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Evaluation of titanium dioxide and chromic oxide as digestibility markers in ponies fed alfalfa hay in relation to marker dosing frequency

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In equines, Cr_2O_3 is widely accepted as an indigestible marker, but there are health concerns regarding the carcinogenic properties of Cr_2O_3. Recently, TiO_2 has been suggested to be an alternative digestibility marker in equines. However, a comparison between Cr_2O_3 and TiO_2 has not been made in equines. Six Welsh pony geldings (initial BW: 254 ± 3 kg; 7 years of age) fed chopped alfalfa hay were used to evaluate the use of TiO_2 (Ti) and Cr_2O_3 (Cr) as markers for calculating apparent digestibility and to investigate the effect of frequency of marker administration on the measurement of digestibility values. Diets contained 4.65 kg dry matter (DM) chopped alfalfa hay supplemented with minerals, vitamins, TiO_2 (3.3 g Ti/day) and Cr_2O_3 (3.2 g Cr/day). Ponies were dosed with either 3.3 g Ti and 3.2 g Cr once daily (DF1) or with 1.65 g Ti and 1.60 g Cr twice daily (DF2). After adaptation to the diets and procedures for 14 days, voluntary voided faeces were collected quantitatively over 7 days and analysed for moisture, ash, Ti and Cr. Apparent total tract DM digestibility (DMD) and organic matter digestibility (OMD) were calculated using the total faecal collection (TFC) and marker method (Ti and Cr). The overall mean cumulative faecal recovery of Cr and Ti (as % of intake) were 102.0% and 96.6%, respectively. Mean daily faecal recoveries of Cr as well as of Ti were not different ($P = 0.323$; $P = 0.808$, respectively) between treatments. Overall daily faecal recovery of Cr differed ($P = 0.019$) from 100% when the marker was dosed once daily, whereas overall daily faecal recovery was similar to 100% for both administration frequencies when Ti was used as a marker. For both markers, the coefficient of variation of the mean faecal marker recovery between horses was lower when the markers were administrated twice per day. Across treatments, cumulative DMD and OMD estimated with Ti were similar ($P = 0.345$; $P = 0.418$, respectively) compared with those values determined by TFC method. When Cr was used, the calculated cumulative DMD tended ($P = 0.097$) to be greater compared with those estimated with TFC, and cumulative OMD values were overestimated ($P = 0.013$). Orally supplemented Ti recovery in the faeces of ponies fed chopped alfalfa hay with Ti administered once or twice daily was close to 100%, making it the preferred marker for digestibility trials in equines.

Keywords: apparent digestibility, equines, Cr_2O_3, TiO_2, total faeces collection

Implications

Use of markers to estimate total tract digestibility of nutrients is less labour intensive than total faeces collection and has been used for many years. In equines, Cr_2O_3 is widely accepted as a marker to determine nutrient digestibility. However, the use of Cr_2O_3 as a food additive is prohibited due to its potential carcinogenicity and TiO_2 is presented as a promising alternative in many animal species. Hitherto, a comparison between Cr_2O_3 and TiO_2 has not been made in equines. Our results indicate that TiO_2 is the preferred marker for digestibility trials in equines.

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Introduction

Use of markers to estimate digestibility of nutrients is less labour intensive than total faeces collection (TFC) and has been used for many years. Chromic oxide (Cr) has been widely accepted as a faecal marker in many animal species, including horses, pigs, poultry, dogs, cattle and sheep. However, due to its suspected carcinogenicity, use of Cr_2O_3

as a food additive is currently prohibited (Sedman *et al.*, 2006; FDA, 2016). A promising alternative is TiO_2 (Ti) as has been shown in pigs (Jagger *et al.*, 1992), cattle (Titgemeyer *et al.*, 2001), sheep (Myers *et al.*, 2006; Glindemann *et al.*, 2009) and equines (Schaafstra *et al.*, 2015, 2017 and 2018). Titanium dioxide is a colour additive (E171) and permitted for application in foods (FDA, 2016). A direct comparison between TiO_2 and Cr_2O_3 has been made in pigs (Kavanagh *et al.*, 2001), cattle (Titgemeyer *et al.*, 2001) and sheep (Guzman-Cedillo *et al.*, 2016), and TiO_2 was shown to have similar recoveries to Cr_2O_3. Such comparison between Cr_2O_3 and TiO_2, however, has not been made in hindgut fermenting equines and will be important in order to compare digestibility values between studies.

A continuous excretion of a marker per unit time is a crucial property when the marker technique to measure digestibility of nutrients is used. Within and between-day variation in the excretion of the marker limits its use in, for example, field studies (Haenlein *et al.*, 1966; Prigge *et al.*, 1981; Holland *et al.*, 1998). In particular, the time after marker administration seems to be the major determinant for the excretion of the marker (Haenlein *et al.*, 1966) and a more frequent administration of the marker tends to reduce variation in recovery (Prigge *et al.*, 1981). In order to accurately determine nutrient digestibility by the use of an orally supplemented marker, a steady-state excretion of the marker is essential. The aim of this study was to compare the recoveries of Cr_2O_3 and TiO_2 when administered as markers and determine the effect of frequency of marker administration (once or twice per day) on the measurement of nutrient digestibility in ponies fed chopped alfalfa hay.

Material and methods

Ethical considerations and animal welfare

The experimental protocol was approved by the Animal Experiments Committee of the Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands (Ethics Committee approval no. 2013.III.12.093). During the experiment, the ponies were exercised once daily for 1 h in a horse walker at a speed of 4.5 km/h during which time the ponies did not have access to feed or water. In addition, environmental enrichment (jolly balls) was provided in the stables, and the animals were groomed twice daily during sample collection periods. A period of 1 month before the study, all ponies were clinically examined, dewormed (Equest, Fort Dodge Animal Health Benelux B.V., Weesp, The Netherlands) and vaccinated (Equilis Prequenza TE, Intervet International B.V., Boxmeer, The Netherlands) by a licensed veterinarian.

Animals, experimental design and housing

According to a cross-over design, six, 7-year-old, Welsh pony geldings with an initial BW of 254 kg (SEM ± 3 kg) were assigned to two treatments. Each experimental period consisted of a 14-day preliminary feeding period followed by a 7-day sample collection period (Schaafstra *et al.*, 2017). The ponies were housed individually in 4 × 4 m boxes on wood shavings during the first 12 days of each experimental period. On day 13, the boxes were thoroughly cleaned, and rubber mats were placed to cover the floor until the end of the collection periods. Throughout the study, ponies had free access to a plastic bin containing fresh water and the BW of each pony was measured and recorded weekly.

Experimental diets

All ponies were fed a mineral and vitamin supplement (Research Diet Services B.V., Wijk bij Duurstede, The Netherlands) and chopped alfalfa hay (*Medicago sativa*) originating from the same batch, which was provided in different bales during the experiment (Table 1). The ponies were fed iso-energetically (on net energy (NE) basis), and energy intake was calculated to be 25.6 MJ NE/day (CVB, 2004) to meet the energy requirement for maintenance and exercise (250 kg BW, 1 h at 5 km/h). The daily amount of feed DM provided to the animals consisted of 4.65 kg chopped alfalfa hay and 0.09 kg mineral and vitamin supplement, including the Cr_2O_3 and TiO_2 markers. The alfalfa hay was offered in three meals at 0900, 1400 and 1800 h. At 0900 and 1400 h, ponies received a quarter of the alfalfa hay mixed with 2.5 l of water; the other half of the alfalfa hay was mixed with 5 l of water and fed at 1800 h. Water was

Table 1 *Chemical composition of the components of the experimental diet and the titanium and chromium-containing supplement provided to the ponies*

	Components of the experimental diet	
Items	Alfalfa	Supplement[1]
DM, % (as fed)	93.5	91.5
OM	90.3	67.8
CP	16.1	15.9
Sugar	4.6	6.8
Starch	0.6	4.8
Crude fat	1.2	3.8
Crude fibre	36.1	9.5
NDF	49.5	15.4
ADF	41.4	13.3
ADL	9.3	3.3
Ash	9.7	32.2
Ti	BDL	3.3
Cr	BDL	3.2
NE[2] (MJ/kg DM)	6.9	6.0

OM = organic matter; BDL = below detection limit of 0.5 g.kg; NE = net energy; DM = dry matter.
Values are expressed as % of DM unless otherwise indicated.
[1]The supplement contributed the following (per kg, as fed basis): NaCl, 100 g; $CoCO_3$, 4 mg; Na_2SeO_3, 9 mg; KI, 20.6 mg; MnO, 2.7 g; $CuSO_4 \cdot 5H_2O$, 1.6 g; $ZnSO_4 \cdot 7H_2O$, 4.45 g; $FeSO_4 \cdot 7H_2O$, 8 g; Cr_2O_3, 50 g; TiO_2, 50 g; thiamine, 0.12 g; riboflavin, 0.1 g; retinol acetate, 20.64 mg; cholecalciferol, 0.33 mg; DL-α-tocoferylacetate, 4 g; molasses 60 g and alfalfa, 718.98 g.
[2]Net energy of feedstuffs was calculated according to the formula: NE (MJ/kg DM) = (k_m × (ME − 31.3 × CFAT) + 0.80 × 31.3 × CFAT)/1000, where NE is net energy, ME is metabolisable energy (MJ/kg) and CFAT is crude fat (g/kg DM) (CVB, 2004).

supplemented to avoid potential obstruction due to the high DM content of the chopped alfalfa hay. The mineral and vitamin supplement was offered by means of a feed bin with intake being monitored by the animal caretaker to ensure complete consumption of the supplement. The supplement was provided either once daily (treatment DF1; 3.3 g Ti and 3.2 g Cr) at 1000 h or twice daily (treatment DF2; 1.65 g Ti and 1.60 g Cr) at 1000 and 1700 h. Actual DM intake was determined as described in Schaafstra *et al.* (2017). During each collection period, water intake was calculated as the sum of the amount of water supplemented with the diet and the daily amount of water offered and refused for each pony each morning at 0600 h.

Collection of samples

Representative samples of the chopped alfalfa hay and supplement fed during each individual collection period were obtained before each experimental period. During the coll ection periods, voluntarily voided faeces of each pony were collected quantitatively for 24 h for 7 consecutive days. The faeces collection devices were emptied every hour from 0600 up until 0100 h every 24-h (Schaafstra *et al.*, 2017). All 24-h collected faeces were weighed per pony and thoroughly mixed using a concrete mixer (Altrad Lescha GmbH, Burgau, Germany). Then, two portions of 10% of each mixed 24-h faeces sample were collected. The first 10% proportion was used to determine the daily recovery of the markers and daily DM digestibility (DMD) and organic matter digestibility (OMD). The second 10% proportion was pooled per pony to determine macronutrient digestibility values. Thereafter, a 1.5 kg sample was stored at −20°C in sealed aluminium trays until further processing. At the end of the experiment, the stored daily and pooled faeces samples were dried, ground and stored until chemical analysis (Schaafstra *et al.*, 2017).

Chemical analysis

Alfalfa hay, the supplement and daily faeces samples were analysed for DM and Ti, as described in Schaafstra *et al.* (2017). In addition, Cr was determined using the spectrophotometric procedure described by Fenton and Fenton (1979). The combined within and between-run precisions of the determinations for Ti and Cr were $\leqslant$2.5% and 3.5% (CV), respectively.

Alfalfa hay, the supplement as well as pooled faecal samples were analysed for DM, ash, CP, crude fat, crude fibre, starch, sugar and combustion value as described in Schaafstra *et al.* (2017). Fibre fractions (NDF, ADF and ADL; expressed without residual ash) were determined according to methods described by Van Soest *et al.* (1991).

Calculations

Organic matter (OM) was calculated as:

$$\mathrm{OM}(\mathrm{g}/\mathrm{kg\,DM}) = 1000 - \mathrm{Ash}(\mathrm{g}/\mathrm{kg\,DM})$$

Apparent total tract DMD and OMD determined by TFC were calculated for each pony using mean daily feed intake (*I*; DM or OM (kg)) and daily faecal output (*FO*; DM or OM (kg)) as:

$$\mathrm{DMD_{TFC}\,or\,OMD_{TFC}}(\%) = [(I - \mathrm{FO})/I] \times 100$$

Recovery of each marker in the faeces (Rf) was calculated using mean marker intake and faecal marker excretion over 7 consecutive days as:

$$\mathrm{Rf}(\%) = [\text{Faecal marker excretion}(\mathrm{g/day}) / \text{Marker intake}(\mathrm{g/day})] \times 100$$

Cumulative faecal recovery (Rf_{cum}) over the 7 consecutive days of faeces collection (days 1 to 7) was calculated as:

$$\mathrm{Rf_{cum}}(\%) = \left[\sum_{i=1}^{n} \text{Faecal marker excretion}(\mathrm{g/day}) \Big/ \sum_{i=1}^{n} \text{Marker intake}(\mathrm{g/day})\right] \times 100$$

where n is the number of faeces collection days with $n \leqslant 7$.

The apparent total tract DMD and OMD using the marker method (Ti or Cr) was calculated as:

$$\mathrm{DMD_{Ti\,or\,Cr}\,or\,OMD_{Ti\,or\,Cr}}(\%) = [1 - (A_{\mathrm{feed}}/B_{\mathrm{faeces}}) \times (\mathrm{XB_{faeces}}/\mathrm{XA_{feed}})] \times 100$$

where *A* and *B* represent the marker concentration (g/kg DM) and XA and XB the DM or OM concentration (g/kg DM) in the feed and faeces, respectively.

Cumulative apparent DMD (%) or OMD (%) over the seven consecutive days of faeces collection (days 1 to 7) was calculated as:

$$\left[\left(\sum_{i=1}^{n} \text{DM or OM intake}_i - \text{Faecal DM or OM excretion}_i\right) \Big/ \sum_{i=1}^{n} \text{DM or OM intake}_i\right] \times 100$$

where n is the number of faeces collection days, with $n \leqslant 7$.

Statistical analysis

All data were subjected to a one-way ANOVA with period and treatment as factors (SPSS Statistics 21.0) following the model:

$$Y_{ijk} = \mu + P_i + T_j + e_{ijk}$$

where Y_{ijk} is the observed value for the treatment, μ the overall mean, P_i the effect of the i^{th} period ($i = 1$ to 2), T_j the effect of the j^{th} treatment ($j = 1$ to 2) and e_{ijk} the error term. Within animals, comparisons on BW, DMD and OMD determined with either TFC, Ti, Cr and Rf (DF1 *v.* DF2) of Cr and Ti were made by paired sample *t*-tests. The latter test was also used to determine whether Rf of Cr and Ti were different from 100%. Differences were considered statistically significant when $P < 0.05$, and trends were declared at $0.05 \leqslant P < 0.1$.

Results

All ponies remained healthy throughout the experiment, and no coprophagia was observed during the experiment. Due to a technical error, faecal samples of three ponies at day 4 of faeces collection were missing. As a result, calculation of the mean daily Rf, DMD and OMD on day 4 is based on three ponies and Rf_{cum}, cumulative DMD and OMD were calculated omitting day 4. Feed refusals for the chopped alfalfa hay and the supplement were not observed throughout the study. Mean BW (± SEM) at the end of the experiment (254 ±4 kg) was not different ($P = 0.656$) to pre-experimental values (254 ± 3 kg). For all treatments combined, mean water intake (including water provided with the feed) was 36.3 l/day (SEM 4.2; $n = 6$).

Faecal recovery of markers

For both treatments and treatments combined, mean daily as well as cumulative faecal recovery (Rf) (percentage of intake) of Cr was greater ($P < 0.001$) compared with Ti. Overall, at day 7 Rf_{cum} of Cr as well as of Ti were not different from 100% for both administration frequencies.

Mean cumulative faecal recovery (Rf_{cum}) of Ti and Cr (Tables 2 and 3) were not different between administration frequencies. The average daily faecal recovery (Rf) of Ti for DF1 (98.3%; CV 13.0) was not different compared with the average daily Rf of Ti for DF2 (97.1%; CV 9.5). For both administration frequencies, daily Rf of Ti was not different to 100%. The greatest daily Rf value (% of intake) was observed for Cr when ponies were administered Cr once per day, that is 113.0% (CV, 11.4%) and no differences were observed when the marker was administered once or twice daily. The average daily faecal recovery (Rf) of Cr was significantly higher than 100% when the marker was dosed once daily.

For both markers, a 3.3 or 3.5 percentage points decrease in CV was observed for average daily Rf of Cr as well as for Ti, respectively, when the marker was provided twice daily compared with once daily.

Digestibility

For all treatments combined, cumulative apparent total tract DMD_{TFC} and OMD_{TFC} were 61.1% (SEM, 0.25) and 60.1% (SEM, 0.31), respectively. Across treatments, cumulative DMD_{Ti} (60.5%, SEM 0.54) and OMD_{Ti} (59.6%, SEM 0.48) were not different compared with corresponding values determined with TFC. When cumulative DMD and OMD were estimated using Cr as a marker for all treatments, DMD_{Cr} (62.2%, SEM 0.43) tended to be greater than DMD_{TFC} ($P = 0.097$). Cumulative OMD_{Cr} (62.0%, SEM 0.42) was greater compared with OMD_{TFC} ($P < 0.013$) (Tables 4 and 5).

Discussion

An indicator method allowing accurate estimation of nutrient digestibility has several advantages over the conventional TFC method; the main one being a simplification of the procedure for a sampling of faeces. In equines, Cr_2O_3 is widely accepted as an indigestible marker to determine nutrient digestibility. However, the use of Cr_2O_3 as a food additive is prohibited due to its potential carcinogenicity (Sedman *et al.*, 2006; FDA, 2016) and TiO_2 is presented as a promising alternative in many animal species (Jagger *et al.*, 1992; Titgemeyer *et al.*, 2001; Myers *et al.*, 2006). Observations in the current study show that the use of TiO_2 as a marker in digestibility trials in equines is more accurate over Cr_2O_3 when total faeces collection is applied.

Table 2 *Mean daily and cumulative faecal recoveries (as % of intake) and corresponding CV obtained using TiO_2 as an external marker administered once or twice daily over 7 days to ponies fed chopped alfalfa hay* ($n = 6$)[1]

	Treatments[2]									
	DF1 recovery					DF2 recovery				
Days	Daily	CV	*P*-value[3]	Cumulative	CV	Daily	CV	*P*-value[3]	Cumulative[4]	CV
1	93.3	18.4	0.383	93.3	18.4	95.4	10.9	0.323	95.4	10.9
2	103.4	15.6	0.630	98.3	6.7	95.7	5.8	0.112	95.5#	5.6
3	97.6	12.3	0.643	98.1	5.8	90.7	13.2	0.130	93.9*	5.1
4[5]	95.9	8.9	0.494	–	–	98.9	5.9	0.775	–	–
5	97.1	3.5	0.092	97.8	4.7	103.2	9.7	0.474	96.2	6.1
6	104.8	13.7	0.446	99.2	6.0	97.2	8.4	0.442	96.4	5.4
7	94.5	13.2	0.335	98.5	4.8	99.4	7.8	0.860	96.9	5.5
Average	98.3	13.0	0.400	–	–	97.1	9.5	0.054	–	–

[1]Mean cumulative faecal recoveries for days 5–7 are calculated excluding day 4.
[2]Treatment: DF1 recovery: once daily administration of marker; DF2 recovery: twice daily administration of marker.
[3]Probability that the mean daily faecal recovery is different from 100.
[4]Values with a # and * denote a trend ($P < 0.1$) and significant ($P < 0.05$) difference from 100.
[5]$n = 3$.

Table 3 *Mean daily and cumulative faecal recoveries (% of intake) and corresponding CV obtained using C_2O_3 as an external marker administered once or twice daily over 7 days to ponies fed chopped alfalfa hay (n = 6)*[1]

	Treatments[2]									
	DF1 recovery					DF2 recovery				
Days	Daily	CV	*P*-value[3]	Cumulative[4]	CV	Daily	CV	*P*-value[3]	Cumulative	CV
1	97.5	16.6	0.725	97.5	16.6	101.4	9.2	0.724	101.4	9.2
2	108.9	14.9	0.235	103.2	5.9	100.9	8.2	0.805	101.2	4.9
3	103.0	13.4	0.615	103.2	4.4	96.0	13.5	0.489	99.5	3.5
4[5]	108.8	5.0	0.108	–	–	107.6	7.7	0.253	–	–
5	103.1	14.1	0.624	103.1	6.1	105.4	8.3	0.192	100.9	3.9
6	113.0	11.4	0.057	105.1#	5.6	105.7	10.1	0.249	101.9	4.1
7	103.7	6.8	0.256	104.9	5.8	104.5	6.7	0.179	102.3	3.7
Average	105.2	12.6	0.019	–	–	102.7	9.3	0.082	–	–

[1]Mean cumulative faecal recoveries for days 5–7 are calculated excluding day 4.
[2]Treatment: DF1 recovery: once daily administration of marker; DF2 recovery: twice daily administration of marker.
[3]Probability that the mean daily faecal recovery is different from 100.
[4]Value with a # denotes a trend ($P < 0.1$) difference from 100.
[5]$n = 3$.

Table 4 *Mean daily digestibility of dry matter (DM) and organic matter (OM) over 7 days obtained using total faeces collection (TFC) and external markers (TiO_2, Cr_2O_3) administered once or twice daily to ponies fed chopped alfalfa hay (n = 6)*

	Treatments[1]								
	DF1 digestibility					DF2 digestibility			
Day	TFC	TiO_2	Cr_2O_3	Pooled SEM		TFC	TiO_2	Cr_2O_3	Pooled SEM
					DM				
1	62.0	59.8	61.1	2.7		61.0	59.8	61.9	1.7
2	60.4	62.2	63.8	2.4		60.6	59.6	61.4	1.4
3	61.2	60.7	62.3	2.6		62.5	59.2	61.1	2.0
4[2]	60.6	59.7	64.1	2.1		59.5	60.0	62.7[a]	1.6
5	62.0	61.5	63.1	2.3		59.9	61.5	62.2	1.9
6	57.9	60.1	62.9[a]	2.0		61.0	60.4	63.3	1.9
7	61.0	59.0	62.6	2.1		59.8	60.0	61.8	1.4
					OM				
1	61.0	59.0	60.9	2.8		60.0	58.8	61.5	1.7
2	59.6	61.5	63.8	2.3		59.5	58.6	61.1	1.4
3	60.3	59.9	62.2	2.5		61.4	58.2	60.7	2.1
4[2]	60.0	59.2	63.9	2.3		59.0	59.5	62.6[a]	1.7
5	61.1	60.6	62.8	2.3		59.1	60.7	62.1	1.8
6	56.6	58.9	62.4[a]	2.0		60.0	59.5	63.1	1.8
7	59.9	57.9	62.2	2.2		58.8	59.1	61.6	1.3

[a]Values differ significantly ($P < 0.05$) from TFC.
[1]DF1 digestibility: once daily administration of marker; DF2 digestibility: twice daily administration of marker.
[2]$n = 3$.

Table 5 *Mean cumulative digestibility of dry matter (DM) and organic matter (OM) over 7 days obtained using total faeces collection (TFC) and external markers (TiO_2, Cr_2O_3) administered once or twice daily to ponies fed chopped alfalfa hay (n = 6)*[1]

	Treatments[2]								
	DF1 digestibility					DF2 digestibility			
Days	TFC	TiO_2	Cr_2O_3	Pooled SEM		TFC	TiO_2	Cr_2O_3	Pooled SEM
					DM				
1	62.0	59.8	61.1	2.7		61.0	59.8	61.9	1.7
2	61.2	61.4	62.8	0.9		60.8	59.8	61.7	1.1
3	61.2	61.3	62.8[a]	1.0		61.4	59.7	61.6	0.9
4	–	–	–	–		–	–	–	–
5	61.4	61.3	62.9	1.1		61.0	60.2	61.6	1.1
6	60.9	61.1	62.9[a]	1.0		61.0	60.2	62.0	1.1
7	60.8	60.9	62.9	1.0		60.8	60.2	62.0	1.1
					OM				
1	61.0	59.0	60.9	2.8		60.0	58.8	61.5	1.7
2	60.3	60.7	62.7	0.9		59.8	58.8	61.4	1.1
3	60.3	60.5	62.7[a]	0.9		60.3	58.7	61.2	0.9
4	–	–	–	–		–	–	–	–
5	60.5	60.5	62.8	1.0		60.0	59.2	61.5	1.0
6	59.9	60.3	62.8[a]	0.8		60.0	59.3	61.8	1.0
7	59.8	60.0	62.7[a]	0.9		59.8	59.3	61.8[a]	1.0

[a]Values differ significantly ($P < 0.05$) from TFC.
[1]Mean cumulative digestibility of DM and OM for days 5–7 are calculated excluding day 4.
[2]DF1 digestibility: once daily administration of marker; DF2 digestibility: twice daily administration of marker.

In the current study, overall cumulative faecal recovery (Rf_{cum}, as % of intake) for Cr was 102.0%, which is greater than recovery rates reported in other studies with horses (98.4%, Haenlein *et al.*, 1966; 99.6%, vander Noot *et al.*, 1967; 94.8%, Parkins *et al.*, 1982; 60%; 81%, Patterson *et al.*, 2002; 71.2%, Oliveira *et al.*, 2003; 83.7%, Lanzetta *et al.*, 2009; 88%, Siqueira *et al.*, 2009). Although the observed recovery rate did not differ from 100%, DMD_{Cr} and OMD_{Cr} yielded higher values compared with the TFC method on several days. These observations are in contrast to results found by Takagi *et al.* (2002), who reported that Cr_2O_3 was found to provide accurate estimates of the digestibility of rations fed to horses. However, the latter study can be criticised as faeces were collected from the floor (covered with sawdust) not allowing total faeces collection and as such provide a lower Rf_{Cr}.

When Ti was used as a marker in the current study, observed overall Rf_{cum} was 96.6% for ponies fed chopped alfalfa hay, which corresponds to recovery rates observed in horses (96.2%, Schaafstra *et al.*, 2015; 97.5%, Schaafstra *et al.*, 2017), maned wolves and dogs (86.7% and 97.0%, Childs-Sandford and Angel, 2006), steers (97.2%, Titgemeyer *et al.*, 2001), dairy cows (98.8%, Hafez *et al.*, 1988), pigs (97.6%, Jagger *et al.*, 1992), growing pigs (92.3%, Kavanagh *et al.*, 2001), sheep (102.2%, Glindemann *et al.*, 2009), cattle (102%, Sampaio *et al.*, 2011) and chickens (99.3%, Short *et al.*, 1996). After adaptation to the marker, recovery of Ti was not different to 100%, and for any of the days, DMD_{Ti} and OMD_{Ti} were not significantly different to corresponding values obtained with the TFC method. Based on these results, it can be concluded that Ti is a preferred indigestible marker compared with Cr to estimate the digestibility of dietary nutrients in horses.

A variation in the excretion of a marker within and between days in conjunction with the amount of faeces collected and its time of collection is important for the accurate determination of nutrient digestibility. Glindemann *et al.* (2009) showed that grab samples obtained at 0900 h had a higher TiO_2 concentration than grab samples obtained at 1700 h in sheep fed herbage. A more frequent administration of the marker tends to reduce this variation (Dove and Mayes, 1991; Peddie *et al.*, 1982). Digestibility values using Ti and Cr have been reported to be higher with twice, compared with once daily administration of the marker in sheep (Myers *et al.*, 2006; Glindemann *et al.*, 2009). Although in the present study a decrease in CV was observed when the marker was provided twice daily, this decrease was not significant. Therefore, the accuracy of the estimated digestibility values was not improved when the marker was administered more frequently. This would imply that administration of these markers once daily to horses is justified, provided that a steady-state excretion is attained.

The total error in estimating digestibility values using a marker method is a result of the faecal recovery of the used marker, the latter which is affected by the accuracy of marker administration, absorption, chemical analysis and the total collection of faeces. Mean cumulative DMD_{Cr} was different to values determined with the TFC method on day 6 when the marker was administered once daily. For OMD_{Cr}, different values from the TFC were observed on days 3, 6 and 7 for DF1 and day 7 for DF2, which is the result of an increased daily faecal recovery of Cr. The time after administration of Cr is considered a major determinant for the recovery of Cr. According to vander Noot *et al.* (1967), an equilibrium of recovery of the ingested Cr_2O_3 has been reached after 4 days in horses, with sheep and cattle requiring 5 or 6 days to adapt to the marker administration (Titgemeyer *et al.*, 2001; Glindemann *et al.*, 2009). In the current study, Cr was administered for 14 days before the measurement of digestibility, and it can, therefore, be assumed that faecal Cr concentration had reached steady-state conditions during the faeces collection periods and could not explain the increased Rf of Cr. In the current study, the mean daily Rf of Cr as well as of Ti did not differ between faeces collection days. For all treatments combined, Rf of Cr for individual animals over 7 days of faeces collection ranged from 79.6% to 121.9% (data not shown). Large individual differences among animals in the recovery of both Cr_2O_3 and TiO_2 were also observed in horses (vander Noot *et al.*, 1967) and steers (Titgemeyer *et al.*, 2001) and could influence mean recovery rates and subsequently digestibility values when insufficient animals are used.

In summary, the mean Rf_{cum} of Cr and Ti was not different from 100% and not affected by administration frequency (once or twice daily). However, DMD_{Cr} and OMD_{Cr} were overestimated, whereas DMD_{Ti} and OMD_{Ti} gave not significantly different values compared with the TFC method. Based on these results, Ti is the preferred marker in the estimation of DMD and OMD in horses fed chopped alfalfa hay, regardless of administration frequency. To fully exploit the advantage of a digestibility marker, a partial collection of faeces is needed to gain insight in the excretion pattern of Ti with faeces. Further studies need to determine the accuracy of Ti as a digestibility marker when grab samples or incomplete faeces collection are used.

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Declaration of interest

There are no conflicts of interest.

Ethics statement

The experimental protocol was approved by the Animal Experiments Committee of the Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands (Ethics Committee approval no. 2013.III.12.093).

Software and data repository resources

Data and models are not deposited in an official repository.

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