected that the administration of TiO_2 in two daily doses would reduce possible variations on its concentration in the feces along the day (PENNING, 2004). Nevertheless, SIBBALD et al. (2000) evaluated the use of n-alkane protocols with two and single daily doses and found no difference between the observed and the estimated intake of markers in both protocols. Conversely, VULICH et al. (1993) concluded that the intake was overestimated by single daily doses of n-alkanes, while estimated and observed values were similar when two daily doses were administered. In the present study, as for the n-alkanes protocol (SIBBALD et al., 2000), the use of a single daily dose did not reduce the accuracy of TiO_2 to estimate the fecal output.

Therefore, it is possible to conclude that the single daily administration of TiO_2 is a potential protocol to estimate fecal outputs and intake in grazing sheep.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This project was submitted and approved by the Committee on Ethics for the Use of Animals (CEUA) of Federal University of Parana under the protocol n° 007/2012.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTION

All authors contributed equally to the design and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

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