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RELIABILITY OF ACID-INSOLUBLE ASH AS INTERNAL MARKER FOR THE MEASUREMENT OF DIGESTIBILITY IN RABBITS

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Abstract: The present study aimed to evaluate acid-insoluble ash (AIA) as an internal marker for the measurement the coefficient of total tract apparent digestibility (CTTAD) in rabbits through two experiments (E1 and E2). In E1, 48 rabbits were used to calculate the CTTAD of the same basal diet according to the European reference method (ERM), the AIA and the titanium dioxide (TiO_2 with 1 g of TiO_2/kg diet) techniques (n=16 rabbits/method). The effect of feed sample quantity on dietary AIA content was investigated and total collection of faeces was carried out to calculate marker recovery. In E2, 48 rabbits were allotted to three groups fed diets with no sugar beet pulp (SBP0) or with 100 (SBP100) and 200 (SBP200) g sugar beet pulp/kg (n=16 rabbits/group). Each group was divided into two subgroups, ERM and AIA (n=8 rabbits/subgroup), in which CTTAD was measured using the European reference and AIA method, respectively. In AIA subgroups, only 10% of the total daily faecal output was sampled from 9:00 to 9:30 am. Feed analysis in E1 showed that increasing sample quantity from 5 to 9 g did not affect the dietary AIA content; however, the analytical error was 7 and 5 times lower ($P<0.05$) for 9 g, when compared to 5 and 7 g samples. Feed analysis also showed 1.030±0.003 g TiO_2/kg diet. Faecal marker recovery was 99.80±0.03 and 96.89±0.16% for AIA and TiO_2, respectively. The CTTAD of dry matter (DM), did not differ between methods in E1, but a 5-fold higher variability ($P<0.05$) was observed for the TiO_2 technique in comparison with the ERM and AIA methods. Also, no differences in the CTTAD of DM between the ERM and AIA methods were found in E2. In conclusion, AIA is a reliable internal marker in rabbits and offers the possibility of measuring the CTTAD of diets with precision, when complete faecal collection or feed intake measurement is not possible.

Key Words: acid-insoluble ash, European reference method, rabbits, titanium dioxide, total tract apparent digestibility.

INTRODUCTION

Coefficient of total tract apparent digestibility (CTTAD) of nutrients and energy is an important measurement for acquiring information on the digestive utilisation of feeds in rabbits. The CTTAD of rabbit diets is most commonly determined using the European reference method (Perez *et al.,* 1995). This method is based on total collection of faeces and as such it requires the precise measurement of feed intake and faecal output during the trial. Although well established as a reference method and very precise, it cannot be carried out when complete faecal collection or feed intake measurement is not possible.

Inert markers overcome the need for precise measurements of feed intake and total faecal output in the traditional total collection methods (Jagger *et al.,* 1992; Kananagh *et al.,* 2001; Sales and Janssens, 2003). Several external markers, such as chromic oxide (Cr_2O_3), titanium dioxide (TiO_2) and rare earth elements have been extensively evaluated through the years in the search for a suitable marker (Sales and Janssens, 2003). There is abundant literature on the most important indicators of reliability of external markers' (such as faecal recovery) in many animal

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species, with quite conflicting results (McCarthy *et al.,* 1974; Jongbloed *et al.,* 1991; Moughan *et al.,* 1991; Jagger *et al.,* 1992; Bakker and Jongbloed, 1994; Hill *et al.,* 1996; Yin *et al.,* 2000; Kavanagh *et al.,* 2001). On the other hand, little and mainly outdated research can be found on the use of external markers in rabbits. Huang *et al.* (1954) found that Cr_2O_3 was a suitable marker in a digestion trial with rabbits. In a recent study, the use of TiO_2 was also suggested as a good alternative to the total collection method (Safwat *et al.,* 2015). However, both studies were limited to a simple comparison of the digestibility values between marker and total collection methods, and did not evaluate the faecal recovery of the marker, which is the most important indicator of its efficacy. Care should be taken when interpreting the results of the literature, because one disadvantage of external markers is that they must be added to the feed. Some feeds do not mix well with markers (Rymer, 2000), and external markers may be partly lost during feeding (Sales and Janssens, 2003) or sample preparation for analyses, thereby giving inaccurate and inconsistent results.

An alternative to external markers is the use of internal markers (natural constituents of feeds), among which acid-insoluble ash (AIA) has gained considerable attention. Sales and Janssens (2003) reviewed 45 studies in different animal species, 26 of which showed good faecal AIA recovery rates and similar results between AIA and total collection methods, whereas 19 studies reported unacceptable faecal recoveries and unrealistic digestibility values compared to the total collection method. In contrast to other species, there is little information on the suitability of AIA as a marker in rabbits. Although AIA has been used to measure digestibility in rabbits (Di Meo *et al.,* 2007; Peiretti and Meineri, 2008; Bovera *et al.,* 2012), its faecal recovery has only been assessed in the study of Furuichi and Takahashi (1981) and Alvarenga *et al.* (2017), with contradictory results. However, the work of Furuichi and Takahashi (1981) was prior to the European reference method (ERM) (Perez *et al.,* 1995), which contains certain guidelines for digestibility trials in rabbits and the study of Alvarenga *et al.* (2017) was not harmonised with the ERM. In the authors' opinion, the suitability of AIA as an internal marker in rabbits should be re-evaluated on the basis of comparison with a reliable and well-established reference method. A suitable AIA technique could be particularly useful in some practical situations, such as commercial rabbitries, group housing and organic farming.

The objective of this study was to confirm the suitability of AIA as an internal indigestible marker for the measurement of digestibility in growing rabbits through two experiments. Experiment 1 aimed to determine the faecal recovery of AIA and compare dry matter digestibility values between AIA and the ERM. In addition, AIA was compared to titanium dioxide, which is a frequently used external marker in many animal species. Experiment 2 sought to investigate whether different diets, typical for growing rabbits, contain sufficient amounts of AIA to allow for precise measurement of digestibility in comparison again with the ERM.

MATERIALS AND METHODS

Experiment 1

Experimental procedures and diets

In experiment 1, all the procedures regarding animals, adaptation and collection period were harmonised with the ERM (Perez *et al.,* 1995), as detailed in Table 1. Briefly, 48 healthy 35-d-old weaned Hyla male animals were properly selected from a commercial breeding farm. Upon arrival at the facilities of the Dept. of Nutritional Physiology and Feeding, rabbits were kept indoors in individual digestibility cages under controlled environmental conditions (Table 1) and were fed the commercial farm diet [containing 9.8 MJ digestible energy, 150 g crude protein (CP), 340 g neutral detergent fibre (NDF) and 175 g acid detergent fibre (ADF) per kg] for 10 d (acclimatisation period). Afterwards, rabbits were allocated into three experimental groups, namely ERM, AIA and titanium dioxide (TiO_2) group, of 16 rabbits each, considering the homogeneity of body weight (BW) within and between groups. The commercial farm diet was then gradually replaced within 3 d by the experimental basal diet, which was formulated to meet the requirements of growing rabbits (de Blas and Mateos, 2010). Rabbits were allowed a 7-d adaptation period to the experimental diet. In TiO_2 group, 1 g of TiO_2/kg [Titanium (IV) oxide, anatase, Sigma-Aldrich, MO, USA] was added to the basal diet. Titanium dioxide was thoroughly mixed with the ground feedstuffs and then the experimental basal diet was pelleted. No diatomaceous earth (as Celite 545™ or in any other form) was added to the basal diet to increase AIA content (Table 2).

Table 1: Harmonization of the experimental procedures in experiment 1 of the present study with the European Reference Method (ERM; Perez *et al.,* 1995).

Description	ERM	Current study
Animals		
Number of replicates (n)	min n=8, optimum n=10 per diet	n=16 per diet
Age	42-56 d (at onset of adaptation), Weaning= min 7 d prior to adaptation, Acclimatisation (for external rabbits)=4 d min prior to adaptation	Weaning age=35 d of age Acclimatisation=10 d Onset of adaptation=(35+10)=45 d of age
Breed	Same genotype (commercial strain or breed recommended), if genotype is not the subject of the study	Same commercial genotype (Hyla)
Sex	Not controlled or balanced	Balanced (all males)
Weight	Homogeneous within and between groups (coefficient of variation; CV<10%)	Homogeneous within and between groups (CV=7.3%)
Litter	The greatest possible number of litters should be used. Within-litter rabbits are selected considering the average litter live weight.	Forty-eight litters were used (each rabbit from different litter). Average litter live weight was considered.
Housing	Individual wire mesh (stainless steel or galvanised) cages. Surface=min 0.06 m^2.	Individual wire mesh (galvanised) cages. Surface=0.13 m^2 (length 0.41 m, width 0.32 m).
Environmental conditions	Temperature=18-22°C (max allowed 15-25°C). Humidity=65-85%. Air NH_3<10 ppm.	Temperature=19.5-24.5°C. Humidity=60-80%. Air NH_3: not measured.
Adaptation period		
Length	min 7 d	10 d (3 d gradual change from commercial to experimental diet +7 d adaptation to the new diet)
Feeding	*ad libitum*, feed intake must be measured	*ad libitum*, feed intake was measured
Collection period		
Length	4 d	4 d
Feeding	*ad libitum*, recorded on the whole 4-d period. Pellets outside the feeder are stored for dry matter analysis. Caecotrophy is not prevented.	*ad libitum*, recorded on the whole 4-d period. Pellets outside the feeder were stored for dry matter analysis. Caecotrophy was not prevented.
Faeces collection	Every day at the same time in the morning.	Every day at 9:00 am.

Starting at the age of 56 d, faeces were collected over a 4-d period from all three experimental groups, according to the ERM (Table 1). Feed intake measurement and total faecal collection in AIA and TiO_2 groups were necessary to determine the faecal recovery of AIA and TiO_2 markers, respectively. Feed samples were also collected from all three groups at the beginning of the collection period, as recommended by Perez *et al.* (1995).

Analytical procedures

Upon preparation, samples of the experimental basal diet were collected and analysed for DM, ash (to calculate organic matter; OM), CP (Kjeltec autoanalyser unit, Foss, Sweden), as well as for neutral detergent (NDF) and acid detergent (ADF) fibre sequentially, according to the guidelines of the European Group on Rabbit Nutrition (EGRAN, 2001).

Table 2: Ingredients and chemical composition of the basal diet in experiment 1.

	Basal diet
Ingredient (g/kg)	
Dehydrated alfalfa meal	294.0
Barley grain	169.0
Wheat bran	284.0
Sunflower meal (280 g CP/kg)	154.0
Citrus pulp	80.0
L-Lysine HCl (80%)	2.6
DL-Methionine (99%)	2.3
L-Threonine	2.0
Sodium chloride	4.1
Ultrafed® (binder)[1]	3.5
Mineral-Vitamin premix[2]	3.5
Analysed chemical composition	
Dry matter (DM, g/kg)	892.0
Organic matter (OM, g/kg DM)	932
Crude protein (CP, g/kg DM)	164
aNDFom (g/kg DM)[3]	368
ADFom (g/kg DM)[4]	218
Calculated chemical composition	
Digestible energy[5] (MJ/kg DM)	11
Total dietary fibre[6] (g/kg DM)	483
Soluble fibre[6] (g/kg DM)	114

[1]Contained >95% palygorskite.

[2]Mineral and vitamin mixture, provided per kg diet: vitamin A, 10000 IU; vitamin D3, 1,800 UI; vitamin E, 60 UI; vitamin K3, 2 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B6, 3 mg; vitamin B12, 0.02 mg; calcium pantothenate, 7 mg; nicotinic acid, 30 mg; folic acid, 0.5 mg; biotin, 0.2 mg; choline chloride, 400 mg; I, 1.5 mg; Mn, 60 mg; Cu, 6 mg; Zn, 80 mg; Fe, 30 mg; Co, 0.35 mg; antioxidant, 0.250 mg; 300 mg Cycostat (60 mg robenidine/kg).

[3]a-amylase treated neutral detergent fibre, corrected for ash.

[4]Acid detergent fibre, corrected for ash.

[5]From tabulated data (FEDNA, 2003).

[6]From tabulated data (Van Amburgh *et al.,* 1999; Jha and Berrocoso, 2015; Gidenne, 2015).

During the digestibility trial, the determination of feed DM content and of the total excretion of DM for the calculation of digestibility was performed according to Perez *et al.* (1995) in all three groups. Feed and faeces samples in AIA and TiO_2 groups were then prepared as recommended by Perez *et al.* (1995) for the determination of AIA and TiO_2 markers, respectively.

As the AIA content of the basal diet was unknown, different feed quantities were analysed for AIA to determine the sample quantity that gives the lowest analytical variance. A quantity of 5.0 (n=8), 7.0 (n=8) and 9.0 (n=8) g prepared feed samples from the ERM (n=4) and AIA (n=4) groups was analysed using the method of Van Keulen and Young (1977) slightly modified. Briefly, samples were weighed into a 100 mL porcelain crucible and then ashed overnight at 450°C. Subsequently, 4N HCl was added slowly, the crucibles were covered with a watch glass and the mixture was boiled gently for 10 min on a ceramic hotplate. The hot hydrolysate was filtered through ashless filter paper (Macherey-Nagel no. MN 1640W, Düren, Germany) and washed free of acid with hot distilled water (85 to 100°C). Mild vacuum was provided by a water aspirator to increase the filtration speed. The filtrate was thoroughly checked for any precipitate. The ash and filter paper were then transferred back into the crucible and ashed overnight at 450°C. The crucible and content were cooled in a desiccator to room temperature and weighed while containing ash and re-weighed immediately after emptying. The ratio of HCl volume to feed sample weight was kept constant to 10:1 i.e., 50, 70 and 90 mL of 4N HCl were added to the ash residue of 5.0, 7.0 and 9.0 g feed sample. For individual faeces, 5.0 g of the prepared samples were analysed for AIA, as described above.

Titanium dioxide was analysed according to Myers *et al.* (2004) in the prepared feed and faeces samples from the TiO_2 group. Briefly, duplicate 0.5 g feed or dried faeces samples were weighed into 250 mL Kjeldahl tubes. A reaction catalyst containing 3.5 g of K_2SO_4 and 0.4 g of $CuSO_4$ was added to the tubes and samples were digested with 13 mL of concentrated (98%) H_2SO_4 at 420°C for 2 h. Following a 30 min cooling of the tubes, 10 mL of 30% H_2O_2 was added slowly to the tubes. After an additional 30 min cooling, the total liquid weight in the tubes was brought up to 100 g using distilled water, and then filtered through Whatman No. 541 filter paper to remove any precipitate. The absorbance was read at 410 nm in the spectrophotometer (Hitachi U3010 Spectrophotometer, Japan). The spectrophotometer was calibrated with working standards, prepared by adding 0, 2, 4, 6, 8, and 10 mg of TiO_2 to blank tubes (no OM) that were prepared as described above. The 0-mg standard was used to zero the instrument. Two feeds and two faecal samples were randomly selected from the AIA group (no added TiO_2) and were analysed for TiO_2, in order to provide background corrections as required by the method of Myers *et al.* (2004).

Table 3: Ingredients and chemical composition of the diets in experiment 2.

	Diets[1]		
	SBP0	SBP100	SBP200
Ingredient (g/kg)			
Dehydrated alfalfa meal	350.0	250.0	150.0
Sugar beet pulp	-	100.0	200.0
Wheat bran	160.0	160.0	154.0
Sunflower meal (320 g CP/kg)	80.0	115.0	150.0
Barley grain	376.0	340.0	310.0
Soybean meal (450 g CP/kg)	20.0	20.0	20.0
L-Lysine HCl, 80%	1.4	1.4	1.0
DL-Methionine, 99%	1.6	1.6	1.6
L-Threonine	1.0	1.0	1.0
Limestone	-	1.0	3.0
Sodium chloride	5.0	5.0	4.4
Mineral-Vitamin premix[2]	5.0	5.0	5.0
Analysed chemical composition			
Dry matter (DM, g/kg)	887	916	908
Organic matter (OM, g/kg DM)	916	925	926
Crude protein (CP, g/kg DM)	158	163	180
aNDFom (g/kg DM)[3]	338	323	321
ADFom (g/kg DM)[4]	199	188	185
Acid-insoluble ash (AIA)[5] (g/kg DM)	21.28	15.84	13.07
Calculated chemical composition			
Digestible energy[6] (MJ/kg DM)	10.6	11.0	11.1
Total dietary fibre[7] (g/kg DM)	447	449	463
Soluble fibre[7] (g/kg DM)	109	125	147

[1]SBP0=diet with no sugar beet pulp (SBP) added; SBP100, SBP200=diets with 100 g and 200 g SBP/kg, respectively.
[2]Mineral and vitamin mixture provided per kg diet: vitamin A (retinol), 12000 IU; vitamin D_3 (cholecalciferol), 2000 IU; vitamin E (α-tocopherol), 70 mg; vitamin K_3 (menadione), 3 mg; vitamin B_1 (thiamine), 2 mg; vitamin B_2 (riboflavin), 6 mg; vitamin B_6 (pyridoxine), 3 mg; vitamin B_{12} (cyanocobalamin), 0.02 mg; pantothenic acid, 15 mg; nicotinic acid, 60 mg; choline, 1000 mg; folic acid, 1.5 mg; biotin, 0.2 mg; I (as KI), 1.1 mg; Mn (as MnO), 65 mg; Cu (as $CuSO_4.5H_2O$), 15 mg; Zn (as ZnO), 80 mg; Fe (as $FeSO_4 \cdot 7H_2O$), 80 mg; Se (as Na_2SeO_3), 0.08 mg; Co (as $CoCO_3$), 1 mg; antioxidant, 0.250 mg.
[3]α-amylase treated neutral detergent fibre, corrected for ash.
[4]Acid detergent fibre, corrected for ash.
[5]Mean of four 9 g samples/diet.
[6]From tabulated data (FEDNA, 2003).
[7]From tabulated data (Van Amburgh *et al.,* 1999; Jha and Berrocoso, 2015; Gidenne, 2015).

Calculations

In the ERM group, the CTTAD of the DM of the diet was calculated based on the classical formula (Perez *et al.,* 1995):

$$\text{CTTAD of DM} = \frac{DM_{intake} - DM_{excreted}}{DM_{intake}} \quad (1)$$

In AIA and TiO_2 groups, the CTTAD of the DM of the diet was calculated according to the following formula:

$$\text{CTTAD of DM} = 1 - \frac{M_{feed}}{M_{faeces}} \quad (2)$$

Where: M_{feed}=marker (AIA or TiO_2) content of feed (g/kg DM) and M_{faeces}= marker (AIA or TiO_2) content of faeces (g/kg DM)

In AIA and TiO_2 groups, the faecal recovery of the markers was calculated according to the following formula:

$$\text{Faecal recovery (\%)} = \frac{DM_{excreted} \times M_{faeces}}{DM_{intake} \times M_{feed}} \quad (3)$$

Where: M_{feed}=marker (AIA or TiO_2) content of feed (g/kg DM) and M_{faeces}=marker (AIA or TiO_2) content of faeces (g/kg DM)

Experiment 2

Experimental procedures and diets

Forty-eight healthy 35-day-old weaned Hyla male animals, other than those used in experiment 1, were selected from a commercial breeding farm according to the ERM guidelines, as in experiment 1. Rabbits were kept indoors in individual digestibility cages equipped with a metal trough and an automatic nipple drinker, under controlled environmental conditions (20±2°C). They were allocated into three groups, balanced for body weight (mean BW per group 1075±69 g; mean±standard deviation) and fed three diets with no sugar beet pulp (SBP0) or with 100 (SBP100) and 200 (SBP200) g sugar beet pulp/kg (n=16 rabbits/group), which were formulated to meet the requirements of growing rabbits (de Blas and Mateos, 2010). The SBP100 and SBP200 diets contained less alfalfa hay (by 100 and 200 g/kg, respectively) compared to the SBP0 diet; it was substituted in equal amounts by sugar beet pulp (Table 3). The objective was a) to modify the dietary soluble fibre and ADF content (Table 3), which are known to affect the digestive utilisation of the diet (Trocino *et al.,* 2013) and b) to investigate whether AIA was present in sufficient amounts in different diets, thereby allowing for accurate calculation of the CTTAD.

The rabbits of each group were divided into two subgroups: ERM and AIA (n=8 rabbits/subgroup), balanced for BW within and between subgroups (CV<10%). In ERM subgroups, the experimental procedures (feed sampling, feed intake measurement and faecal collection) were harmonised with the guidelines of Perez *et al.* (1991), as described in experiment 1 (Table 1).

In AIA subgroups, a small fraction of faeces, (approximately 10%) was sampled once daily (between 9:00 and 9:30 am) from the whole faeces produced by each rabbit during the previous night. Samples were grabbed randomly from the collection trays, i.e. without any mixing of the whole quantity of the excreted faecal pellets. Four samplings (for 4 consecutive days) were performed and the samples were then pooled for analyses. After each sampling, the collection trays were prepared for the next day by discarding the remainder faeces. The rationale behind this was to indirectly simulate the grab sampling performed in earlier digestibility studies (McCarthy *et al.,* 1977; Moughan *et al.,* 1991; Bakker and Jongbloed, 1994; Kavanagh *et al.,* 2001). Feed intake was not recorded and feed samples (for DM and AIA determination) were randomly collected (by grab sampling) from 4 feeders of each AIA subgroup at the beginning of the collection period.

Analytical procedures and calculations

Immediately after the preparation of the experimental diets, samples were collected and analysed for DM, ash (to calculate OM), CP (Kjeltec autoanalyser unit, Foss, Sweden), as well as for NDF and ADF sequentially, following the guidelines of the European Group on Rabbit Nutrition (EGRAN, 2001).

During the digestibility trial, determination of DM content of the experimental diets and of the total excretion of DM for the calculation of digestibility was performed according to Perez *et al.* (1995) in the ERM subgroups.

In AIA subgroups, the DM content of the experimental diets was determined according to the European Group on Rabbit Nutrition (EGRAN, 2001). Faeces were pre-dried (DM>85%) and submitted to analysis after grinding at 1 mm. The determination of AIA was carried out in 9 g feed and 5 g faeces samples using the slightly modified method of Van Keulen and Young (1977), as described in experiment 1.

The CTTAD of the DM of the experimental diets was calculated using formula 1 and formula 2 (described in experiment 1) for the ERM and AIA subgroups, respectively.

Statistical analysis

Data were analysed using the SPSS statistical package (version 17.0). Prior to analysis, data were tested for normality using the Kolmogorov–Smirnov test. In experiment 1, dietary AIA contents in different sample quantities were analysed by a one-way (sample quantity) ANOVA, whereas faecal recovery of markers was analysed by a one-way ANOVA with marker (AIA or TiO_2) as fixed effect. The CTTAD of DM was analysed by a one-way ANOVA with method (European Reference, AIA or TiO_2) as fixed effect. In experiment 2, the CTTAD of DM was analysed using a one-way ANOVA with the combination of diet and method (6 combinations, namely SBP0-ERM, SBP0-AIA, SBP100-ERM, SBP100-AIA, SBP200-ERM and SBP200-AIA) as fixed effect. Differences in variances of the above parameters were investigated using Levene's test statistic in both experiments. When variances were not homogeneous, the Welch's ANOVA was considered. Rabbit was the experimental unit and statistical significance was set at $P<0.05$ for all tests in both experiments.

RESULTS

Experiment 1

The absolute AIA content increased ($P<0.001$) with increasing sample quantity as was expected (Table 4). The mean dietary AIA content was similar for the three sample quantities analysed and averaged 12.9 g/kg DM. However, the variance decreased ($P<0.001$) with increasing sample quantity, as indicated by Levene's statistic test. The variance of the dietary AIA content in the 9 g samples was 4.6 and 7 times lower in comparison with the 7 and 5 g samples, respectively. Therefore, faecal recovery and CTTAD values in AIA group were based on the analysis of 9 g feed samples.

The mean dietary TiO_2 content in TiO_2 group was 1.03 g/kg DM, somewhat lower than the expected value of 1.12 g/kg DM (Table 4). Faecal recovery of AIA was estimated at 99.8% in the AIA group. Although not significantly different from the 96.9% determined for TiO_2, the standard error in faecal AIA recovery was approximately 5 times lower ($P<0.001$) than that of TiO_2. This was likely the result of the difference in marker variances between feed and faeces. In the AIA group, faecal AIA variance was similar to the dietary AIA variance, whereas in the TiO_2 group faecal TiO_2 variance was approximately 40 times higher than dietary TiO_2 variance, as indicated by the standard error of means (Table 5).

There were no significant differences in the CTTAD of DM between the ERM and the AIA, and TiO_2 techniques (Table 5). The CTTAD of DM calculated by the TiO_2 technique was numerically lower and had a 5-fold higher ($P<0.01$) variance compared to those of the European reference and AIA methods, as indicated by Levene's statistic test (Table 6).

Experiment 2

Acid-insoluble ash content was different between the experimental diets. With the change of ingredients from SBP0 to SBP100 and SBP200 diets, the AIA content decreased by 26 and 39%, respectively (Table 3). The CTTAD of DM was approximately 7% higher ($P<0.05$) for SBP100 and SBP200 diets in comparison with SBP0 (Table 7). The CTTAD of DM either measured by the ERM or calculated by the AIA technique was similar (Table 7).

Table 4: Acid-insoluble ash (AIA) dietary content according to the sample quantity (means±standard error of mean).

	Feed sample quantity (g)			*P*-value	
	5 (n= 8)	7 (n= 8)	9 (n= 8)	ANOVA[1]	Levene[2]
Sample quantity	5.01±0.02	7.02±0.03	9.06±0.04	-	-
Mean AIA (mg)	57.7^{a}±1.1	80.3^{b}±0.9	106.6^{c}±0.4	<0.001	0.019
Mean AIA (g/kg DM)	12.87±0.25	12.94±0.17	12.91±0.04	0.988	<0.001

[1]*P*-value of Welch's analysis of variance (ANOVA); Welch ANOVA is used when there is no homogeneity of variances between groups.
[2]Levene's statistic tests the homogeneity of variances.

Table 5: Dry matter (DM) intake and excretion during the 4-day faecal collection period, analysed marker in feed and faeces, and faecal recovery (%) of acid-insoluble ash (AIA) and titanium dioxide (TiO_2) in experiment 1 (means±standard error of mean).

	Group[1]			*P*-value	
	ERM (n=16)	AIA (n=16)	TiO_2 (n=16)	Welch[2]	Levene[3]
DM intake (g/d)	134.0±3.6	127.6±3.4	133.8±3.4	0.367	0.663
DM excretion (g/d)	55.0±1.7	52.2±1.3	54.6±1.4	0.200	0.268
Marker (g/kg DM)					
Feed[4]	-	12.94±0.07	1.030±0.003 (1.17)[5]	-	-
Faeces	-	31.57±0.03	2.45±0.12	-	
Faecal recovery	-	99.80±0.03	96.9±0.2	0.652	<0.001

[1]ERM: European reference method, AIA: acid-insoluble ash, TiO_2: titanium dioxide.

[2]*P*-value of Welch's ANOVA for the faecal recovery of marker only. Welch ANOVA is used when there is no homogeneity of variances between groups.

[3]Levene's statistic tests the homogeneity of variances.

[4]Mean±standard error of mean of 4 analysed feed samples (of 9 g each) per group; AIA was determined in AIA group; TiO_2 was determined in TiO_2 group.

[5]Expected value in the parenthesis.

DISCUSSION

An ideal marker must fulfil certain requirements; it must be non-toxic, unaltered during its passage through the digestive tract, with no influence on the digestive processes, flowing at an identical rate as the nutrients and totally recovered in the faeces (Kotb and Luckey, 1972; De Silva, 1985; Marais, 2000).

Total faecal recovery is generally considered the most important criterion to be met by any marker, as it indicates its efficacy in accurately calculating the digestibility (Sales and Janssens, 2003). In experiment 1 of the present study, faecal recovery of AIA was 99.8% and the CTTAD of DM was similar between the ERM and AIA techniques. Similar results have previously been reported in rabbits (Furuichi and Takahashi, 1981), where AIA was totally recovered in faeces and gave digestibility values similar to those of the total collection method. However, another study in rabbits (Alvarenga *et al.,* 2014) obtained recovery rates ranging from 87 to 135% depending on the form of the diet (muesli, pelleted, extruded) and reported digestibility values different from those of the total collection method. Titanium dioxide in the present study was used as means of comparison of AIA with an external marker and had a good recovery rate of approximately 97%; however, its efficacy as an external marker in rabbits was not conclusive. Although TiO_2 gave DM digestibility values not significantly different from those of the ERM and AIA techniques, they were underestimated by approximately 7%. Moreover, the standard error of the faecal recovery of TiO_2 was 5 times greater than that of AIA, and this resulted in the calculation of CTTAD of DM with higher error compared to both the ERM and AIA techniques. This indicates that digestibility in rabbits may be not measured accurately using 1 g TiO_2/kg diet. Alternatively, much higher quantities of TiO_2 may be added to diet (e.g. >2-3 g TiO_2/kg diet) to compensate for the increased variability in CTTAD measurements. For instance, Safwat *et al.* (2015) found that the digestibility of nutrients in rabbit diets

Table 6: Coefficient of total tract apparent digestibility (CTTAD) of dry matter (DM) using the European reference method and the marker techniques in experiment 1 (means±standard error of mean).

	Group[1]			*P*-value	
	ERM (n=16)	AIA (n=16)	TiO_2 (n=16)	Welch[2]	Levene[3]
DM	0.590 ±0.004	0.592 ±0.004	0.581 ±0.021	0.970	<0.001

[1] ERM= European Reference Method (CTTAD calculated with formula 1), AIA= acid-insoluble ash (CTTAD calculated with formula 2), TiO_2= titanium dioxide (CTTAD calculated with formula 2).

[2] *P*-value of Welch's ANOVA; Welch ANOVA is used when there is no homogeneity of variances between groups.

[3] Levene's statistic tests the homogeneity of variances.

Table 7: Coefficients of total tract apparent digestibility (CTTAD) of dry matter (DM) in experiment 2 (means±standard error of mean).

Diet[1]	SBP0		SBP100		SBP200		*P*-value	
Method[2]	ERM (n= 8)	AIA (n= 8)	ERM (n= 8)	AIA (n= 8)	ERM (n= 8)	AIA (n= 8)	ANOVA[3]	Levene[4]
DM	0.571[a] ±0.009	0.573[a] ±0.010	0.607[b] ±0.009	0.605[b] ±0.007	0.612[b] ±0.008	0.617[b] ±0.007	<0.001	0.695

Means with different superscripts differ significantly ($P<0.05$).

[1]SBP0= diet with no sugar beet pulp (SBP) added; SBP100, SBP200= diets with 100 g and 200 g SBP/kg, respectively.

[1]ERM= European Reference Method (CTTAD calculated with formula 1), AIA= acid-insoluble ash (CTTAD calculated with formula 2).

[3]*P*-value of ANOVA.

[4]Levene's statistic tests the homogeneity of variances.

determined either by the total collection method or by the TiO_2 method yielded similar results. Most likely, the addition of 4 g TiO_2/kg diet in the work of Safwat *et al.* (2015) resulted in lower variation and higher accuracy in TiO_2 determination compared to the 1 g TiO_2/kg diet employed in our study. When using external markers, it is usually assumed that there is a homogeneous dispersion of the marker in the feed and consequently in the digestive tract. However, rabbit feeds consist of heterogeneous raw materials and most likely the 1 g of TiO_2 was not homogeneously distributed over the entire mass of the diet, thus resulting in a stratification within the digestive tract. This may explain the higher variability in the faecal TiO_2 content compared to the dietary TiO_2 content observed in our study. In addition to this, it is not unlikely that during sample preparation for analysis, the grinding of the feed and faecal pellets containing TiO_2 may have resulted in an additional loss of TiO_2, which contributed to the low dietary TiO_2 content (1.03 *vs.* the 1.12 g/kg DM expected) and further increased the variability in the faecal TiO_2 recovery.

The above problems of faecal recovery rate associated with external markers are reduced when using internal markers such as AIA. Nevertheless, inconsistencies can be observed between studies in rabbits (Furuichi and Takahashi, 1981; Alvarenga *et al.,* 2014) as well as in other species (Sunvold and Cochran, 1991; Bakker and Jongbloed, 1994; Goachet *et al.,* 2009), as mentioned above. There may be many reasons for this, including the contamination of diets and faeces with soil and dust, which contain high amounts of AIA and may introduce great errors during the analysis of diets or faeces (Piaggio *et al.,* 1991; Marais, 2000 or improper experimental and analytical procedures. Notably, most digestibility studies where AIA had not met the expectations of a suitable marker did not attribute this to marker failure, but to analytical inaccuracy due mainly to low dietary and faecal AIA content that introduced a large margin of error to the calculation of CTTAD (Van Keulen and Young, 1977; Jones and De Silva, 1998; Sales and Janssens, 2003). Thonney *et al.* (1985) recommended that the dietary AIA content should exceed 7.5 g/kg on a DM basis in order to achieve accurate measurements. In the present study, the dietary AIA content in both experiments ranged from 13 to 21 g/kg DM, exceeding the minimum content recommended by Thonney *et al.* (1985). Rabbit diets contain high amounts of forages as fibre sources, the most common being dehydrated alfalfa meal, which has a relatively high AIA content varying from 37 to 98 g/kg DM (Undersander *et al.,* 1987; Sunvold and Cohran, 1991). This was evidenced in experiment 2, where dietary AIA content decreased with decreasing dehydrated alfalfa meal. Nevertheless, AIA was present at sufficient amounts in the diets even when alfalfa meal was reduced to a minimum of 150 g/kg diet in experiment 2. This allowed us to determine the dietary AIA content accurately and as a result, the digestibility values were similar to those obtained by the ERM. These findings indicate that typical rabbit diets contain adequate amounts of AIA to allow for a precise measurement of digestibility and there is no need for any dietary AIA addition, e.g. in the form of diatomaceous earth. However, based on our findings, the precision of AIA determination does not depend only on its minimum dietary content, but also on the quantity of feed sampled for AIA analysis. Increasing feed sample quantity from 5 to 9 g gave identical AIA contents, but significantly decreased the analytical error, resulting in more accurate determination of dietary AIA. Acid-insoluble ash is determined gravimetrically (Van Keulen and Young, 1977). Hence, greater sample quantities contain greater absolute AIA amounts and reduce the analytical error. In both experiments in the present study, we used 9 g of feed samples to determine AIA and the precision of the calculated CTAAD was satisfactory. Both the average digestibility values and their standard errors were similar between the ERM and AIA techniques. Presumably, the use of 5 or 7 g feed samples for AIA analysis and CTTAD calculation

would have yielded similar digestibility values, but with lower accuracy because of the higher variance in the mean dietary AIA content compared to the 9 g samples. Such high quantities were not necessary for faecal samples, as they had higher AIA contents (approximately 2.4 times the dietary content in both experiments). Hence, 5 g samples of faeces, as recommended by Van Keulen and Young (1977), were sufficient to produce precise results, but the 5 g of feed suggested may introduce errors in the dietary AIA determination, thereby affecting the precision of digestibility values.

In experiment 1, total faeces collection was indispensable for calculation of the faecal recovery of AIA, but did not allow us to investigate the practical value of the AIA technique, which does not require precise measurements of feed intake and total faecal output. This was investigated in experiment 2; feed intake was not determined and only a fraction (10%) of the total faecal output was collected, thereby simulating the grab sampling employed in other studies. The similar CTTAD between the reference method and the AIA technique indicated high precision, making the use of a portion of faeces possible in rabbits. This may be attributed to the homogeneous dispersion of AIA in the feed and the digesta, which reduces the diurnal variations in the excreted AIA. Indeed, Furuichi and Takahashi (1981) found small diurnal and daily variations (CV<4%) in the AIA content of faecal samples collected twice daily over 8 consecutive days and similar results have been reported in other animal species (Vogtmann *et al.,* 1975; McCarthy *et al.,* 1977; Van Keulen and Young, 1977; Thonney *et al.,* 1985; Cuddeford and Hughes, 1990; Kavanagh *et al.,* 2001), where grab sampling was carried out instead of total faeces collection. This, in combination with the precise digestibility values using a small (n=8 in experiment 2) or large number (n=16 in experiment 1) of replicates, indicates that AIA offers the possibility to conduct reliable digestibility trials in situations where feed intake and faecal excretion cannot be measured, such as commercial rabbitries, group housing and organic farming.

However, the digestibility trials when using AIA as marker should follow some of the standard procedures recommended by the ERM, which are of great importance for the precision of the technique. In detail, all experimental procedures regarding animals (minimum replicates, age, breed, sex, body weight, litter of origin, housing and environmental conditions) and length of adaptation must conform to ERM guidelines (Table 1). Any deviation from these recommendations may introduce errors to the calculation of digestibility that are not related to the efficacy of AIA *per se*. Feed intake measurement is not necessary during the adaptation or the collection period. Sampling a portion of the faecal output (random grab sampling) is adequate for the precise determination of digestibility, provided that it will be carried out in a manner reducing the contamination with soil or dust. The collection period should be kept at 4 d, while further research will indicate whether the collection period can be reduced without affecting the efficacy of the technique. Additionally, future work should explore alternative methods for the determination of AIA. Although simple in terms of analytical procedures and equipment, AIA determination is time consuming and does not allow for a high sample throughput, and also requires large quantities of sample and HCl.

CONCLUSIONS

Acid-insoluble ash could be used as an internal marker to calculate the digestibility of a diet precisely, and could be an alternative to total faecal collection in rabbits. AIA reached a sufficiently high level in rabbit feed to allow a high degree of analytical precision and an almost complete faecal recovery rate. These advantages of AIA may allow samples to be obtained from rabbits under commercial or organic farming conditions, provided that the experimental procedures, with the exception of feed intake measurement and total faecal collection, conform to the ERM guidelines. Further research is necessary to determine whether the duration of the faeces collection period can be reduced and to explore alternative analytical procedures for acid-insoluble ash.

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