Evaluation of chromium oxide and titanium dioxide as inert markers for calculating apparent digestibility in sheep

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ABSTRACT
The objective of the present study was to evaluate two markers: chromium oxide (Cr$_2$O$_3$) and titanium dioxide (TiO$_2$). We evaluate the interaction between Cr$_2$O$_3$ and TiO$_2$ and the techniques used to determine it, using atomic absorption spectrophotometry (AAS) and photometry simple (PS). We used six growing sheep distributed in a replicated Latin square 3 × 3 design, with adjustment for the residual error effect. The TiO$_2$ and Cr$_2$O$_3$–TiO$_2$ produced values similar to those obtained by total faecal collection (TFC) or the use of Cr$_2$O$_3$ alone, determined by AAS and PS. Digestibility of the marker/TFC ratio was similar (p > .05) between markers and technique. The use of TiO$_2$ alone or in combination with Cr$_2$O$_3$ seems to be a suitable alternative to TFC and Cr$_2$O$_3$ to calculate apparent digestibility of the total digestive tract determined in sheep by PS and AAS.

1. Introduction
Chromium oxide (Cr$_2$O$_3$) has been widely used as an external digestibility marker in digestion trials with ruminants (Amor-Bor-cho et al. 2009; Delagarde et al. 2010; Al Alami et al. 2014). The recovery of this marker in ruminants’ fed forage has revealed variations in faecal recovery (Titgemeyer et al. 2001); on the other hand, the use Cr$_2$O$_3$ is restricted in diets for animals because it possesses carcinogenic properties (Peddie et al. 1982; Sedman et al. 2006). Titanium dioxide (TiO$_2$) is presented as an alternative, but its study in small ruminants is scarce (Titgemeyer et al. 2001; Glindeman et al. 2009). Additionally, the combined use of these markers in vivo presented differences in digestibility coefficients but had similar recoveries (Kavanagh et al. 2001; Titgemeyer et al. 2001).

Several analyses for these markers differ for colorimetric and spectroscopy techniques, the latter being the most commonly recommended for the least amount of interference present (AAFCO 2004). However, in vivo studies of these two procedures showed no differences (Carciofi et al. 2007). For TiO$_2$ we do not know whether there are differences between these two methods: colorimetric and spectroscopy.

The aim of this study was to evaluate the use of Cr$_2$O$_3$ and TiO$_2$ as digestibility markers, as well as their interaction Cr$_2$O$_3$ + TiO$_2$, as determined by atomic absorption spectrophotometry (AAS) and colorimetric methods, and to compare digestibility coefficients obtained from total faecal collection (TFC) in sheep.
by the Kjeldahl method (AOAC 1990); chromium oxide suspension was performed according to Hill and Anderson (1958), and titanium dioxide according to Titgemeyer et al. (2001). Both were analysed through a photometry simple (PS) (Thermo Fisher model Scientific Genesyis 10 Vis) with a wavelength of 430 nm for Cr2O3 and 410 nm for TiO2; an AAS (Perkin Elmer 3110) was used to determine Cr2O3 and TiO2 with a Perkin Elmer lamp for Cr (part#303-6021 Serial H235571) and Ti (part#303-6075 Serial H167419) for each marker, respectively. The calibration curve was performed with standard solutions for Cr2O3 PS with 0.05, 0.01, 0.15 and 0.5 mg/ml ($y_{ppm} = 0.481x_{OD} - 0.006$; $R^2 = 0.99$), and 20, 50 and 100 ppm for AAS ($y_{ppm} = 206.19x_{ABS} - 3.7113$; $R^2 = 0.99$) (standard for AAS from J.T. Baker 1000 µg/ml CAS 6449-04 for Cr), as for TiO2 PS with 0.005, 0.01, 0.075, 0.15 and 0.2 mg/ml ($y_{ppm} = 0.3226x_{OD} - 0.008$; $R^2 = 0.98$) and for AAS 10, 20 and 40 ppm ($y_{ppm} = 0.1756x_{ABS} + 1.5$; $R^2 = 0.99$) (standard for AAS from J.T. Baker 1000 µg/ml CAS 6472-04 for Ti), where ABS is the absorption wavelength value and OD is the optical density value. The combinations of Cr2O3 + TiO2 differ in that the two markers are mixed, but we only suspended and analysed one of the compounds.

The proportion of DM and OM degraded was calculated using the following equation:

$$\text{Digestibility} = \frac{\text{intake} - \text{excretion}}{\text{intake}} \times 100,$$

where intake and excretion are expressed in g/d, and x represents DM and OM content, respectively.

The recovery of the markers was determined using the following equation:

$$\text{Marker recovery} (%) = \frac{\text{total faecal excretion (DM, g/d) with marker}}{\text{total faecal excretion (DM, g/d) using TFC (g/d)}} \times 100.$$

The faecal excretions of chromium and titanium dioxide were estimated by multiplying the total DM excretion by the marker content in the representative faecal sample, according to the following equation:

$$\text{MFE} = \text{FM} \times [\text{M}]_{RS},$$

where MFE is the faecal excretion of marker (kg/d), FM is the faecal mass obtained by total collection (g/d) and $[\text{M}]_{RS}$ is the marker content in the representative faecal sample (g/kg).

From the assumptions of Equation (3), the faecal recovery of the markers was calculated as the ratio of faecal excretion to the intake of marker, described as:

$$\text{FR} = \frac{\text{MFE}}{\text{D}} \times 100,$$

where FR is the faecal recovery of marker (%), MFE is the faecal excretion of marker (g/d) and D is the daily dose of external marker (g/d).

The faecal excretion was also determined by using the faecal content of markers according to the following equation:

$$\text{FE}_{RS} = \frac{\text{D}}{[\text{M}]_{RS}},$$

where FE$_{RS}$ is the faecal excretion estimated by using the marker content in the faecal representative sample (kg/d), D is the daily dose of external marker (g/d), and $[\text{M}]_{RS}$ is the marker content in the representative faecal sample (g/kg) (Lippke 2002). The values of total tract apparent digestibility in each treatment were subjected to analysis of variance using a design of two replicated Latin squares with adjustment for residual error, including three treatments in six animals. Treatment sequences within each Latin square were organized to balance the effects of carryover such that each treatment followed every other treatment one time within each square using the GLM procedure SAS (1999), following the model:

$$Y_{ijkl} = _{1} \mu + P_{j} + A_{k} + T_{i} + e_{ijkl},$$

where $Y_{ijkl}$ is the dependent variable, $_{1} \mu$ is the overall mean, $P_{j}$ is the effect of period $j$, $A_{k}$ is the effect of animal $k$, $T_{i}$ is the effect of treatment $i$, and $e_{ijkl}$ is the residual error.

Furthermore, in order to evaluate the technical values of total tract apparent digestibility and the digestibility of the markers/TFC ratio (DM and OM), a factorial arrangement 4 × 2 with six replicates for each one (Kuehl 2000) was performed using the statistical program Statistical Package for Social Sciences (SPSS, version 15.0, November 2006),

$$Y_{jk} = _{1} \mu + M_{j} + T_{j} + M \times T_{jk} + e_{jk},$$

where $Y_{jk}$ is the dependent variable, $_{1} \mu$ is the overall mean, $M_{j}$ is the effect of marker treatment $j$, $T_{j}$ is the effect of determination technique $k$ (PS vs. AAS), $M \times T_{jk}$ is the interaction $jk$, and $e_{jk}$ is the residual error. Differences between the means of the least squares were considered significant at $p < .05$, and differences were considered to indicate a trend towards significance at $.05 > p < .10$. 

### Table 1. Ingredients and chemical composition (g/kg DM) in the diet using Cr2O3, TiO2, and their interaction used in growing lambs.

| Ingredients (g/kg DM) | Cr2O3 | TiO2 | Cr2O3 + TiO2 |
|----------------------|-------|------|--------------|
| Sorghum grain        | 720.0 | 720.0| 720.0        |
| Alfalfa hay          | 61.8  | 61.8 | 61.8         |
| Oat hay              | 123.5 | 123.5| 123.5        |
| Molasses             | 54.6  | 54.6 | 54.6         |
| Urea                 | 10.0  | 10.0 | 10.0         |
| Calcium, 38%         | 11.5  | 11.5 | 11.5         |
| Phosphate 18/20      | 2.0   | 2.0  | 2.0          |
| Magnesium oxide      | 2.1   | 2.1  | 2.1          |
| Na Bicarbonate       | 5.0   | 5.0  | 5.0          |
| NaCl                 | 5.0   | 5.0  | 5.0          |
| Minerals mix         | 0.5   | 0.5  | 0.5          |
| Cr2O3                | 4.0   | ND   | 4.0          |
| TiO2                 | ND    | 4.0  | 4.0          |

Chemical composition

| DM | 917 | 921 | 919 |
| OM | 871 | 870 | 871 |
| NDF | 166 | 163 | 164 |
| N | 15 | 15 | 15 |

aMinerals mix: Ca 15.0%, P 8.0%, Mg 0.5%, K 0.35%, S 0.2%, Fe 450 mg/kg, Zn 1900 mg/kg, Mn 1800 mg/kg, Se 15 mg/kg, I 30 mg/kg, Co 25 mg/kg.

bND, no data; the marker is not part of the diet.
3. Results and discussion

Diet (g/kg DM) averaged 919 g/kg DM; OM, 871 g/kg DM; NDF, 165 g/kg DM, and N, 15 g/kg DM, consisting of the same amount of ingredients for each marker (Table 1).

The recovery percentage (Table 2) was similar between markers (p > .05), and the techniques used do not affect the TiO2 determination (this may be due to the inclusion of the Cr2O3 level used in the present study). Therefore, it can be assumed that chronic oxide and titanium dioxide achieve the necessary requirement for an ideal marker, because faecal recovery of both markers seems to be unaffected by different feeding conditions. From this, assuming that there is total faecal recovery and there are no diet effects on recovery, both external markers can be presumed to be similar to each other. Thus, titanium dioxide can be assuredly used as a substitute for chronic oxide, which is more desirable because it has not been reported as a carcinogenic precursor.

Although the recovery of Cr2O3 in the presence of TiO2 is the highest numerically (Table 2), no statistical difference was found (p = .388) from those markers analysed by PS and AAS. It remains unclear as to how the presence of Ti could improve the assessment of Cr recovery. The values differ from Titgmeyer et al. (2001), who recovered 112% of Cr2O3 + TiO2, analysed by AAS using the technique proposed by Williams et al. (1962). In another study, Kavanagh et al. (2001) showed 96% recovery in pig diets adding Cr2O3; however, Jagger et al. (1992) obtained recoveries of 74% and 80% for diets supplemented with 1 g and 5 g Cr2O3/kg respectively, not in combination with TiO2, using the colorimetric method of Fenton and Fenton (1979). Using the same technique, Carciofi et al. (2007) recovered 106 ± 0.044 and 101 ± 0.045% of Cr2O3 with AAS and PS, respectively. The recovery percentages differ from those of Jagger et al. (1992), who reported recoveries of uncombined Cr2O3 with TiO2 of 98.3% and 96.9%, respectively, for additions of 1 g and 5 g/kg of marker; moreover, Kavanagh et al. (2001) found 92.3% recoveries with 1 g/kg when combined with Cr2O3 + TiO2. Titgmeyer et al. (2001) found recoveries of 95% adding 5 g TiO2/kg DM. Hafez et al. (1988) obtained 99% recovery in concentrate-based diets and corn silage forage diets.

Different methodologies for determining TiO2 vary according to the use of substance for the wet samples. Leone (1973) recommended 10 ml of concentrated H2SO4, but Jagger et al. (1992) and Short et al. (1996) modified this technique, using twice as much H2SO4. Titgmeyer et al. (2001) modified this technique using 7.4 M H2SO4 and 10 ml of 30% H2O2. However, Myers et al. (2004) changed the use of the ashing procedure prior to the suspension, by the wet suspension of the sample with concentrated H2SO4. The wavelength used was different in each technique, varying from 400 nm by Short et al. (1996) and Kavanagh et al. (2001), to 408 nm by Jagger et al. (1992) and Leone (1973) and 410 nm by Titgmeyer et al. (2001) and Myers et al. (2004). The wavelength used in each technique may affect the sensitivity obtained for TiO2. Myers et al. (2004) founded better recoveries when using 409 vs. 410 nm; in the present study TiO2 was determined at 410 nm.

DM and OM intake were similar among treatments (Table 3), due to the feed intake being restricted to 2.2% of live weight; DM digestibility (g/d) was higher (p < .05) for the combination TiO2 + CrO3 than for either CrO3 or TiO2 alone. The OM digestibility showed a rising trend between markers (p = .087), being higher for the combination TiO2 + CrO3. The DM and OM digestibility (g/g) and the digestibility marker/TEC ratio were similar between markers (p > .05). DM digestibility (g/d) comparing PS vs. AAS showed a rising trend (p = .062), being higher (1.8%) for AAS than PS, and similar (p > .05) when data

Table 2. Markers recovery percentage in faeces, using Cr2O3, TiO2 and Cr2O3+TiO2, estimated by PS and AAS.

| Markers  | CrPS  | CrAAS | TiPS  | TiAAS  |
|----------|-------|-------|-------|--------|
| Cr2O3    | 140.8 | 138.3 | ND    | ND     |
| TiO2     | ND    | ND    | 148.7 | 121.1  |
| Cr2O3 + TiO2 | 148.2 | 162.9 | 143.5 | 128.4  |
| SEM      | 8.0   | 6.5   | 8.3   | 10.8   |

Table 3. Intake (g DM/d), digestibility (g/g) and digestibility marker/TEC ratio, obtained by Cr2O3, TiO2 or combined determined by PS and AAS.

| Item      | Cr2O3 | TiO2 | Cr2O3 + TiO2 | TiO2 + Cr2O3 | PS  | AAS  | SEM  | M  | T   | M × T |
|-----------|-------|------|--------------|--------------|-----|------|------|----|-----|-------|
| Intake, g/d | 909   | 909  | 909          | 909          | 909 | 909  | 909  | 909| 909   | 909  |
| OM        | 863   | 860  | 862          | 862          | 977 | 791  | 822  | 822| 822   | 822  |
| Digestibility, g/d | 746a | 751a | 752abc       | 774b         | 749 | 763  | 3.8  | .041| .062  | .625  |
| OM        | 727   | 731  | 729          | 748          | 729 | 739  | 3.3  | .087| .115  | .569  |
| Digestibility, g/g | 0.85 | 0.83 | 0.84         | 0.83         | 0.83| 0.84 | 0.85 | .070| .258  | .264  |
| OM        | 1.00  | 0.99 | 1.02         | 1.02         | 1.01| 1.01 | 1.00 | .367| .927  | .271  |

Note: Means with the different letter in the same row are significantly (p < .05) different.

1TEC: total faecal collection.
2Markers, 0.4% Cr2O3, 0.4% TiO2, 0.4% Cr2O3 + 0.4% TiO2.
3Estimated digestion based on titanium.
4Estimated digestion based on chromium.
5SEM, standard error of mean.
are expressed in OM; the DM and OM digestibility and the digestibility marker/TFC ratio was similar between techniques (PS and AAS; p > .05). Across treatments, DM digestion averaged 74.35%; this agrees closely with values reported previously (74.1%, Almaraz et al. 2010 and 75.25%, Cabral Filho et al. 2013) with lambs fed a sorghum-based diet, even though the chemical composition of the diets was different.

This results are similar to Carciofi et al. (2007), who obtained no differences in digestibility using Cr2O3 compared with TFC determined by PS or AAS, while Titgmeyer et al. (2001) overestimate the digestibility using Cr2O3 and underestimate with TiO2 when compared with TFC. Kavanagh et al. (2001) obtained similar values for TFC and Cr2O3, while the values obtained with TiO2 were lower compared with TFC and Cr2O3.

The observed difference between DM digestibility (g/d) by ASS vs. PS may be due to variations in the estimation of digestibility caused by a lack of certainty in the analysis of the marker (TiO₂ + Cr₂O₃), as the use of the wet sample proposed by Hill and Anderson (1958) includes HClO₄, which could saturate the solutions when it is mixed with TiO₂. Kavanagh et al. (2001) and Titgmeyer et al. (2001) found TFC values similar to those using Cr2O3 mixed with TiO2, determined by the technique proposed by Williams et al. (1962).

The results of the present study regarding titanium dioxide agree with Ferreira et al. (2009), Batista Sampaio et al. (2011), and Glindeman et al. (2009), who reported similar results for the marker/TFC ratio obtained with titanium dioxide. Additionally, Marcondes et al. (2008) and Ferreira et al. (2009) observed that titanium dioxide could be accurately used for estimating the individual intake of concentrate in group feeding when it was associated with other markers.

4. Conclusions

These experiments show that the use of TiO₂ or TiO₂ with Cr₂O₃ is a reliable marker for calculating TFC and for determining the apparent digestibility of the total digestive tract in growing lambs, as determined by AAS and colorimetric methods.

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