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# Validating the alkane pair technique to estimate dry matter intake in equids

D. G. SMITH[1]*, R. W. MAYES[2], T. HOLLANDS[3], D. CUDDEFORD[4], H. H. YULE[1], C. M. MALO LADRERO[1] AND E. GILLEN[5]

[1] *Department of Agriculture and Forestry, University of Aberdeen, Aberdeen, Scotland, AB24 3FX, UK*
[2] *Macaulay Institute, Craigiebuckler, Aberdeen AB15 8QH, UK*
[3] *Dodson and Horrell Ltd, Kettering Road, Islip, Northamptonshire NN14 3JW, UK*
[4] *Animal Health and Welfare Division, Royal Dick School of Veterinary Studies, Easter Bush Veterinary Centre, Roslin, Midlothian, Scotland, EH25 9RG, UK*
[5] *ILPH Belwade Farm, Aboyne, Aberdeenshire AB34 5DJ, UK*

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## SUMMARY

The estimation of dry matter intake (DMI) using the alkane pair technique has been validated in ruminants, but not in equids. The current paper reports the finding of three comparative validation studies carried out using a total of 12 cattle, 29 donkeys and 10 horses during which directly measured intake was compared to estimated intake using the alkane pair technique. Two methods were developed to dose the even chain alkanes that were used as external markers. Study I, carried out in Zimbabwe, compared the accuracy of estimated intake with measured intake in cattle and donkeys using hexatriacontane ($C_{36}$) as the external marker. Studies II and III were carried out in the UK with horses and donkeys and compared the accuracy of estimated intake with measured intake using dotriacontane ($C_{32}$) as the external marker. Study III also tested the effect on the accuracy of intake estimates of two marker dosing levels (mean daily dose of 224 mg per animal and 448 mg per animal) and two dosing frequencies (2× and 3× daily). Twice daily dosing of even-chain alkane at the lower dose level provided an estimate of DMI similar to that obtained by thrice daily dosing at this low level. The higher dose level given twice daily tended to produce large variation in faecal concentrations of dosed even-chain alkanes, this variation was reduced when dosing frequency was increased to thrice daily. The accuracy of estimated intake improved progressively as the number of faecal sampling days was increased from one to six with no significant difference between estimated intake based on day 5 or 6 of faecal sampling.

The results of all three studies indicate that the alkane pair technique provides a robust method of estimating intake in equids with no significant difference between measured and estimated values in all but one case. Using $C_{31}$ as an internal marker provided a more accurate estimated intake than using $C_{33}$ as the internal marker in all cases. Faecal recoveries of alkanes in equids do not appear to show the same influence of carbon chain length that has been observed in ruminant studies.

## INTRODUCTION

Little is known about the voluntary food intake of pasture-fed equids or what sward factors influence intake. In order to avoid common health problems that have an underlying nutritional aetiology, such as obesity, laminitis, hyperlipaemia and developmental orthopaedic disorders, it is essential to have a reliable estimate of the contribution of pasture to the overall nutrient intake of equids. In the past, accurate estimation of the DMI of pasture-fed animals has been problematic because of the lack of reliable methodologies. Recently the use of the alkane pair technique to estimate DMI at pasture has been successfully developed and validated in a variety of ruminant species including sheep (Mayes *et al.* 1986, 1988) and cattle (Malossini *et al.* 1996). To a limited extent this method has also been validated in horses by O'Keefe & McMeniman (1998), Stefanon *et al.*

* To whom all correspondence should be addressed. Email: d.smith@napier.ac.uk

Table 1. *The dry matter, organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), crude protein (CP) composition and in vitro and in vivo dry matter digestibility (DMD) coefficients of herbages fed during Studies I, II and III*

| | Feed | Dry matter content (g/kg) | Nutrient content of dry forage (g/kg DM) | | | | DMD | |
|---|---|---|---|---|---|---|---|---|
| | | | OM | NDF | ADF | CP | *In vitro* | *In vivo* |
| Study I | Hay | 904 | 946 | 785 | 497 | 30 | 0·35 | 0·42 |
| Study II | Hay | 902 | 972 | 584 | 334 | 40 | 0·59 | 0·52 |
| | Straw | 919 | 979 | 779 | 454 | 30 | 0·46 | |
| Study III | Haylage | 683 | 936 | 656 | 392 | 97 | 0·52 | – |

(1999), Ordakowski *et al.* (2001) and Stevens *et al.* (2002). However, under pasture conditions where horses had an opportunity to graze selectively, Friend *et al.* (2004) observed a substantial difference between intake estimated using the $C_{31}/C_{32}$ and $C_{32}/C_{33}$ alkane pairs in one of their treatments. This may have been attributed to the dosing regime and/or the sampling regime. Hence there is a need to determine the most appropriate alkane dosing and sampling regimes for accurate intake estimations in horses.

Accurate estimation of faecal output is dependent on obtaining daily representative samples of faeces with minimal variation in the concentration of external marker between samples (Dove & Mayes 1996), which is achieved by delivering a steady flow of marker into the gut. In ruminants this is usually accomplished by using an intra-ruminal controlled release device that provides a constant trickle-dose of external marker over a period of 12 days or more (Mayes & Dove 2000). Because they lack a rumen, the use of such devices in equids is impossible and therefore alternate methods of delivery of external marker need to be developed and validated.

The current paper presents the results of three validation studies of the n-alkane technique to estimate DMI in equids and cattle. The objectives of the studies were to compare estimated DMI (E-DMI) made using the alkane pair technique with measured DMI (M-DMI) determined using conventional gravimetric techniques in pen-fed cattle, donkeys and horses. Faecal recovery values of dosed and selected natural alkanes in cattle and donkeys were also measured. Furthermore, optimal faecal sampling regimes, optimal dosing frequency, and effective alkane dose level were determined in horses.

## MATERIALS AND METHODS

### *Location, animals, feeds and treatments*

#### *Study I*

The objective of the study was to estimate DMI of pen-fed donkeys and cattle using $C_{36}$ alkane as an external marker and compare these to the values of M-DMI. On this occasion $C_{36}$ was selected as the external marker, because at the time of the study there was a worldwide shortage of $C_{32}$ alkane (which is more commonly used). Faecal recoveries of dosed $C_{36}$ alkane, natural odd chain alkanes ($C_{25}$–$C_{35}$) and natural $C_{32}$ were also determined. The levels of even-chain alkanes (except for $C_{32}$) were too low to be measured accurately using the conventional method for alkane analysis. Relative recoveries of natural, odd-chain alkanes ($C_{25}$–$C_{35}$) in relation to $C_{31}$ were also determined to allow comparison between studies.

The study was carried out at Matopos Research Station located 30 km south of Bulawayo, Zimbabwe. Twelve mature male castrate donkeys (initial mean live weight 153 kg) and twelve 2-year-old, male castrate cattle of the Tuli breed (initial mean live weight 233 kg) were housed in individual pens for a period of 24 days in February 1996. During this time, they were fed *ad libitum* on a diet of poor quality hay, and given free access to water (food composition is shown in Table 1). Approximately 0·50 of the estimated daily food allowance was placed in the feeding bins at 8.00 h, immediately after the previous day's refusals had been removed from the bin. The feeding bins were topped up with a known amount of food at 12.00, 16.00 and 20.00 h.

After a period of 12 days adaptation, the animals were dosed twice daily at 08.00 and 16.00 h for 12 days with a 5 g alkane-labelled fibre (ALF) bolus (preparation method described below). The estimated daily dose of $C_{36}$ ALF given in Study I was 308·3 mg day, with a coefficient of variation (CV) of 0·97 % ($n = 5$).

Daily DMI was measured during the final 7 days of the dosing period by calculating difference between the dry weight of food offered and the dry weight of food refused. A total faecal collection was made for the final five days, from which daily faecal output was calculated on a dry weight basis.

Dry weights of food offered, food refused and faecal output were calculated on a daily basis by measuring the weight of fresh materials and calculating the dry

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matter content of daily samples of each of these fresh materials. The dry matter content of a sample of the food offered, food refused and faeces were measured by recording the change in weight of samples after drying to constant weight in a forced-draught oven at 100 °C. *In vivo* dry matter digestibility (DMD) was calculated from the average DMI measured over day 6–10 of the study and average faecal output measured over day 8–12 of the study.

Sub-samples (100–200 g) were taken from pooled samples of feed offered and food refusals and faecal samples that had been collected daily over the final 5 days of the study and were dried to constant weight in a forced-draught oven at 60 °C. Sub-samples of the feed offered were retained for analysis in duplicate for their organic matter (OM), crude protein (CP), neutral detergent fibre (NDF), and acid detergent fibre (ADF) composition at the Royal (Dick) School of Veterinary Studies laboratory in Edinburgh according to the methods reported by the Association of Official Analytical Chemists (AOAC) (1990). Dried sub-samples of feed offered and faeces were analysed for their alkane content at the Macaulay Institute, Aberdeen using the method described by Ali *et al.* (2004). *In vitro* DMD of the feed offered was determined by the neutral cellulase plus gamanase technique (ANKOM 1998).

*Study II*

The objectives of the study were to estimate DMI of stable-fed donkeys using $C_{32}$ as an external marker and compare these to the values of M-DMI. Faecal recoveries of dosed $C_{32}$ alkane and natural odd-chain alkanes ($C_{25}$–$C_{35}$) were also determined. As in Study I, natural, even-chain alkanes were not present in sufficient concentrations to determine faecal recoveries. Relative recoveries of natural, odd-chain alkanes ($C_{25}$–$C_{35}$) in relation to $C_{32}$ were also determined.

The study was undertaken at The Donkey Sanctuary, Sidmouth, Devon over a period of 12 days during February 2004. Twenty mature donkeys (ten castrate male and ten female) aged between 10 and 26 years and weighing between 149 and 223 kg were used in the study. The donkeys were introduced to their experimental routine 19 days before the start of the trial. During the study three donkeys were removed from the trial as a precaution to prevent the reoccurrence of minor illnesses, resulting in 17 donkeys (seven castrate male and ten female) completing the trial. An automatic drinking system was provided in each stable allowing unrestricted access to water. Each animal was provided with free access to a commercial mineral and vitamin block (Uniblock, Dodson and Horrell Ltd, Kettering, UK).

The donkeys were provided with a diet of hay and barley straw, the composition of these forages is shown in Table 1. Donkeys received a total daily dry matter allowance equivalent to 0·025 of their body weight; hay and straw were fed in a ratio of 7:3. The ration was fed in three meals at 09.30, 13.00 and 19.00 h each day. Each donkey received approximately 0·25 of the daily ration in each of meals one and two, and the remaining 0·50 for meal three. Feed refusals were collected at 09.00 h the following morning.

Daily DMI of each donkey was recorded for a period of 12 days using the method described in Study I. Daily faecal output was recorded for each donkey by carrying out a total faecal collection for the final 5 days of the 12-day study, using the method described in Study I. Pooled samples of food offered and faeces collected over the final 5 days of the study were dried at 60 °C to constant weight, and retained for the same laboratory analysis as described in Study I. *In vivo* DMD was determined from the average DMI measured over day 6–10 of the study and faecal output averaged over day 8–12 of the study using the same method as in Study I.

In order to calculate E-DMI using alkanes, on each day of the 12-day study every animal was handfed three times per day at 07.00, 13.00 and 19.00 h with one bite-sized Weetabix® (BSW) (Weetabix Ltd, Kettering, UK) labelled with $C_{32}$ alkane. The actual daily estimated dose of $C_{32}$ given was 127·1 mg/day (CV = 1·09 %, $n = 5$).

*Study III*

The objective of the study was to determine the optimum $C_{32}$ alkane dosing level and frequency when used to determine E-DMI in stabled horses.

The study was carried at the International League for the Protection of Horses, Belwade Centre in Aboyne, Aberdeenshire. Twelve mature horses were used in the study with live weights at the start of the study of between 228 and 496 kg. Horses were housed in individual stables each fitted with self-filling water troughs. Each horse received its normal haylage allowance (nutrient composition is shown in Table 1) during the course of study based on approximately 0·02 of the animals' live weight on a dry matter basis, offered as three meals per day at 8.00, 12.00 and 16.00 h. Food refusals were collected at 07·30 h the following morning.

In order to determine the alkane-dosing regime that gave the most accurate E-DMI, two batches of BSW were produced, one heavily labelled batch (nominally 150 mg of $C_{32}$) and one lightly labelled batch (nominally 100 mg of $C_{32}$). On subsequent analysis the heavily labelled BSW was found to contain 110·7 mg per BSW (CV 0·81 %, $n = 5$) and the lightly labelled BSW contained 75·7 mg per BSW (CV 1·95 %, $n = 5$). Although the actual amount of $C_{32}$ given was lower than planned the proportionality between the dose treatments was preserved.

Horses were randomly allocated to one of four groups. Each group of three animals underwent one

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of the following dosing treatments for a period of 13 days.

(i) Low dose, twice daily (L × 2) – received 1 BSW heavily labelled with $C_{32}$ alkane twice daily at 09.00 and 17.00 h; a total daily dose of 221·4 mg.
(ii) Low dose, thrice daily (L × 3) – received 1 BSW lightly labelled with $C_{32}$ alkane thrice daily at 09.00, 13.00 and 17.00 h; a total daily dose of 227·1 mg.
(iii) High dose, twice daily (H × 2) – received 2 BSW heavily labelled with $C_{32}$ alkane twice daily at 09.00 and 17.00 h; a total daily dose of 442·8 mg.
(iv) High dose, thrice daily (H × 3) – received 2 BSW lightly labelled with of $C_{32}$ alkane thrice daily at 09.00, 13.00 and 17.00 h; a total daily dose of 454·2 mg.

As in Study II alkane-labelled BSW was hand fed to each horse. Daily DMI of each animal was recorded for the final 7 days of the 13-day study using the methods described for Study I. Faecal samples were collected at 7·30 h for the final 6 days of the 13-day study from each animal and were dried at 60 °C to constant weight, and retained for laboratory analysis as described in Study I. Pooled weekly samples of food offered were dried at 60 °C to constant weight and retained for laboratory analysis as described in Study I. Faecal output was estimated using $C_{32}$ as an external marker (Dove & Mayes 1996), as a total faecal collection was not carried out. This allowed the relative recovery of natural, odd–chain alkanes ($C_{25}$–$C_{35}$) and $C_{36}$ to be determined in relation to $C_{32}$.

*Preparation of alkane marker pellets*

Two different methods of dosing external marker were developed. In Study I, ALF was used to dose $C_{36}$ absorbed into a carrier fibre prepared from hay. The ALF was prepared using 3 kg of fibre produced by boiling ground (4 mm screen) perennial ryegrass hay for 1 h in commercial washing detergent (Persil, Unilever Ltd, UK). After thorough rinsing and drying, $C_{36}$ alkane was absorbed into the fibre, using a method modified from Mayes *et al.* (1986). A solution of pure alkane ($C_{36}$) in n-heptane (97 g/l) was prepared and sprayed, for a period of 10 min with the aid of a hand-spray gun, on to 1 kg batches of fibre while it was being spun in a small rotary cement mixer. The fibre was placed on trays and dried at room temperature. The batches of fibre were then placed in an oven at 100 °C for 10–20 min to melt the alkane on to the fibre. Five 5 g samples of ALF were analysed for their individual alkane concentration. These data were used to calculate the dosing level of the finished fibre and the CV between samples.

The ALF was given to the animals in the form of a 5 g bolus. Boli were formed using 50 ml plastic centrifuge tubes into which 5 g of ALF was loosely compressed and 5 ml of warm 5% gelatine solution added. The contents of the tube were then compressed into a moderately compact bolus and placed in a refrigerator at 4 °C until required.

Boli were given to the animals with the aid of a specially enlarged Foulk's balling gun. Animals were restrained with the aid of a neck yoke. In the case of cattle the bolus was placed over the tongue into the back of the throat. In donkeys, the throat was too narrow to allow effective dosing in this way. When placed at the back of the tongue the donkeys were frequently able to recover the boli and eject them from their mouths. To overcome this problem, a drop of peppermint oil was added to each bolus to make it more palatable; the soft texture and palatability of the bolus then made rejection less likely. Animals were released from the neck yoke and observed closely for 2–3 min to confirm that the boli had been swallowed.

Studies II and III were carried out at equine welfare centres in Devon and Aberdeenshire and frequent application of restraint was not permitted. In these studies $C_{32}$ marker was administered via individual BSW, which could be readily hand fed without the need for restraint.

The alkane-labelled BSW was prepared in a fume cupboard. Twenty-five g of $C_{32}$ was fully dissolved in 2·5 litres of heptane, using a hotplate stirrer set to a low heat. The resultant solution contained a concentration of 10 mg of $C_{32}$ per ml of heptane. Small foil dishes (nominal capacity of 20 ml) were laid out on metal oven shelves and an individual BSW (approximately 2 g FW) was placed in each dish.

A Gilson pipette was then used to transfer a given volume of the $C_{32}$/heptane solution on to individual BSW within the foil dishes. The precise volume of solution transferred depended on the desired final quantity of alkane required on the BSW. In practice BSW with a final $C_{32}$ dose of either 50, 100 or 150 mg were required, so typically 5, 10 or 15 ml of solution were transferred. However, experimentation showed that up to 300 mg could be added with no effect on BSW palatability. These volumes of solution would gradually become absorbed by the BSW during the subsequent evaporation and baking processes.

Following the transfer of the $C_{32}$/heptane solution on to the BSW, all the heptane was allowed to evaporate in the fume cupboard after which the oven shelves containing the foil dishes and BSW were put into a forced-draught oven at 70 °C for 2 h. After 2 h the oven temperature was raised to 100 °C and the BSW were baked overnight allowing the molten $C_{32}$ to be fully absorbed. The prepared BSW were then ready for use in the studies with at least five per batch being retained for laboratory analysis to determine $C_{32}$ dose rate.

*Alkane analysis*

The alkane analysis of feed and faecal samples took place at the Macaulay Institute, Aberdeen using a method identical to that described by Ali *et al.* (2004).

Alkane was extracted from marker pellets (ALF and BSW) using the following method. Five marker pellets were placed into separate 100 ml borosilicate glass bottles (Duran), which were capped and weighed. Heptane (70 ml) was added to each bottle and re-weighed. The Duran bottles plus the contents were heated at 55 °C for 2 h in an ultrasonic bath to dissolve the alkane. Duplicate samples (0·5 ml) of warmed solution were removed from the Duran bottles to empty, tared scintillation vials, and then capped and weighed. Internal standards were added to each 10 ml vial ($C_{22}$ = 0·59887 mg/g; $C_{34}$ = 0·74085 mg/g) and the vial re-weighed. A volume of 0·1 ml of solution from each scintillation vial was placed into 0·8 ml flat bottom gas chromatography vials. Dodecane (0·25 ml) was added to each vial, which was then capped with a polyethylene plug. The concentration of $C_{32}$ in each vial was determined by gas chromatography using the conditions described by Ali *et al.* (2004).

*Calculations of DMI using markers and marker recovery*

DMI was calculated using measurements of the herbage and faecal concentration of consecutive even- and odd-chain alkanes using the equation derived from Mayes *et al.* (1986).

Natural, odd-chain alkane recovery was determined by calculating the mean daily input of the natural alkanes from their concentration in the feed and M-DMI. Mean daily output of odd chain alkanes was estimated from faecal concentration and mean daily dry faecal output. Faecal recoveries were calculated according to the method of Momont *et al.* (1994). In the case of the dosed, even-chain alkane account had to be taken of both the large amount of dosed alkane and the small amount of marker that occurred naturally in the forage, this was done using Eqn (1).

$$Rj = \left(\frac{Fj}{Dj + Hj}\right) \quad (1)$$

where Rj is the proportion of even-chain alkane recovered in the faeces, Dj is the amount of even-chain alkane dosed per day, Fj is daily faecal output of even chain alkane and Hj is the daily intake of natural even chain alkane derived from herbage. Faecal recoveries of natural alkanes relative to $C_{32}$ were also calculated to allow comparisons between studies I, II and III. It was assumed that both dosed and natural alkanes were recovered equally.

*Data analysis*

Paired *t*-tests (Minitab Release 14.20) were used to make statistical comparison of E-DMI obtained using the alkane technique with those obtained by direct measurement. In Study III, a two-way ANOVA (Minitab Release 14.20) was used to compare statistically the effect of dose level, dosing frequency and any interaction between these two treatments using the difference between directly measured values and those estimated using the alkane technique. Where appropriate Least Significant Difference was calculated at $P = 0{\cdot}05$, 0·01 and 0·001 based on the pooled residual error across the whole experiment and where $n = 6$ for the main treatment effects (dose level and dosing frequency) and $n = 3$ for the interaction effect.

## RESULTS

Comparisons of E-DMI made with various odd-chain alkanes with M-DMI are shown in Table 2. In Study I, an additional E-DMI was calculated using $C_{35}$ (E-DMI-$C_{35}$), as in this study it was the odd-chain alkane adjacent to the dosed even chain ($C_{36}$). The only E-DMI that significantly differed from M-DMI was E-DMI-$C_{33}$ during Study II (Table 2). However, in all cases the differences between E-DMI-$C_{31}$ and M-DMI were smaller than those between E-DMI-$C_{33}$ and M-DMI. In Study I, the differences in E-DMI-$C_{35}$ and M-DMI were larger than the differences between M-DMI and E-DMI using either $C_{31}$ or $C_{33}$. Further analysis only included comparisons between E-DMI-$C_{31}$ and M-DMI, as these values proved to be the most reliable.

In Study III, E-DMI based on faecal and herbage samples pooled over a 6-day period (Table 2) showed no significant difference between dose treatments (L and H) or between dosing frequency (2 or 3 times per day). The numbers of animals in Study III was limited to three per treatment, resulting in large standard errors within treatments and no significant differences between measured and estimated values were obtained. Relative differences between E-DMI-$C_{31}$ and M-DMI were greatest for the L × 3 treatment (16·8% overestimation) and smallest for the H × 2 and H × 3 treatments (1·3% underestimation) (Table 2).

The number of faecal sampling days used to provide a pooled sample for alkane analysis had a very large effect on the accuracy of E-DMI (Fig. 1). When E-DMI was based on a single faecal sample, very large differences between E-DMI-$C_{31}$ and M-DMI were evident, these differences diminished as the number of days included in the pooled sample increased from one to six (Fig. 1).

There was a large effect of dose level when faecal samples were pooled over a period of 3 or less days. The differences between E-DMI-$C_{31}$ and M-DMI

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Table 2. *Comparison of the dry matter intake (DMI) of cattle, donkeys and horses measured conventionally using direct techniques or estimated with the n-alkane technique using $C_{31}$, $C_{33}$ or $C_{35}$ as internal markers and either $C_{32}$ or $C_{36}$ as external markers*

| | | | | | | DMI (kg/day) mean ± S.E. | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Alkane estimated using internal marker | | |
| Study | Species | External marker | Dose level | Dosing frequency | *n* | Directly measured | $C_{31}$ | $C_{33}$ | $C_{35}$ |
| I | Cattle | $C_{36}$ | | ×2 | 12 | 3·15 ± 0·089 | 3·13 ± 0·120 | 3·35 ± 0·132 | 3·36 ± 0·338 |
| | Donkeys | $C_{36}$ | | ×2 | 12 | 2·25 ± 0·135 | 2·21 ± 0·220 | 2·31 ± 0·227 | 2·47 ± 0·299 |
| II | Donkeys | $C_{32}$ | | ×3 | 18 | 3·01 ± 0·132 | 2·96 ± 0·097 | 2·62 ± 0·081 | |
| III | Horses | $C_{32}$ | Low | ×2 | 3 | 4·65 ± 0·475 | 4·42 ± 0·553 | 3·73 ± 0·206 | |
| | | $C_{32}$ | Low | ×3 | 3 | 4·67 ± 0·766 | 4·00 ± 0·366 | 3·48 ± 0·267 | |
| | | $C_{32}$ | High | ×2 | 3 | 6·14 ± 0·645 | 6·22 ± 1·506 | 6·59 ± 1·405 | |
| | | $C_{32}$ | High | ×3 | 3 | 5·99 ± 0·969 | 6·07 ± 1·231 | 5·71 ± 0·789 | |

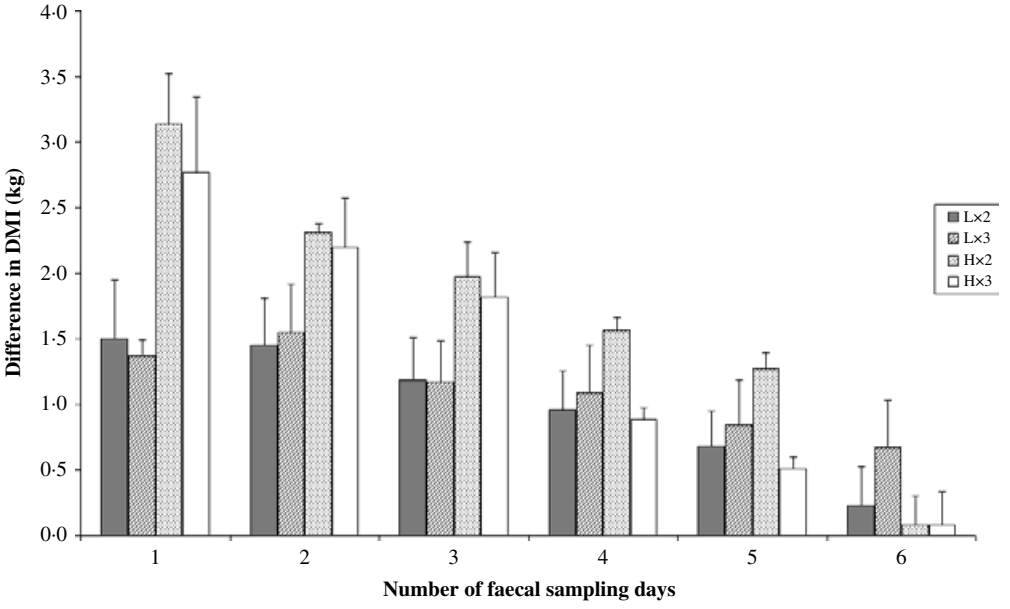


Fig. 1. The effect of the number of faecal sampling days on the difference between directly measured and alkane estimated (using $C_{31}/C_{32}$ alkane pair) DMI in horses receiving either a low (L) or high (H) dose of $C_{32}$ alkane dosed either twice (×2) or three times (×3) daily. Error bars indicate standard error.

obtained in H treatment groups showed significantly ($P<0·05$) greater differences than those obtained in the L treatment groups (Fig. 1). Increasing the frequency of dosing to animals in groups H did not significantly affect the accuracy of E-DMI-$C_{31}$ until the number of faecal sampling days that contributed to the pool exceeded 3 days. E-DMI from animals on the H × 3 treatment were significantly ($P<0·05$) more accurate than animals on the H × 2 treatment when calculations were based on faecal samples pooled over 4 and 5 days, but not over 6 days (Fig. 1). There was no significant effect of dosing frequency on the accuracy of E-DMI-$C_{31}$ for animals on L treatments.

In Study III, comparison between the CV of M-DMI and the CV of faecal concentration of $C_{32}$ showed a statistically similar amount of variation in the case of treatments L × 2, L × 3 and H × 3 but in the case of H × 2 there was a statistically ($P<0·05$) greater amount of variation in the faecal concentration of $C_{32}$ than in M-DMI (Fig. 2). Treatment H × 3 had the smallest difference in CV between

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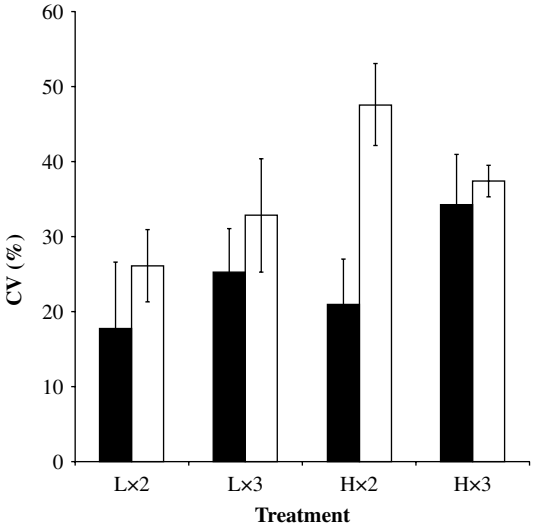


Fig. 2. The coefficient of variation (%) of directly measured daily DMI (■) and daily faecal concentration of $C_{32}$ alkane (□) in horses receiving either a low (L) or high (H) dose of $C_{32}$ alkane dosed either twice (×2) or three times (×3) daily ($n=6$). Error bars indicate standard error.

M-DMI and the faecal concentration of $C_{32}$, whilst the two L treatments had similar differences in the CV of M-DMI and the faecal concentration of $C_{32}$ (Fig. 2).

Table 3 shows the herbage concentration and faecal recoveries of odd-chain ($C_{25}$–$C_{35}$) and dosed, even-chain alkanes ($C_{32}$ and $C_{36}$). Faecal recoveries of odd-chain alkanes ($C_{25}$–$C_{35}$) relative to $C_{32}$ are also shown in Table 3. In Studies II and III, donkeys and horses had similar ($P>0{\cdot}05$) faecal recoveries to one another. In these cases, alkane carbon chain length had a small effect on relative recovery. The most abundant odd-chain alkanes ($C_{27}$–$C_{33}$) had statistically similar ($P>0{\cdot}05$) recoveries to $C_{32}$ (Table 3). Alkanes that were only present in small quantities in herbage ($C_{25}$ and $C_{35}$) were recovered to a significantly ($P<0{\cdot}01$) lesser degree than $C_{32}$ in both donkeys and horses.

## DISCUSSION

Administering alkane marker by absorbing them into palatable and readily consumed carrier substances like BSW was a successful method for frequent dosing of relatively tame animals such as donkeys and horses. Animals would readily approach the person dispensing the BSW anticipating that they would receive a treat. Under pasture conditions, this is a useful feature of using BSW, because animals do not need to be captured to administer the dose. Dosing with ALF was more problematic, because animals had to be restrained whilst being dosed and there was a risk that animals could regurgitate the bolus.

Friend *et al.* (2004) dosed horses with $C_{32}$-labelled grass particles suspended in xanthan gum. The suspension was successfully administered to the animals using a standard drenching gun. Marais *et al.* (1996) showed that this was an effective method of dosing with a CV of 2·6%, slightly greater than the mean CV achieved with BSW in the present study (1·8%). The use of a dosing gun to administer the $C_{32}$ does not overcome the need to capture and restrain animals to deliver the marker, nor does it entirely eliminate the risk of animals regurgitating some of the marker once released. Stefanon *et al.* (1999) fed horses once daily with alkane-labelled ($C_{32}$ and $C_{36}$) shredded paper mixed with concentrate feed, and sampled faeces three times daily after 8 days of dosing. With this method faecal concentration of dosed alkane varied widely between days and never reached the anticipated levels.

Dose level had an important effect on the accuracy of E-DMI in horses in the current study. The accuracy of E-DMI at the lower dose level was less dependent on the number of faecal sampling days than at the higher dose level; especially when the marker was only given twice per day. This effect was probably due to incomplete mixing of the marker with the gut contents, resulting in a large circadian variation in the faecal concentration of marker. The absolute scale of this variation is greater at higher dose levels, because of the larger difference between the daily 'peak and trough' of faecal marker output. When dosing frequency of the H treatment was increased to three times per day E-DMI was more accurate than either L treatments provided that the number of faecal sampling days exceeded three (Fig. 1). The present study has shown that high dose levels do not necessarily improve the accuracy of E-DMI; indeed at lower dosing frequency, higher dose levels have a detrimental effect on the accuracy of E-DMI. Mayes & Dove (2000) had suggested that the ideal level of dosed alkane is that which produces similar faecal concentrations of both alkanes in the marker pair. Setting a dose level for a particular investigation requires knowledge of the likely level of intake of the animal, the approximate DMD of the diet and the concentration of odd-chain alkane in the herbage prior to the start of the study. In most cases, this information could be estimated from the live weight of the animal, *in vitro* determination of DMD and published values of herbage alkane concentrations.

A major source of variation in E-DMI is the daily fluctuation in faecal concentrations of dosed alkane. The two principal components that affect faecal concentrations of marker are variation in the flow of dosed alkane through the gut (caused by the intermittency of the dosing schedule and/or incomplete

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Table 3. *The herbage concentration (mg/kg DM) of selected alkanes with their faecal recovery (%) and faecal recovery relative to $C_{32}$ as measured in cattle, donkeys and horses during Studies I, II and III*

| Alkane | Study I | | Study II | Study III |
|---|---|---|---|---|
| Concentration in diet (mg/kg DM) | | | | |
| $C_{25}$ | 10·3 | | 10·5 | 42·3 |
| $C_{27}$ | 29·0 | | 16·0 | 72·4 |
| $C_{29}$ | 62·4 | | 64·7 | 125·0 |
| $C_{31}$ | 143·5 | | 115·6 | 188·1 |
| $C_{32}$ | 9·2 | | 6·6 | 14·9 |
| $C_{33}$ | 72·7 | | 38·4 | 61·7 |
| $C_{35}$ | 8·8 | | 9·0 | 9·0 |
| $C_{36}$ | 0·0 | | 0·0 | 3·8 |
| | Cattle | Donkeys | Donkeys | |
| Faecal recovery (%) | | | | |
| $C_{25}$ | 52·7 ± 7·01 | 76·0 ± 4·50 | 67·8 ± 1·96 | |
| $C_{27}$ | 73·0 ± 3·54 | 78·9 ± 2·75 | 78·3 ± 2·25 | |
| $C_{29}$ | 74·0 ± 3·59 | 78·1 ± 2·84 | 80·9 ± 2·03 | |
| $C_{31}$ | 86·6 ± 3·49 | 87·6 ± 3·31 | 77·0 ± 2·02 | |
| $C_{32}$ | 79·0 ± 4·08 | 86·1 ± 5·35 | 81·1 ± 3·30 | |
| $C_{33}$ | 92·1 ± 2·25 | 93·0 ± 3·83 | 72·6 ± 1·98 | |
| $C_{35}$ | 92·8 ± 6·88 | 95·0 ± 6·67 | 44·3 ± 1·25 | |
| $C_{36}$ | 88·8 ± 2·85 | 91·4 ± 3·81 | 0·0 | |
| | Cattle | Donkeys | Donkeys | Horses |
| Faecal recovery relative to $C_{32}$ | | | | |
| $C_{25}$ | 1·05 ± 0·113 | 1·23 ± 0·083 | 0·85 ± 0·029 | 1·03 ± 0·053 |
| $C_{27}$ | 1·31 ± 0·063 | 1·43 ± 0·088 | 0·97 ± 0·033 | 1·01 ± 0·053 |
| $C_{29}$ | 1·35 ± 0·067 | 1·44 ± 0·093 | 0·99 ± 0·038 | 0·95 ± 0·050 |
| $C_{31}$ | 1·00 ± 0·042 | 1·04 ± 0·067 | 0·95 ± 0·037 | 0·92 ± 0·054 |
| $C_{33}$ | 1·06 ± 0·032 | 1·09 ± 0·069 | 0·89 ± 0·031 | 0·87 ± 0·056 |
| $C_{35}$ | 1·06 ± 0·089 | 1·14 ± 0·089 | 0·54 ± 0·018 | 0·80 ± 0·047 |
| $C_{36}$ | 1·00 ± 0·000 | 1·09 ± 0·065 | 0·0 | 1·01 ± 0·057 |

mixing of the marker with gut contents) and differences in the daily faecal output of the animal. When E-DMI was based on five or more faecal sampling days and the marker was dosed at low levels or at least three times per day there was no significant difference between the CV of M-DMI and the faecal concentration of marker (Fig. 2), implying that much of the variation in the faecal concentration of the marker is explained by daily variation in faecal output. Increasing faecal sampling frequency improves the accuracy of faecal marker concentration measurement. In other published alkane studies with horses, faecal sampling occurred more frequently than in the present study or, a total faecal collection was carried out (O'Keefe & McMeniman 1998; Stefanon *et al.* 1999; Ordakowski *et al.* 2001; Stevens *et al.* 2002). Frequent sampling at pasture is problematic in practice, and the present study has shown that once-daily faecal sampling can produce accurate E-DMI determinations, provided that faecal samples are collected and pooled for a minimum of 5 days (Fig. 1).

The current study did not provide an unequivocal picture of the effect of alkane chain length on faecal recovery in equids. In Study I with donkeys, alkanes showed a similar pattern of recovery to the cattle in the same study (Table 3) and to that reported by Dove *et al.* (1989) and Casson *et al.* (1990) for sheep. In Studies II and III, faecal recoveries of the major odd-chain alkanes ($C_{27}$–$C_{33}$) were not affected to the same extent by carbon chain length (Table 3). The difference in recovery of alkanes between equids and ruminants has important practical ramifications. In ruminant intake studies it is important to select odd-chain alkanes that are adjacent to the dosed even-chain alkane when estimating DMI because faecal recoveries are most similar. In equids, this consideration appears less important because the faecal recoveries of odd-chain alkanes are very similar to one another, enabling a large number of alkane pairs to be used.

The results of the present study, together with those of previously published studies, indicate that the alkane pair technique provides a robust method of estimating DMI by equids, provided that adequate faecal samples are pooled over a period of at least 5 days. Twice-daily dosing of even-chain alkane at low

levels provided an accurate estimate of DMI, as did thrice-daily dosing. Higher dose levels given twice daily tended to produce large variations in faecal concentrations of dosed, even-chain alkanes; this variation was reduced when dosing frequency was increased to thrice daily. Faecal recoveries of alkanes in equids do not show the same influence of carbon chain length that is evident for ruminants.

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