A dual marker technique to estimate individual feed intake in young pigs

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Abstract
Accurate estimation of individual feed intake (FI) of pigs could help better understand the variation in performance between individual animals. We studied dual marker methods to estimate individual FI in pigs. This method is based on the measurement of the ratio between two indigestible markers in faeces. Twelve 6.5-week-old individually housed male pigs were assigned to one of three oral dosing treatments supplying 180 mg of ytterbium chloride (YbCl₃)/day and 111 mg of dotriacontane (C₃₂)/day as reference markers, either once (R₁), three times (R₃) or five times (R₅) daily. Pigs were offered a diet containing 0.46 g/kg of chromium chloride (CrCl₃) and 0.15 g/kg of hexatriacontane (C₃₆) as in-feed markers. The experiment lasted for 10 days: days 5/0: adaptation; days 1–3: dosing of reference marker; days 2–4: total faecal collection. Spot faecal samples were taken on day 3 at 1200 h, 1700 h and on day 4 at 0700 h. Pigs were fed restrictedly three times daily, at 133.6 g/kg BW⁰.⁶⁰. Individual measured FI was recorded daily and was compared to predicted FI using the ratio of the dual marker pairs (Yb:Cr and C₃₂:C₃₆), both in total faecal collection and spot samples. Due to unequal variance, R₁ pigs were omitted from the statistical treatment comparison. When using total faecal collection samples, the absolute prediction error (APE) (predicted FI minus measured FI) in R₃ and R₅ pigs was numerically lower than in R₁ pigs, regardless of the marker pair used. The APE measured by C₃₂:C₃₆ was numerically lower than measured by Yb:Cr at all frequencies, and significantly (P = 0.039) in R₃ pigs (C₃₂:C₃₆: 0.15 ± 0.02 kg/day; Yb:Cr: 0.29 ± 0.04 kg/day). This was related to a larger difference in faecal recovery between Yb and Cr compared with C₃₂ and C₃₆. When using C₃₂:C₃₆ to predict feed intake, pooled, but not single spot samples gave similar APEs compared with total faecal collections. Therefore, we recommend dosing the reference markers three times per day for 2 days on days 1 and 2, combined with pooled spot faecal sampling collected on days 3 and 4. In this way, absolute prediction errors of 10%-15% of simultaneously measured intakes of multiple nutrient resources in a complex housing system are feasible using the dual marker technique.

Introduction
Accurate estimation of individual feed intake of pigs could help us to better understand the variation in performance between individual animals. Although technically demanding, the introduction of individual feeding stations has yielded valuable information in this respect (Kim et al., 2010). In the farrowing pen, however, measurement of individual feed intake of pigs is often considered impossible, and observations have been restricted to the classification of piglets into eaters and non-eaters (Van Nieuwamerongen et al., 2015), or time spent eating (Middelkoop, 2020). Moreover, in more spacious rearing systems which catering for the increasing population density of sows and piglets, measurement of individual feed intake is even more challenging. Therefore, the use of nutritional markers is a promising alternative to assess individual feed intake in pigs.
demand for animal welfare, individual nutrient intake from various sources is even more difficult to estimate. These systems include for example outdoor organic pig farming (Von Borell and Sørensen, 2004) and multi-suckling systems (Van Nieuwamerongen et al., 2017).

Trivalent metal markers such as ytterbium chloride ($\text{YbCl}_3$) (Mavromichalis et al., 2001) and chromium oxide (Clawson et al., 1955) have been successfully used for the estimation of nutrient digestibility in pigs. Kim et al. (2010) estimated individual feed intake in postweaned pigs by using two indigestible metal markers, one of which was Lanthanum oxide ($\text{La}_2\text{O}_3$) which was orally administered to pigs, the other one was Yttrium oxide ($\text{Y}_2\text{O}_3$) supplemented in diets. The individual feed intake was then calculated based on the measurement of the ratio between the two metal markers in faeces. However, there are some constraints about the use of markers. The daily manipulation of the animals can cause stress and thus biases data. In addition, the excretion of markers might not be stable since their passage rate through the digestive tract is not uniform. In addition, spot sampling of the faecal output has to be representative. Moreover, the measurement of markers requires complex and costly chemical analyses. For the use of double marker technique, the constraint would be that external markers (e.g. chromium oxide) may exhibit a different passage rate behaviour compared with intrinsic or mordanted markers (de Vries and Gerrits, 2018). Additionally for metal markers, some of them are limited in use since the resulting manure can cause pollution of the environment (e.g. chromium) (Coetzee et al., 2020).

From the 1980s, the double n-alkane technique has been used as a reliable method for estimating individual intake in herbivores, due to their low digestibility, non-toxicity to animals, and natural presence in a wide range of herbage species (Wright, 2017). In herbivores, various dual marker techniques have been used for the determination of individual intake of forages, grass, clover and browse (Andriarimalala et al., 2020). These methods are based on the measurement of the ratio between two indigestible markers in faeces. The external marker (hereafter referred to as reference marker) is dosed frequently and independent from the forage of study which is used to determine the faecal output. The internal marker (hereafter referred to as in-feed marker) is associated with the forage of interest (Chavez et al., 2011). The ratio measured quantitatively reflects forage intake and can be estimated from the analysis of spot faecal samples, obtained in a steady state (Chavez et al., 2011).

Around 90% of the naturally present n-alkanes are odd-chained. Therefore, odd-chained alkanes are often used as in-feed markers, and synthetic even-chained alkanes are often used as reference markers (Chavez et al., 2011). It is important to note that n-alkanes are not completely indigestible and that digestibility varies between and within animal species (e.g. the physiological status of animals) as well as diet type. Faecal recovery is an important indicator of the reliability of a marker, which is the quantity recovered from the total collection of faeces expressed as a proportion of that consumed in diet (Morais et al., 2011). In goats, for example, faecal recoveries of the alkane C25 ranged from 43.0% to 72.4%, while C35 recoveries ranged from 87.9% to 99.9% (Hilburger, 2017). The complete faecal recoveries of markers are important to attain unbiased estimates of intake in digestibility studies. However, it is not a requirement for dual marker technique, as the main assumption of the technique is that the faecal recoveries between the reference and in-feed marker used are similar (de Vries and Gerrits, 2018).

Dual marker methods might be well-suited to estimate individual feed intake of group housed pigs, particularly in a setting where multiple ration components are offered, e.g. in outdoor farming or in multi-suckling systems. Prior to application of such a dual marker method for the estimation of individual feed intake in pigs, assumptions regarding the steady state conditions, involving dosing frequency of the reference marker, collection procedure of spot faecal samples, and the faecal recoveries of the selected markers, need to be verified. In choosing marker pairs, it is important that these markers can be analysed using a single procedure, e.g. gas chromatography for n-alkanes of different chain lengths (Smit et al., 2005), or inductively coupled plasma optical emission spectrometry for metal oxides or chlorides (Williams et al., 1962). For estimation of each additional source of nutrients, an extra marker can be added.

There have been very few attempts of the application of the dual alkane method to estimate individual feed intake in pigs: Mendes et al. (2007) and Ferraz de Oliveira et al. (2006) investigated dual alkane technique to estimate intake of roughages in growing pigs. They also investigated the minimum dosing duration to get a steady concentration of alkanes in faeces. Kanga et al. (2012) applied the dual alkane technique to determine individual voluntary forage intake in 8-week-old male pigs which were fed a mixed forage and concentrate diet. So far, alkane faecal recovery data are mostly available in sows (Wilson et al., 1999) and growing pigs (Ferraz de Oliveira et al., 2006; Ribeiro et al., 2007), but few cases in postweaned pigs (Staals et al., 2007). Observations from other species indicate that faecal recoveries of n-alkanes increase curvilinearly with increasing carbon chain length (Wright et al., 2020). However, in pigs, an increase in faecal recovery with increased chain length of long-chain alkanes is not apparent from the limited data available (Wilson et al., 1999; Ferraz de Oliveira et al., 2006).

The aim of the current study was to assess the dual marker method to estimate individual feed intake of young pigs. To achieve this, we explored the effects of the frequency and duration of oral administration of the reference marker on the accuracy of the feed intake estimation using two n-alkanes (dotriacontane (C32) and hexatriacontane (C36)) and two metal chlorides ($\text{YbCl}_3$ and Chromium chloride ($\text{CrCl}_3$)) as marker pairs. As spot sampling is key to the success of the dual marker method, we compared variation among single and pooled spot samples. We also compared spot faecal sampling methods with quantitative faecal collections to estimate feed intake. Marker pair comparison and duration of faecal sampling were also investigated.

**Material and methods**

**Experimental design**

The experiment was conducted at the research farm ‘Laverdonk’ of Agrifirm Innovation Center (Heeswijk-Dinther, the Netherlands). Pigs were housed individually in pens of 0.76 × 2.28 m, but they could smell, see, hear and touch (e.g. nose to nose contact) the animals in neighbouring pens. The pens consisted of a solid (rubber) floor and a slatted (approximately 1/6 of total area) floor. The temperature was kept at 24–26 °C. Lights were turned on from 0700 h to 1900 h, and during feeding events or bolus administration outside this period.

Twelve individually housed male pigs (Tempo × Topigs 20, Topigs Norsvin, 18.8 ± 0.6 kg) of 6.5 weeks of age were used in this experiment for a period of 10 days, including an adaptation period of 6 days (days −5 to 0) and an experimental period of 4 days (days 14). Pigs were grouped into four blocks of three pigs in each block based on initial BW. Within block, pigs were assigned to one of three treatments that differed in the frequency of administration of the reference marker: once (R1), three (R3) or five (R5) times a day. Two pairs of dual markers were tested: $\text{YbCl}_3$ with $\text{CrCl}_3$ and the n-alkanes C32 with C36. $\text{YbCl}_3$ (180 mg/d) and C32 (111 mg/d) were considered as reference markers and were pro-
vided with a small amount of feed (~2 g/feeding bolus). CrCl₃ (460 mg/kg of feed) and C36 (150 mg/kg of feed) were considered as in-feed markers and were mixed into a commercial mash feed (net energy: 9.6 MJ/kg, CP: 160 g/kg) (Big Rendement 1, Agrifirm, NL), providing 150 mg of Cr and 150 mg of C36/kg of feed. The marker dosage was calculated based on the expected concentration in faeces of 1 000 mg/kg, assuming feed intake of 750 g/d and a DM digestibility of 85%. The sensitivity of metal chlorides and alkane analytical methods is then well-suited to pick up differences in this concentration range. Feeding boluses were prepared fresh by mixing the preweighed amount of reference markers and a small amount of feed (~2 g/bolus) and kneaded to a firm bolus using a few drops of sucrose-based lemonade syrup (Karvan Cevitam, Amsterdam, NL).

From day 5 onwards, pig diet was supplied restrictedly, at 133.6 g/kg BW⁰.⁶⁰, in three equal meals per day, at 0700 h, 1200 h and 1700 h. The restricted feeding was used for controlling the variation of feed intake between individual pigs in order to reduce the variation of the excretion of markers in pigs at different administration frequencies. The restricted feeding levels were set around 80% of the estimated ad libitum feed intake of the pigs, assuming that the estimated ad libitum feed intake is 3.5 times the maintenance energy requirement, according to National Research Council (1998). Each meal was mixed with 400 mL of water to stimulate feed intake. During the adaptation period, pigs were allowed access to the feed for a maximum of 45 minutes per meal to make pigs get used to being fed restrictedly. During the experimental period, pigs were allowed access to the feed for a maximum of 30 minutes per meal, after which feed refusals were removed and weighed.

Water was freely available during 24 h/day. Boluses with reference marker were given to the pigs by hand for three consecutive days (days 1, 2, 3 of the experimental period), at 0900 h (R1); at 0900 h, 1500 h and 2100 h (R3); at 0600 h, 0900 h, 1500 h, 1800 h and 2100 h (R5). For R1 and R3 pigs, placebo boluses without reference markers were provided at the timepoints that they did not receive boluses containing reference markers, as indicated in Fig. 1.

**Faecal sampling**

During adaptation period, faeces were collected quantitatively using faecal collection bags attached to a patch with ring, glued around the anus using adhesive spray (Hollister, Libertyville, USA) on day 3. The faeces collected during adaptation period was discarded. During the experimental period, faeces were collected quantitatively for 72 h on days 2, 3 and 4, as indicated in Fig. 1: the faecal bags were replaced twice per 24-h period at 0700 h and 1700 h and three times on day 3 for an extra timepoint at 1200 h. The faecal bags were pooled and stored per day per pig. In addition, for all pigs, three spot samples of about 50 g were taken from the faecal collection bags at three points in time, immediately after removing the bags: spot sample 1: day 3 at 1200 h; spot sample 2: day 3 at 1700 h; spot sample 3: day 4 at 0700 h. The three spot samples per pig were stored separately. All faecal samples were stored at −20°C for further laboratory analysis.

**Chemical analyses and calculation**

The faecal samples were oven-dried at 65°C and ground to pass a 1 mm screen. The concentrations of Yb and Cr in faeces and feed were determined using inductively coupled plasma optical emission spectrometry as described by Williams et al. (1962). N-alkanes in faeces and feed were extracted by heptane and analysed by gas chromatography according to the method of Smit et al. (2005) using tetraatriacontane (C34) as an internal standard. The feed intake was estimated using total faecal collection samples and spot faecal samples separately. For the feed intake estimation using total faecal collection samples, the removal of markers by removing spot sampling from the faecal collection bags was corrected.

The estimated feed intake (kg/d), percent difference between measured and estimated feed intake (%), faecal recovery of markers (%) and within marker pair faecal recovery difference (%) were calculated for the averaged value from day 1 till day 3. The absolute prediction error (APE, kg/d) was calculated both for the averaged value from day 1 till day 3 and for each day separately. For days 1 to 3, the APE was calculated from the faecal samples collected on days 2 to 4.

The calculations were as follows:

**Estimated feed intake (kg/day)**

\[
\text{Estimated feed intake (kg/day)} = \frac{\text{daily intake of in-feed marker (mg/kg)}}{\text{daily intake of reference marker (mg/kg)}} \times \frac{\text{concentration of in-feed marker in diet (mg/kg)}}{\text{concentration of reference marker in faeces (mg/kg)}}
\]

Absolute prediction error (kg/day)

\[
\text{Absolute prediction error (kg/day)} = |\text{predicted feed intake (kg/day)} - \text{measured feed intake (kg/day)}|
\]

Percent difference between measured and estimated feed intake (%)

\[
\text{Percent difference between measured and estimated feed intake (%) = \left( \frac{\text{absolute prediction error (kg/day)}}{\text{measured feed intake (kg/day)}} \right) \times 100}
\]

**Faecal recovery of marker (i) (%)**

\[
\text{Faecal recovery of marker (i) (%) = } \frac{\text{excretion of marker (i) in faeces on days 2-4 (g)}}{\text{intake of marker (i) on days 1-3 (g)}} \times 100
\]

**With marker (i) referring to Yb, Cr, C32 or C36.**

**Within marker pair faecal recovery difference (%)**

\[
\text{Within marker pair faecal recovery difference (%) } = \left( \frac{\text{in – feed marker recovery (%) - reference marker recovery (%)}}{\text{in – feed marker recovery (%)}} \right)
\]

**Estimated feed intake (kg/d) correcting for the differences in faecal recoveries of in-feed and reference marker:**

\[
\text{Estimated feed intake (kg/d) = daily intake of reference marker (mg/day)} \times \frac{\text{concentration of in-feed marker in faeces (mg/kg)}}{\text{faecal recovery of in-feed marker (%)}} \times \frac{\text{concentration of reference marker in faeces (mg/kg)}}{\text{faecal recovery of reference marker (%)}} \times \frac{\text{concentration of in – feed marker in diet (mg/kg)}}{\text{concentration of in – feed marker in diet (mg/kg)}}
\]

**Statistics**

SAS 9.4 was used for all statistical analyses. The data were analysed as a randomised complete block design, with the treatment as the fixed effect and the pig as the experimental unit. The effect of the frequency of administration of the reference markers on the APE of feed intake was analysed by ANOVA, using a model that included BW block and frequency of reference marker administration and all two-way interactions as fixed effects. R1 pigs were excluded from statistical comparison with R3 and R5, as the R1 pigs showed much larger variation in APEs of feed intake than R3 and R5 pigs. Due to the single replication of all treatments within block, Tukey 1df test was used to check non-additivity of treatment × block interaction to determine whether the interaction is present prior to ANOVA (Montgomery, 2017; Marasinghe and Koehler, 2018). As the interaction was not significant in Tukey
1 df test in either total faecal collection samples (Yb:Cr: $P = 0.765$; C32:C36: $P = 0.806$) or pooled spot faecal samples (Yb:Cr: $P = 0.654$; C32:C36: $P = 0.540$), it was omitted from the model. Also as BW block was not significant in ANOVA in either total faecal collection samples (Yb:Cr: $P = 0.144$; C32:C36: $P = 0.087$) or pooled spot faecal samples (Yb:Cr: $P = 0.246$; C32:C36: $P = 0.326$), it was omitted from the model. After removing R1 pigs and block effect, the comparison of APEs of feed intake between R3 and R5 pigs was analysed by two-sample t-test using PROC TTEST. The normality of the model was checked using PROC UNIVARIATE with Skewness-Kurtosis test and Shapiro-Wilk test. The assumption of homogeneity of variance was checked using PROC TTEST with folded F statistic, according to SAS/STAT® 15.1 user’s guide (SAS®, 2018). The results showed that the assumption of normality and homogeneity of variance was met in total faecal collection samples and pooled spot faecal samples.

The effects of faecal sampling method (quantitative collection samples vs spot samples) on APEs of feed intake were analysed by one-way repeated measures ANOVA including pig as repeated subject. The Dunnett test was used for the model testing differences between faecal sampling methods to compare each of spot faecal samples with total faecal collection samples as a control. For the comparison of APEs among days 1, 2, 3 within treatment, one-way repeated measures ANOVA including pig as repeated subject and the Tukey-Kramer test were used. The effects of marker pair types (metals vs alkanes) and the effects of day of faecal collection (days 2–4 vs days 3–4) on APEs of feed intake were analysed by paired t-test. The comparison of faecal recovery between Yb and Cr between C32 and C36 within treatment was analysed by paired t-test as well. R1 pigs were excluded from all analyses; however, the descriptive results of R1 pigs are reported in tables and figures.

In all analyses, the normality of model residuals was checked using PROC UNIVARIATE with Skewness-Kurtosis test and Shapiro-Wilk test. For models using one-way repeated measures ANOVA, when the assumption that normality of residuals was met, PROC MIXED with REPEATED statement was used, with type = cs option to specify a compound symmetry covariance structure; when the assumption that normality was not met, the lognormal distribution in PROC GLIMMIX with RANDOM statement was used, with RESIDUAL and type = cs option, according to SAS/STAT® 15.1 user’s guide (SAS®, 2018), (Westfall et al., 2011). The specific analysis used in each model is given in Supplementary Table S1.

Feed intake was kept constant relative to BW$^{0.6}$, hence, some variation appeared in the absolute rate of feed intake between pigs resulting from differences in BW. Prediction of this variation was evaluated by linear regression.

**Results**

The effects of dosing frequency and faecal sampling method on absolute prediction error

The predicted feed intake overestimated the measured feed intake in all treatment groups (Table 1) and in each pig (Fig. 2). For Yb:Cr, feed intake from total faecal collection samples was overestimated by 43.2 ± 15.5%, 37.1 ± 4.5%, 21.4 ± 4.0% in R1, R3 and R5 pigs, respectively, while feed intake from pooled spot samples (spot123) was overestimated by 59.2 ± 20.6%, 17.1 ± 3.1%, 5.1 ± 2.2% in R1, R3 and R5 pigs, respectively. For C32:C36, feed intake from total faecal collection samples was overestimated by 40.8 ± 22.3%, 19.5 ± 1.9%, 17.9 ± 2.5% in R1, R3 and R5 pigs, respectively, while feed intake from pooled spot samples was overestimated by 59.0 ± 26.2%, 23.9 ± 5.2%, 9.6 ± 3.2% in R1, R3 and R5 pigs, respectively. There was no significant difference ($P > 0.05$) in APE between R3 and R5 pigs in total faecal collection samples for either marker pair (Table 1). Comparing pooled spot samples with total faecal collection samples for R3 and R5 pigs yielded some significant differences in both directions: the APEs in pooled spot samples were lower (Yb:Cr in R3 pigs, $P = 0.004$) or not significantly different (Yb:Cr in R5 pigs, $P = 0.086$; C32:C36 in R3 pigs, $P = 0.966$; C32:C36 in R5 pigs, $P = 0.283$), compared with total faecal collection samples. When correcting for the differences in faecal

![Fig. 1.](image-url)
recoveries of in-feed and reference marker, for C32:C36, APEs were reduced to zero in R3 and R5 pigs (Table 1).

Comparison between metal pair and alkane pair in total faecal collection samples

In the total faecal collection samples, the APE was significantly lower in C32:C36 than in Yb:Cr in R3 pigs \((P = 0.039)\), while it did not differ significantly in R5 pigs \((P = 0.414)\) (Fig. 3).

Marker recovery and within marker pair recovery difference

The faecal recovery of Yb and Cr averaged over days 1–3 was 54.0 ± 5.2% and 74.3 ± 2.2% \(\text{R1}\), 55.6 ± 2.2% and 76.2 ± 2.9% \(\text{R3}\) and 62.8 ± 2.0% and 76.5 ± 3.0% \(\text{R5}\) (Fig. 4a), being significantly lower in Yb compared to Cr within treatment \((\text{R3: } P = 0.004, \text{R5: } P = 0.013)\). The faecal recovery of C32 and C36 averaged over days 1–3 was 57.2 ± 8.6% and 74.9 ± 4.1% \(\text{R1}\), 60.8 ± 1.5% and 72.6 ± 0.8% \(\text{R3}\), and 63.5 ± 1.2% and 75.1 ± 2.3% \(\text{R5}\), being significantly lower in C32 compared to C36 \((\text{R3: } P = 0.002, \text{R5: } P = 0.006)\). The faecal recovery difference between Cr and Yb was 20.2 ± 4.4% \(\text{R1}\), 20.6 ± 2.4% \(\text{R3}\), 13.7 ± 2.6% \(\text{R5}\); the faecal recovery difference between C36 and C32 was 17.7 ± 6.1% \(\text{R1}\), 11.8 ± 1.2% \(\text{R3}\), and 11.7 ± 1.6% \(\text{R5}\) (Fig. 4b). There was a strong relationship between APE and the difference in faecal recovery rates of the marker pairs used (Supplementary Figure S1), with \(R^2 = 0.84\) \(\text{R3}\) and 0.89 \(\text{R5}\) for Yb:Cr and \(R^2 = 0.78\) \(\text{R3}\) and 0.73 \(\text{R5}\) for C32:C36.

Comparison between faecal collection days

Using total faecal collection samples, the APE in Yb:Cr was significantly lower on day 3 (faeces collected on day 4) than on day 1 (faeces collected on day 2) in R3 pigs \((P = 0.008)\), while there was

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Table 1

| Item | Frequency administration of reference marker | R1 | R3 | R5 | SEM of R3 + R5 | Comparison R3-R5 |
|------|-----------------------------------------------|----|----|----|----------------|-----------------|
| Measured DFI (kg/day per pig) | | 0.78 ± 0.03 | 0.77 ± 0.02 | 0.78 ± 0.02 | – | – |
| APE for Yb:Cr | Faecal sampling method | 0.33 ± 0.11 | 0.29 ± 0.04 | 0.17 ± 0.03 | 0.04 | 0.056 |
| TFC | | 0.30 ± 0.15 | 0.15 ± 0.02 | 0.14 ± 0.02 | 0.02 | 0.732 |
| Spot1 | | 0.28 ± 0.21 | 0.12 ± 0.02 | 0.27 ± 0.03 | – | – |
| Spot2 | | 0.75 ± 0.36 | 0.19 ± 0.05 | 0.12 ± 0.02 | – | – |
| Spot3 | | 1.03 ± 0.47 | 0.21 ± 0.09 | 0.06 ± 0.02 | – | – |
| Spot123 | | 0.44 ± 0.18 | 0.18 ± 0.04 | 0.08 ± 0.03 | 0.03 | 0.068 |
| SEM | | – | 0.05 | – | – | – |
| APE for C32:C36 | Faecal sampling method | 0.30 ± 0.11 | 0.15 ± 0.02 | 0.14 ± 0.02 | 0.02 | 0.732 |
| TFC | | 0.28 ± 0.21 | 0.12 ± 0.02 | 0.27 ± 0.03 | – | – |
| Spot1 | | 0.75 ± 0.36 | 0.19 ± 0.05 | 0.12 ± 0.02 | – | – |
| Spot2 | | 1.03 ± 0.47 | 0.21 ± 0.09 | 0.06 ± 0.02 | – | – |
| Spot3 | | 1.03 ± 0.47 | 0.21 ± 0.09 | 0.06 ± 0.02 | – | – |
| Spot123 | | 0.44 ± 0.18 | 0.18 ± 0.04 | 0.08 ± 0.03 | 0.03 | 0.068 |
| SEM | | – | 0.05 | – | – | – |
| APE in TFC assuming reference markers have equal faecal recovery with in-feed markers | | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | – | – |

1 TFC = total faecal collection on days 2, 3 and 4; Spot 1, 2 and 3: spot faecal samples on day 3 at 1200 h and 1700 h and day 4 at 0700 h, respectively; Spot123: pooled spot samples.

2 Data are presented as mean ± SEM, \(n = 4\) for each treatment.

3 Comparison between R3 and R5 pigs using two-sample \(t\)-test.

4 APE: absolute prediction error of DM feed intake (kg/day); Absolute prediction error (kg/day) = | predicted DM feed intake (kg/day) – measured DM feed intake (kg/day)|.

5 Comparison between TFC samples and other types of faecal samples using Dunnett’s test within treatment R3 and R5 within the same marker pair. \(X, Y\) values with different letters differ significantly \((P < 0.05)\); \(x, y\) values with different letters tend to be different \((0.05 < P < 0.10)\).
no significant difference of APE between day 2 and day 3 in R3 pigs (Fig. 5a). In C32:C36 (Fig. 5b), the APE on day 3 tended to be lower than that on day 1 in R3 pigs \((P = 0.061)\), while there was no significant difference of APE between day 2 and day 3 in R3 pigs. There were no significant differences in APE among the three days in R5 pigs, predicted either by Yb:Cr or by C32:C36.

Using total faecal collection samples, the APE averaged over days 2 + 3 (faeces collected on days 3 + 4) tended to be lower than that on day 1 in R3 pigs \((P = 0.061)\), while there was no significant difference of APE between day 2 and day 3 in R3 pigs. There were no significant differences in APE among the three days in R5 pigs, predicted either by Yb:Cr or by C32:C36.

Using total faecal collection samples, the APE averaged over days 2 + 3 (faeces collected on days 3 + 4) tended to be lower than that when averaged on days 1 to 3 (faeces collected on days 2 + 3 + 4) in R3 pigs, predicted either by Yb:Cr \((P = 0.064)\) or by C32:C36 \((P = 0.069)\) (Fig. 6). Using total faecal collection samples, there was no significant difference of APE between days 2 + 3 and days 1 + 2 + 3 either predicted by Yb:Cr or by C32:C36 in R5 pigs.

Discussion

The effect of dosing frequency on absolute prediction error and estimation accuracy

In our study, we tested the extent to which a fixed amount of reference marker in a single daily dose (R1) or split in three (R3) or five (R5) daily dose allows accurate estimates of feed intake of pigs. Increasing the frequency of administration of the reference marker by definition leads to more stable excretion patterns of the reference marker, which is beneficial for the feed intake estimation. This is of particular importance when applying this technique under complex housing conditions, in which feed intake patterns are unknown. Increasing the frequency, on the other hand, results in increased labour requirements and may disrupt the animals.

We excluded R1 pigs from statistical comparison with R3 and R5, as the R1 pigs showed much larger variation in APEs of feed intake than R3 and R5 pigs: i.e. the provision of the reference marker once daily apparently leads to erratic excretion patterns. Hence, even in the total faecal collection samples, APEs of feed intake in R3 and R5 pigs were numerically lower than in R1 pigs. It corroborates the findings of Kim et al. (2010), who concluded that dosing a reference marker La2O3 three times per day to post-weaned pigs showed reasonable accuracy \((R^2 = 0.85)\) of individual feed intake estimation, while once per day showed weak accuracy \((R^2 = 0.47)\). Ferraz de Oliveira et al. (2006) found that dosing a reference marker (C32) twice daily to growing pigs reduced variation in faecal C32 concentrations compared to dosing once daily. We conclude that dosing the reference marker three or five times per day improves estimation of the feed intake compared with dosing once per day. No improvements in APE were observed when increasing the frequency from three to five times per day.

The effect of dosing duration on absolute prediction error

Administering the reference marker long enough prior to the start of faecal sampling is important for obtaining steady faecal excretion rates and thereby obtaining accurate estimates of feed intake. With regard to feasibility, particularly when using the dual marker approach in a complex housing system, a shorter duration of administration is preferred. When using the total faecal collection samples, for R3 pigs, feed intake prediction errors were larger at day 1 compared with days 2 and 3 for both marker pairs. For R5 pigs, this effect was not observed. Therefore, two dosing days would likely be the minimum requirement for a reference marker to reach a steady excretion pattern when dosing it to pigs three times daily.

Other studies have reported stabilised alkane excretion on the third day of dosing for once or twice daily in growing pigs (Ferraz de Oliveira et al., 2006), on the fifth day of dosing alkanes for once or twice daily in growing pigs (Ribeiro et al., 2007), after 7 days of dosing alkanes with unknown frequency in adult sows.
as observed by (Sehested et al., 1999) as cited by (Ribeiro et al., 2007). The required number of dosing days naturally depends on the mean retention time of digesta inside the gastrointestinal tract. This varies with age (Wilson and Leibholz, 1981) and feeding level (Schop et al., 2019). We conclude that, the different minimum dosing days reported in these studies is related to the different dosing frequency and different mean retention time of digesta in gastrointestinal tract in pigs. When applying the dual marker technique, this should be carefully considered, or tested prior to the measurement, e.g. via a pulse-dose of a coloured marker and monitoring the time of first appearance in faeces.

A potential effect of a difference in diurnal variation in faecal marker excretion between reference and in-feed markers on feed intake estimates should be considered. The reference markers were administrated once, three and five times per day at different intervals between each dosing, which could create fluctuations in excretion pattern that would differ from the in-feed markers. The limited number of daily defaecations, however, complicates the measurement of such effect.

Comparison between metal pair and alkane pair

In our study, the APE using C32:C36 was numerically lower than using Yb:Cr at all frequencies, and significantly in R3 pigs, indicating that using C32:C36 leads to improved estimates of feed intake compared with Yb:Cr. This is caused by the higher similarity in faecal recovery of C32 and C36 compared with Yb and Cr. Similarly, a strong relationship was found between the prediction error of feed intake and the difference in faecal recovery of the alkane pair in cattle (R² = 0.75, P < 0.001 for both C31:C32 and C32:C33) (Bezabih et al., 2012). In addition, a meta-analysis in ruminants showed a strong relationship between the difference in faecal recovery of n-alkane pairs (=1-natural/dosed) and feed intake prediction error (1-predicted/measured), with the adjusted R² of 0.83 (P < 0.001) for C31:C32 and adjusted R² of 0.93 (P < 0.001) for C32:C33 (Andriarimalala et al., 2020). In our study, C32:C36 seems to have better estimation accuracy of feed intake than Yb:Cr. However, the final choice of marker pairs for estimation of feed intake also depends on the availability of equipment and the precision of analytical procedures.

The effect of faecal sampling method on absolute prediction error

For successful application of the dual marker technique in a complex housing environment, quantitative faecal collections are impossible to perform, and the technique has to be applied using faecal spot sampling. In our study, pooled faecal spot samples had similar or lower SEM of APE than single spot samples in most cases. It was found that in growing pigs, feed intake estimates from morning and evening spot faecal samples underestimated the measured feed intake by 16.8% (±2.79%) and 20.4% (±6.30%), while from the average concentrations of the two faecal samples underestimated by 6.1% (±2.66%) (Méndez Cante, 2013). Circadian variations of marker faecal concentrations were suggested to explain the discrepancy of feed intake estimation between pooled and single spot samples (Méndez Cante, 2013). In addition, single spot samples may also introduce a lot of random variation, particularly so at lower frequencies of administration of the reference marker.
In our study, the APEs were similar or lower in pooled samples compared to total faecal collection samples, reaching statistical significance only for Yb:Cr in R3 pigs. This may be related to the later timing of the first spot sample, compared with the onset of total faecal collection.

**Marker faecal recovery and estimation accuracy of feed intake**

Differences in faecal recovery between reference and in-feed markers lead to feed intake prediction errors, but recoveries deviating from 100% are not necessarily a problem, if faecal recoveries of the reference and in-feed marker are similar or if the difference can be corrected for (de Vries and Gerrits, 2018). Marker recoveries have been reported variable in metals (Köhler et al., 1990) and alkanes (Bachmann et al., 2018). In our study, the faecal recovery of Yb in R3 and R5 pigs averaged 59.2% while it was higher (83%) in postweaned piglets along the gastrointestinal tract (Low et al., 2020); the recovery of Cr in R3 and R5 pigs averaged 76.4%, while it was higher (averaged 87.3%) in gastrointestinal compartment in milk-fed veal calves (Pluschke et al., 2016) and similar (73%) along the gastrointestinal tract in postweaned piglets (Low et al., 2020).

In our study, the faecal recovery of the reference marker C32 in R3 and R5 pigs averaged 62.1%, which was rather low compared to the 72.6% (Ferraz de Oliveira et al., 2006) and to the value even exceeding 100% (Ribeiro et al., 2007) found in growing pigs; the faecal recovery of in-feed C36 in R3 and R5 pigs averaged 73.9% in our study, which was close to the recovery of pulse dosed C36 of 69.1% (Ferraz de Oliveira et al., 2006) but was rather low compared to the value even exceeding 100% (Ribeiro et al., 2007) found in growing pigs.

In our study, the faecal recoveries of both in-feed markers were higher than that of both reference markers. A plausible explanation for this could be the longer adaptation period to get steady faecal excretion rates of the in-feed markers. In addition, the in-feed markers may have been better mixed in digesta than the reference markers. The longer chain length of C36 than C32 could also explain the higher recovery of C36 (Wright et al., 2020). It is remarkable and consistent with Méndez Cante (2013) that the errors in the prediction are merely related to erroneous faecal recoveries of the reference rather than that of the in-feed markers.

**Correcting feed intake estimations for differences in faecal recoveries of reference and in-feed markers**

If the inherent assumption of equal faecal recoveries of the reference and in-feed markers is violated, correcting estimations for differences in recoveries can be done provided that there is reason to assume these differences are consistent across studies. For the metal markers, as discussed above, this is not the case, and hence, corrections are not considered. For n-alkanes, observations from other species indicate that faecal recoveries of n-alkanes increase curvilinearly with increasing carbon chain length (Wright et al., 2020). For pigs, information from literature is more ambiguous: in pregnant sows, faecal recoveries of alkanes increased only numerically with increasing chain length from 71% (C29) to 79% (C35) (Wilson et al., 1999). Ferraz de Oliveira et al. (2006) found in growing pigs that mean faecal recoveries of n-alkanes did increase curvilinearly with increasing carbon chain length (C25 to C36) from 38% to 69%, with little differences among C29, C32, C33 and C36.

In our study, the difference in faecal recovery between C32 and C36 was 11.8 ± 1.2% (R3) and 11.7 ± 1.6% (R5). In our study, in C32, the APEs from total faecal collection samples were 0.15 kg/day and 0.14 kg/day in R3 and R5 pigs, respectively. When assuming equal faecal recoveries, for C32 and C36, APEs were reduced to zero in R3 and R5 pigs. This is further supported by the strong linear relationship between the APEs and the difference in faecal recovery rates of C32:C36 (Supplementary Figure S1).

**Comparison between faecal collection days**

In total faecal collection samples of R3 pigs, the APE using total faecal collection samples collected on days 3–4 (the third and fourth day after the first dosing day) tended to be lower (Yb:Cr, P = 0.064; C32:C36, P = 0.069) than using total faecal collection samples collected on days 2–4 (the second till fourth day after the first dosing day). Apparently, the start of the collection period on day 2 caused additional variation, and appeared to be too soon after the onset of pulse dosing the reference marker. Not surprisingly, this effect was absent in R5 pigs. It is concluded that properly timed faecal spot sampling is key to reducing the bias of estimation. It makes especially sense for the estimation of individual feed intake in pigs reared in free-grazing conditions, which could help to minimise the labour for oral administration duration and faecal sampling duration.

**Conclusion**

In our study, the absolute prediction error measured by C32: C36 was numerically lower than measured by Yb:Cr at all frequencies, and significantly in R3 pigs (0.15 ± 0.02 kg/day vs 0.29 ± 0.04 kg/day). This was related to a larger difference in faecal recovery between Yb and Cr compared with C32 and C36. For successful application of the dual marker technique in a complex housing environment, marker administration and the timing of spot sampling relative to the onset of pulse dosing the reference marker are crucial. Our study indicates that dosing the reference marker three times per day for 2 days on days 1 and 2, combined with pooled spot faecal sampling, collected on days 3 and 4 appeared the minimum requirement for obtaining acceptable estimates of feed intake. In this way, absolute prediction errors of 10%-15% are feasible using the dual marker technique. Hence, this technique is promising to simultaneously provide semi-quantitative estimates of the intake of nutrients from various sources for individual pigs in a complex housing environment.

**Supplementary material**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100451.

**Ethics approval**

All experimental procedures were approved by the Dutch Central Committee of Animal Experiments (the Netherlands) under the authorisation number AVD401002015196.

**Data and model availability statement**

All data generated or analysed during this study are available from the corresponding author upon reasonable request.

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Declaration of interest
Erik Bruininx was the employee of Agrifirm. Emilie-Julie Bos is the employee of Agrifirm. Tianyue Tang, Carola van der Peet-Schwering, Nicole Soede, Bjorge Laurensen, and Walter Gerrits declare no conflicts of interest.

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