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Evaluation of the n-alkane technique for estimating herbage dry matter intake of dairy cows offered herbage harvested at two different stages of growth in summer and autumn.

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Highlights:

- The n-alkane technique provided good estimates of herbage intake of individual dairy cows.
- The accuracy of the technique was not affected by herbage mass or season.
- The alkane pair C_{33}/C_{32} provided the most precise estimates of feed intake.

ABSTRACT

The n-alkane technique for estimating herbage dry matter intake (DMI) of dairy cows was investigated in this experiment. Eight Holstein-Friesian dairy cows were offered perennial ryegrass ad libitum that had been harvested at two different herbage masses and during two

different seasons, in order to assess the effect of herbage mass and season on the accuracy of the n-alkane technique. Two pre-harvested herbage mass treatments (low, target 1500 kg DM/ha versus high, target 4000 kg DM/ha, measured above 4 cm), were investigated in a crossover factorial arrangement within each of two seasons (summer versus autumn), in Ireland. Each season consisted of two periods, each 12 days in length. Cows were housed in individual metabolism stalls to allow for accurate determination of measured DMI. Herbage DMI was estimated, with the n-alkane technique, by dosing cows twice daily with a C_{32} n-alkane. Pre-harvest herbage mass and season did not affect the n-alkane estimated DMI, although lack of season and herbage mass effects may have been masked by variation that occurred between swards within the same herbage mass and season. However, there were a number of differences between summer and autumn in the fecal recovery rates of a number of n-alkanes suggesting that the effect of season requires further investigation prior to the application of recovery rates from literature values when investigating diet selection and botanical composition. Overall, the n-alkane technique provided good estimates of DMI; the discrepancy had a standard deviation due to sward of 1.2 and 1.0 kg DM/cow per day, and hence potential bias of up to twice this, and a measurement error standard deviation of 1.3 and 1.0 kg DM/cow per day, for the C_{33}/C_{32} and C_{31}/C_{32} n-alkane pair methods respectively. Two n-alkane pairs were tested, and C_{33}/C_{32} n-alkane provided the most precise estimates of DMI, compared with the C_{31}/C_{32} n-alkane pair. This research provides some strong evidence for future use of the n-alkane technique including that the accuracy of the technique has not been influenced by contemporary changes to herbage management, is not affected by seasonal changes, and overall is an accurate and precise technique for estimating DMI.

Key words: n-alkane, dry matter intake, dairy cow, pre-grazing herbage mass.

INTRODUCTION

Researchers have for many years faced the challenge of accurately measuring dry matter intake (DMI) of individual cows managed in pasture-based dairying systems. Determination of individual intake would allow the identification of cows that efficiently convert feed into milk, the effect of various herbage characteristics on intake and milk production, and would also enable herbage intake to be complemented more accurately with supplementary feeds (Dillon, 1993, Mayes and Dove, 2000). Given that the estimation of herbage intake in cows grazing in a herd is difficult, most measurements of intake are made as group averages from estimations of pre- and post-grazing pasture masses. However, group averages do not quantify the variation in intake that is likely to occur between cows within a given herd. Some techniques for measuring individual pasture DMI have been developed, but many disrupt normal grazing behavior or cannot be used when cows consume heterogeneous herbage swards. These techniques include determination of changes in bodyweight (BW), water intake methods, back calculation from milk production and BW and condition scores, sward difference measurements, feeding behavior methods, and indigestible marker methods (Dillon, 1993, Dove, 2010).

The n-alkane technique is an indigestible marker method that has become more commonly used in research as a result of the presence of n-alkanes in the cuticular wax of a range of plant species, ease of analysis and unique patterns of concentrations of alkanes in different species, allowing the estimation of diet selection in grazing animals (Dove, 1993). Additionally, this method does not require an independent assessment of digestibility (Dillon, 1993). Natural n-alkanes are long-chain hydrocarbons (C_{25}-C_{35}) found in components of plant cuticular wax, and odd-chain length n-alkanes are in greater quantities than even-chain length n-alkanes in herbage species (Dove and Mayes, 1991). The double n-alkane technique requires animals to be dosed with a synthetic even-chain length n-alkane, at a known amount, paired with a naturally occurring odd-chain length n-alkane that is present in the herbage consumed (Dove and Mayes, 1991). The best estimates of DMI are achieved using n-alkane pairs that differ by one carbon atom because of their similar fecal recovery rates. For instance,

the n-alkane pair C_{33}/C_{32} has been shown to be one of the most accurate n-alkane pairs to use for this reason (Dillon, 1993). Similarities of the n-alkane pair in terms of fecal recovery rates are essential for the accurate estimates of DMI. Importantly, feeding amount, concentrate supplementation, stage of lactation and feeding frequency do not impact recovery rates (Dillon, 1993) allowing their use under a variety of conditions. Previous studies have documented the accuracy of the n-alkane technique for estimating DMI (Mayes et al., 1986a, Mayes et al., 1986b, Dillon, 1993, Dove et al., 2002). For example, Dove and Mayes (1996) reviewed nine scientific articles evaluating the n-alkane technique in both cattle and sheep and reported that the largest discrepancy between known and estimated herbage DMI, at a group level, was 2.6%. Although the n-alkane technique was developed with the purpose of measuring average DMI of a group, the present research explores the potential to describe individual intake. While this is not the first research to use the n-alkane technique for determining individual intake, this research is novel in that it investigates the effect of herbage quality on estimates of individual intake.

A challenge with the use of synthetic, and natural herbage n-alkanes for accurately estimating herbage DM on modern dairy farms is that pasture management has changed since the technique was first published 30 years ago (Mayes et al., 1986a) and subsequently evaluated in dairy cows (Dillon, 1993). Since then, there have been changes to traditional pasture management, resulting in changes in the physical structure and quality of the herbage on offer to grazing cows. New pasture management guidelines suggest that *Lolium* herbage should be defoliated at the 3-leaf stage (Fulkerson and Donaghy, 2001), which often results in a reduction in the previously recommended pre-grazing herbage mass (Creighton et al., 2011) with traditional pasture management. The resulting change in leaf, true stem, pseudo-stem and dead material proportions could mean differences in the concentration and pattern of n-alkanes being consumed. Reduced concentrations of longer chain-length alkanes could impact the accuracy of this technique as longer chain-length alkanes have higher recovery rates. The concentrations of alkanes are of particular importance when determining diet fractions, these

measurements may be prone to measurement error when alkane concentrations are below 50mg (Brosh et al. 2003). For example, the concentration of odd-chain length n-alkanes present in the leaf component of *graminae* species is greater than the stem, due to increased cuticular wax alkane levels in leaf lamina (Dillon, 1993). Similarly, n-alkane concentrations may also differ with seasonal changes in pasture. It has been widely demonstrated that the nutritive characteristics of perennial ryegrass varies throughout the growing season (Jacobs et al., 1999). However, the accuracy and precision of the n-alkane technique in different seasons have not been investigated.

The objectives of this experiment were as follows: (1) to determine the accuracy and precision of estimating herbage DMI using the n-alkane technique at two herbage masses; and (2) to determine the accuracy and precision of estimating herbage DMI using the n-alkane technique in two seasons (defined in the materials and methods section).

MATERIALS AND METHODS

This experiment was conducted at Teagasc Moorepark Research Centre, Fermoy, Co. Cork, Ireland (52° 09'N; 8°16'W). All experimental procedures were carried out in accordance with European Union Directive 2010/63/EU and S.I. No. 543 of 2012. The experiment was conducted in late spring/early summer (April/May; termed summer) and in late summer/early autumn (July/August; termed autumn).

Animals and Experimental Design

This experiment was conducted concurrently with an experiment investigating the digestibility of the herbage mass treatments (Garry et al., 2015). In the current experiment a crossover design was used to investigate two pre-harvest herbage mass treatments (low and high) in each of two seasons (summer and autumn). The targeted pre-harvest herbage masses (measured from 4 cm above ground level) were 1500 kg DM/ha (low herbage mass) and 4000 kg DM/ha (high herbage mass). In each season, there were two consecutive periods of 12

days, which each included a 6-day adaptation period followed by a 6-day measurement period. Cows changed treatments after the first 12 d period. Four lactating cows were allocated to one of two groups. Groups were balanced for bodyweight (BW), milk yield, and milk solids yield (sum of fat and protein yield), within each season. The same cows were used in both seasons and cows averaged 26 ± 3.4 DIM (mean ± s.d.) at the start of the summer experiment. Each cow was weighed at the start of each season using electronic portable scales and the WinWeigh software package (Tru-test limited, Auckland, New Zealand). Before the experiment, all cows had *ad libitum* access to a predominantly perennial ryegrass (*Lolium perenne* L.) pasture, and also received 3 kg DM/cow per day of concentrate in the dairy during milking. During the experiment, all cows had access to the same forage a*d libitum* while housed in individual metabolism stalls, and were fitted with a fecal harness and urine separator. Cows had *ad libitum* access to water and each cow was provided with a salt block (Nutribio Mineral Salt Lick, Cork Ireland). No concentrates were fed during the experimental periods.

Milk production

Cows were milked twice daily, at around 0800 and 1600 h, with milk yields of individual cows recorded at each milking using an in-line milk metering system (Dairymaster, Causeway, Co. Kerry, Ireland). Four days per week during the measurement period of each period, representative milk samples were collected from consecutive p.m. and a.m. milkings. These were analyzed separately for concentrations of fat and protein using an infrared milk analyzer (Milkoscan 03; DK-3400, Foss Electric, Hillerød, Denmark). This information was used with milk yield at each milking to determine daily concentrations of fat and protein. Energy-corrected milk (ECM), standardized to 4.0% fat and 3.3% protein, was calculated using the formula of Tyrrell and Reid (1965):

ECM (kg/cow per day) = milk yield kg × (376 × fat% + 209 × protein % + 948) /3,138.

(Equation 1)

Determination of herbage mass and feeding

To determine pre-harvest herbage mass, herbage was cut to 4 cm twice a week using Gardena hand shears (Accu 60, Husqvarna AB, S561 82, Husqvarna, Sweden) within a 0.25 m^2 quadrat, in accordance with the procedure of O'Donovan et al. (2002). Pre- and post-cutting sward heights were determined daily using a rising plate meter (diameter 355 mm and weight 3.2 kg/m^2; Jenquip, Fielding, New Zealand). Herbage for each treatment was harvested each day at 0830 h using a Pottinger Nova cat 266 F mower (Alois Pottinger Maschinenfabrik GmBH, Grieskirchen, Germany) and transported using a Pottinger Europrofi 1 Euromatic self-loading forage wagon (Alois Pottinger Maschinenfabrik GmBH, Grieskirchen, Germany). Cows were offered 110% of the previous days DMI. Herbage was offered three times per day at 0830 h, 1600 h and 2100 h. Approximately half the daily allocation was fed in the morning and the other half was divided between the afternoon and evening feedings with herbage stored in a cool-room at 4°C prior to feeding.

Nutritive characteristics of herbage

Representative daily samples of each of herbage offered and refused were collected for each cow and then bulked for each herbage mass treatment, and stored immediately at -20°C, then subsequently freeze-dried. Following freeze drying, herbage samples were milled through a 1 mm screen using a Cyclotech 1093 Sample Mill. Samples were then composited per pre-harvest herbage mass treatment per period (6 days), within season, before being analyzed for nutritive characteristics. The offered and refused composited herbage samples were analyzed for ash, crude protein (CP) (Leco FP-528; Leco Corporation, St Joseph, MI, USA), and neutral detergent fiber (NDF) and acid detergent fiber (ADF) using the Ankom Fiber Analyzer (Ankom Technology Corporation, NY, USA) (AOAC International, 2000). Amylase and sulphite were used in the analysis of NDF, and ADF and NDF were expressed including ash on a DM basis. Actual herbage masses, cutting height and nutritive characteristics of the herbage are presented in Table 1.

Measured dry matter intake (DMI)

The quantity of herbage offered and refused was weighed and recorded at each feeding (three times a day) for each cow. Three representative 50 g samples of offered and refused herbage were collected three times per day, at each feeding, for each pre-harvest herbage mass treatment. These samples were oven-dried at 120°C for a minimum of 4 h using a Gallenkamp Hotbox oven (Thermo Fisher Scientific INC., Waltham, MA, USA) for DM determination. While DM samples for herbage refused was collected at each feeding, refused pasture was only removed once a day.

Estimation of dry matter intake (DMI) using the n-alkane technique

Dry matter intake was estimated for each cow from day 7-12 using the double n-alkane technique developed by Mayes et al. (1986a). For the duration of the experiment, cows were dosed twice daily (following morning and afternoon milkings) with paper pellets (Carl Roth GmbH and Co.KG, Karlesruhe, Germany) each containing 378 mg of dotriacontane (C_{32}). Dotriacontane was dissolved using heptane solvent, the solution was then pipetted onto the pellets, and then the pellets were left for the solvent to evaporate prior to oven drying. Two n-alkane pairs were investigated, where C_{32} was paired with either tritriacontane (C_{33}-alkane) or hentriacontane (C_{31}-alkane) as the herbage n-alkanes, to determine the most accurate n-alkane pair (C_{33}/C_{32} or C_{31}/C_{32}) for estimating individual intake when cows were offered the dietary treatments described in this research. Cows were fitted with fecal harnesses (custom made at Warragul Auto Interiors and Upholstery, Victoria, Australia) to enable the total collection of feces. Urine and feces were separated using urine separators (made at Agriculture Victoria's Ellinbank Centre, Victoria, Australia) attached to each cow. Feces from each cow was mixed thoroughly and representative a.m. and p.m. samples collected daily. The a.m. and p.m. fecal samples were composited per cow per day of the experiment. Fecal samples were stored at -20°C after collection. At the conclusion of each season, the daily fecal samples for each cow were defrosted and dried for 48 h at 40ºC, then analyzed for n-alkane concentrations. Daily herbage samples representative of the herbage offered were collected for each herbage mass

treatment for the analysis of n-alkane concentrations. Herbage samples were prepared using the same method as described for nutritive characteristics.

The n-alkane (pentacosane (C_{25}- alkane), hexacosane (C_{26}-alkane), heptacosane (C_{27}-alkane), octacosane (C_{28}-alkane), nonacosane (C_{29}-alkane), triacontane (C_{30}-alkane), C_{31}-alkane, C_{32}-alkane, C_{33}-alkane, and pentatriacontane (C_{35}-alkane)) concentrations in the feces and herbage were analyzed by gas chromatography (GC) using a modification of the method of Mayes et al. (1986b), which used direct saponification (Dillon, 1993). Peak areas were converted to amounts (mg/kg DM) of n-alkane by reference to the internal standard (tetratriacontane (C_{34}-alkane)). These n-alkanes were used to provide reserve n-alkanes if the concentrations of the selected n-alkanes for the calculations were present in minimal concentrations and also to enable the determination of recovery rates of each of these n-alkanes. The herbage DMI/cow per day was estimated using the following modified equation (Mayes et al. 1986):

$$\text{Intake (kg DM/cow per day)} = \frac{F_i D_j}{F_j H_i - F_i H_j}$$

(Equation 2)

Where F_i and H_i represent the concentrations (mg/kg DM) of odd-chain fecal and herbage n-alkanes respectively, and F_j and H_j are the respective concentrations (mg/kg DM) of even-chain length fecal and herbage n-alkanes. The daily dose (mg DM) of even-chain alkane (C_{32}) is represented by D_j. Fecal n-alkane recovery rates were also determined, using the equation below:

$$\text{Recovery rate} = (F_{n\text{-}alkane} \times \text{F DM output}) / (H_{n\text{-}alkane} \times \text{H DM intake})$$

(Equation 3)

Where $F_{n\text{-}alkane}$ and $H_{n\text{-}alkane}$ represent the concentrations (mg/kg DM) of an n-alkane in the feces (F) and herbage (H). Fecal (F) DM output and herbage (H) DMI expressed as kg DM/cow per day. Equation 3 was also used for the dosed n-alkane (C_{32}), however in this case the dose of the n-alkane was added to the denominator.

Statistical Analyzes

Measured DMI, fecal n-alkane concentrations, alkane estimated DMI, and milk production data, were each analyzed with a mixed model with factorial fixed effects for pre-harvest herbage mass by season (main effects, and interaction) and random effects for sward (the combination of herbage mass, season and period), cow within season, and residual (cow within period). Similarly, herbage n-alkane concentration data, and n-alkane recovery rates, were analyzed with a mixed model with factorial fixed effects for herbage mass by season (main effects, and interaction) and random effects for sward (the combination of herbage mass, season and period), and day within sward as residual. Effects of season and herbage mass on all herbage and fecal n-alkane concentrations were presented graphically on a single scale by re-expressing each mean as a percentage deviation from the grand mean of that herbage or fecal n-alkane. These were graphed with corresponding 5% least significant intervals, constructed so that non-overlap of interval indicates a significant difference at 5% level.

Average daily DMI were calculated using each n-alkane pair C_{32} with C_{31} or C_{33} (from this point on the pairs will be labelled according to the natural n-alkane), for the 8 cows in each of the two periods. These data and measured DMI (n=32) were used to calculate Lin's concordance correlation coefficients between the measured and estimated DMI. Lin's concordance correlation is a measure of agreement that is the product between a bias factor, β, and a Pearson correlation coefficient, ρ. The bias factor measures systematic deviation in either mean or slope from the line of agreement between estimated and measured DMI, with a value of unity for perfect agreement. Pearson correlation coefficient, ρ, measures linearity and decreases with variance and/or non-linearity.

The same data were used to examine factors associated with discrepancies between the measured and estimated DMI. The differences, n-alkane estimated DMI minus the measured DMI, were calculated and analyzed using two mixed-effects models. The first model was designed to test effects of herbage mass and season on the discrepancy, using sward as the experimental unit. This model had fixed effects that were factorial in herbage mass by season

(main effects and interaction), and random effects for sward, cow within season, and cow within period within season. The second model was designed to test the effects of sward against experimental units for cow within season and cow within period. This model had fixed factorial effects for period-nested-within-season, by herbage mass, and random effects for cow within season, and cow within period within season. The fixed effects of these models estimate bias, and the random effects estimate imprecision. The mixed model analyses were performed using REML in GenStat software for Windows (Genstat release 18; VSN International Ltd., Hemel Hempstead, UK). A further bivariate analysis of the differences between DMI measured by the n-alkane methods C_{31} and C_{33}, and measured DMI was used to estimate the magnitude of biases due to sward, expressed as a component of variance, and to measure correlations between sward effects, and correlation between measurement errors, for the two n-alkane methods. For this the discrepancy (D) between n-alkane method (Y_{31} or Y_{33}) and measured DMI (Y_A) was modelled using the following mixed effects, bivariate, model:

$$D_{31} = Y_{31} - Y_A = \alpha_{31} + \beta_{31} + \varepsilon_{31}, \text{ and } D_{33} = Y_{33} - Y_A = \alpha_{33} + \beta_{33} + \varepsilon_{33}. \qquad \text{(Equation 4)}$$

where $\alpha = (\alpha_{31}, \alpha_{33})^{\mathrm{T}}$ was the fixed effect representing the mean bias of each n-alkane method, and $\beta = (\beta_{31}, \beta_{33})^{\mathrm{T}}$ and $\varepsilon = (\varepsilon_{31}, \varepsilon_{33})^{\mathrm{T}}$ were random effects for sward and measurement error, with unstructured covariances,

$$\mathrm{Cov}(\beta) = \begin{bmatrix} \varphi_{31}^2 & \varphi_{31,33} \\ \varphi_{31,33} & \varphi_{33}^2 \end{bmatrix} \text{ and } \mathrm{Cov}(\varepsilon) = \begin{bmatrix} \sigma_{31}^2 & \sigma_{31,33} \\ \sigma_{31,33} & \sigma_{33}^2 \end{bmatrix}, \text{ respectively.}$$

Correlations of these random effects between n-alkane methods were calculated as

$$\rho_\beta = \frac{\varphi_{31,33}}{\varphi_{31}\varphi_{33}} \text{ and } \rho_\varepsilon = \frac{\sigma_{31,33}}{\sigma_{31}\sigma_{33}}.$$

Equation 4 was fitted using ASREML software in R, as a bivariate mixed model. Fixed effects were tested using Wald tests, and random effects and covariance were tested by likelihood ratio tests between nested models.

RESULTS

Dry matter intake

Measured and estimated DMI are presented in Table 2, demonstrating that there was a season by herbage mass interaction on measured DMI (P = 0.026). There were higher DM intakes with the low pre-harvest herbage mass compared with the high pre-harvest herbage mass in autumn (16.9 and 14.3 kg DM/cow per day, respectively). In contrast there was no difference between the two herbage masses in summer (14.6 and 14.7 kg DM/cow per day, respectively). Season and herbage mass did not influence intakes estimated with the n-alkane technique with either n-alkane pair (C_{31} and C_{33}).

Accuracy and precision of the n-alkane technique in estimating herbage DMI

Lin's concordance correlation coefficients contain measures of accuracy, with a bias correction factor, and precision, using Pearson correlation coefficient. Lin's concordance correlation coefficient for the relationship between measured and estimated herbage DMI was 0.69, (95% confidence interval 0.46 to 0.84, bias factor 0.99) for the C_{31} pair method, and 0.74 (95% confidence interval 0.53 to 0.87, bias factor 1.00) for the C_{33} pair method. The Lin's concordance correlation coefficient between the two n-alkane methods was 0.97 (95% confidence interval 0.94 to 0.98, bias factor 1.00). The relationships between the two n-alkane pairs estimates of DMI and measured DMI are presented in Figure 1 and the relationship between the two n-alkane pairs estimates of intake are presented in Figure 2.

There were no significant effects of herbage mass, season, nor their interaction, on the discrepancy between n-alkane measured and estimated DMI, for either pair method when these were tested against variance between swards. However, there were significant sward (the combination of herbage mass by season by period) effects on the discrepancy, for C_{31} (P =0.002), and for C_{33} (P < 0.001), relative to cow and within-sward components of variance (Figure 3).

The bivariate analysis of the discrepancy between measured and n-alkane estimated DMI, for C_{33} and C_{31}, showed that the overall bias, α , was not significantly different to zero (P = 0.67), nor mean bias differ significantly between the two n-alkane pairs (bias ± SE (kg/day), 0.25 ± 0.16 C_{31}/C_{32}, and 0.16 ± 0.14, C_{33}/C_{32} (P = 0.41). The sward standard deviation (1.20 and 1.01 kg DM/cow per day for C_{31} and C_{33}, respectively) were not significantly different (P = 0.19), but they were significantly greater than zero (P < 0.001). The sward random effects were highly correlated between the two n-alkane methods ($\rho_\beta = 0.96$, P = 0.004). The measurement error standard deviations for the two methods (1.27 and 1.03 kg DM/cow per day for C_{31}/C_{32} and C_{33}/C_{32}, respectively) were significantly different from each other (P < 0.001), and the measurement errors were highly correlated between the two n-alkane methods ($\rho_\varepsilon = 0.98$, P < 0.001).

Herbage and fecal n-alkanes

The effects of herbage mass and season on herbage and fecal n-alkane (C_{25}-C_{35}) concentrations are presented in Figure 4. In herbage, the concentration of C_{31} alkane was higher in the high herbage mass treatment in autumn. In feces, the low herbage mass treatment had higher fecal concentrations of C_{28} and C_{30}. Season influenced a number of herbage and fecal n-alkane concentrations including an increase in herbage C_{25} and C_{27}, and a reduction in C_{26}, C_{28}, C_{30}, C_{31}, C_{32}, C_{33}, and C_{35}, in summer compared with autumn. The fecal n-alkane concentrations of C_{25}, C_{27}, C_{29}, and C_{32} were higher in summer compared with autumn but fecal concentrations of C_{26}, and C_{35} were lower in summer than in autumn, as with C_{33} however, only for the high herbage mass treatment.

Fecal n-alkane recovery rates

Fecal recovery rates of various n-alkanes (C_{25}-C_{35}) were investigated to determine the influence of season and herbage mass on recovery rates (Table 3). The fecal n-alkane recovery rates of C_{31}, C_{32}, and C_{33} were not influenced by herbage mass or season, and were

similar 0.87, 0.86, and 0.86, respectively. Herbage mass alone did not influence the recovery rates of n-alkanes (C_{25}-C_{35}). In summer, the recovery rate (0.64) of C_{25} was lower than autumn (0.83). However, fecal n-alkane recovery rates were higher in summer compared with autumn for two even-chain length alkanes C_{28} (1.06 versus 0.69 for summer and autumn, respectively) and C_{30} (0.78 versus 0.68). There was a herbage mass by season interaction for the recovery rate of C_{35}.

Milk production

Mean milk, ECM, fat, and protein yields, and concentrations of milk fat and protein for cows offered different pre-harvest herbage masses are presented in Table 4. Milk production variables were not affected by pre-harvest herbage mass. Yields of milk, ECM, fat, and protein and the concentration of fat were higher in summer than in autumn.

Discussion

Pre-harvested herbage mass did not affect the accuracy or the precision of the n-alkane technique. The effect of herbage mass on the accuracy and precision of the n-alkane technique has not been previously published, but Dillon (1993) found that high quality herbage (higher metabolizable energy, crude protein, and lower fiber concentration) had higher concentrations of all n-alkanes (C_{27}-C_{35}) due to a higher proportion of leaf versus stem in the high quality herbage. However, in the current experiment, C_{31} was the only alkane that occurred in different concentrations as a result of the pre-harvest herbage mass treatments (low 1,800 versus high 4,200 kg DM/ ha). Surprisingly, this n-alkane was lower in concentration in the low herbage mass treatment compared with the high herbage mass treatment despite the low herbage mass sward having higher CP concentration and lower NDF and ADF concentrations. In addition, a concurrent experiment by Garry et al. (2015), demonstrated that the low herbage mass had an increased proportion of leaf (65% versus 45%) and a reduced proportion of stem (6% versus 23%) compared with the high herbage mass. Overall, differences in herbage mass did not influence the ability of the n-alkane technique to estimate DMI possibly as a result of minimal differences in n-alkane concentrations between the herbage mass treatments.

There was no significant effect of season on the accuracy of the n-alkane technique. Although season did not influence the accuracy of the n-alkane technique in terms of the agreement between measured and estimated DMI, the current experiment did show differences in the n-alkane herbage profiles between seasons. The concentrations of several herbage n-alkane concentrations were lower in summer, when compared with autumn. This was presumably the result of the altered leaf to stem ratio reported by Garry et al. (2015), where the summer herbage had a lower leaf proportion than autumn herbage. In *graminae* species higher concentrations of n-alkanes are found in the leaf component compared with the stem component, because leaf lamina have higher cuticular wax concentrations (Dillon, 1993). Longer chain length alkanes (C_{31}-C_{35}) are associated with the leaf fraction of the plant and shorter chain length n-alkanes are associated with the stem fraction (Dillon, 1993) which is supported by the higher concentrations of herbage C_{25} and C_{27} in summer in the current experiment. The effects of season on the concentrations of n-alkanes have not been extensively investigated. In the current experiment, fecal n-alkane concentrations differed between summer and autumn. Interestingly, while concentrations of C_{31} and C_{32} were lower in summer herbage compared with autumn, concentrations of C_{31} and C_{32} were greater in the feces in summer compared with autumn; the reason for this finding is uncertain.

The variability in the recovery rates of various n-alkanes with season emphasizes the requirement for controlled studies to determine fecal n-alkane recovery rates under various conditions prior to the application of the n-alkane technique in field-based research. The recovery rates of synthetic and natural n-alkanes must be the same when estimating DMI with the double n-alkane technique, and minimal difference between calculated and actual recovery rates is very important when using the n-alkane technique to estimate diet composition, where more n-alkanes are required than the number of plant species present (Dove and Mayes, 1991).

Overall, the n-alkane technique provided good estimates of DMI when cows were fed *ad libitum* herbage. These estimates were accurate but imprecise, however this imprecision was within an acceptable margin (on average less than 5%). The double n-alkane technique provided estimates of

herbage DMI that were, on average, unbiased when either of C_{33} or C_{31} was used as the herbage n-alkane in combination with C_{32}, as the dosed n-alkane. The two n-alkane pair estimates of DMI showed no systematic differences associated with season, nor with herbage mass, between the estimates of DMI with the n-alkane technique and measured DMI in cows. In addition, there was little difference between the intake estimates with the two n-alkane pairs having very high Lin's concordance of 0.97. Concordance for each method with measured DMI, however, were moderate (0.69 for C_{31} and 0.74 for C_{33}) despite both having negligible overall bias and bias factors of 0.99 or 1.00, for C_{31} and C_{33}, respectively. Other research has demonstrated the accuracy of the n-alkane technique when comparing intake estimates derived with other techniques. Pérez-Ramírez et al. (2012) compared the n-alkane technique to ytterbium/fecal index for estimating the herbage intake of dairy cows fed herbage and maize silage and found that the n-alkane technique estimated intake more accurately than the ytterbium method and the authors of this study recommended the n-alkane technique method for estimating herbage intake. Malossini et al. (1996) compared the n-alkane technique to the chromic oxide method for estimating the herbage intake of grazing dairy cows and found the capacity of both techniques to estimate intake was similar. Wright (2017) collected data from 26 published scientific papers and found that the mean difference between measured and estimated DMI with the n-alkane technique was ~0.23 kg DM /animal per day or 6.1%.

The current research found that the inability to detect systematic effects of season and/or herbage mass on their bias, could be traced to two sources of error. Firstly, precision depended on sampling of herbage sward. The experiment included eight different herbage swards and these affected the discrepancy between estimates and measured DMI significantly. These sward differences had estimated standard deviations of 1.12 and 1.01 kg DMI/cow per day for C_{31} and C_{33}, respectively (Figure 3).

The second source of imprecision is the measurement error due to cows and associated with fecal sampling within swards. This measurement error had standard deviations of 1.24 and 1.03 kg DMI/cow per day for C_{31} and C_{33}, respectively. These were of a similar magnitude to those of sward,

and their effects are similarly concerning to the method if its purpose is to estimate DMI for individual cows, though less concerning if the purpose is to estimate group DMI.

The C_{33} n-alkane pair was more accurate than the C_{31} pair, showing slightly smaller components of variance for both sward and measurement error, and a somewhat higher concordance with measured DMI. Therefore, this experiment is in agreement with the recommendation of Dove and Mayes (1991), that when using the double n-alkane technique, the preferred n-alkane pair is C_{33}/C_{32} because of similar fecal recovery rates. The accuracy of the DMI estimates from the two n-alkane pairs depends on the agreement between the fecal n-alkane recovery rates for the dosed n-alkane, C_{32} and herbage n-alkanes, C_{33} and C_{31}. However, when concentrations of C_{33} are low in the herbage, C_{31} can be used as the herbage n-alkane for DMI estimation, with a possible slight reduction in the intake estimation accuracy (Laredo et al., 1991). The recovery rates of C_{32} and C_{33} in the current research were the same, 0.86 while the recovery rate of C_{31} was 0.87. The marginally higher recovery rate of C_{31} explains the larger difference between the n-alkane estimate of DMI with C_{31}/C_{32} and measured DMI, compared with the DMI estimated with C_{33}/C_{32}. It is well understood that fecal n-alkane recoveries are incomplete but when using the double n-alkane method for DMI estimation, incomplete recovery is accounted for in the calculations as long as the recovery rate of the dosed and herbage n-alkanes are similar (Mayes et al., 1986a). As mentioned, in the current experiment, the C_{31}/C_{32} n-alkane pair had slightly higher sward and measurement variances.

The strong correlation between the two n-alkane pair estimates of DMI, whether measured by Lin's concordance correlation coefficient (0.97), and apparent in Figure 2, was expected considering n-alkane concentrations were derived from the same samples. Both methods incorporated herbage n-alkane concentrations from the same herbage samples, and fecal concentrations from the same fecal samples and cows within periods. Likewise, discrepancies between estimated and measured DMI were associated with this sampling structure. The size and direction of the difference between estimated and measured DMI depended on sward. The standard deviation for these differences was just over 1 kg DMI/cow per day, which was of a similar order of magnitude to the difference

associated with measurement error associated with cow. This was the case of both n-alkane pairs and these were highly correlated both in sward effects (correlation 0.96) and measurement error (correlation 0.98). The variance components were similar for sward deviations as for measurement error, for the estimates with each of the n-alkane pairs. The similar magnitude of the deviations and the high correlation of the deviations between the two n-alkane pairs are apparent in Figure 3 for sward effects. The sward variances were not significantly different between the two n-alkane pairs. However, the measurement error variances were significantly different between the two n-alkane pairs, C_{31}/C_{32} being slightly more variable. The sward differences on the discrepancies between n-alkane estimated and measured DMI are not easily explained. They may be a result of sampling or laboratory error. It is also important to consider that the measured DMI are not without error as they rely on subsampling to determine DM content, and there are also errors associated with not collecting offered and ort DM contents of the feeds presented to each individual cow.

CONCLUSIONS

This experiment applied the n-alkane technique to a herbage-only feeding system to determine the accuracy and the precision of the technique when pre-harvested herbage-mass differed, and in different seasons. At the two pre-harvested herbage masses investigated (1770 versus 4220 kg DM/ha), herbage mass did not influence the ability of the n-alkane technique to estimate DMI. Change in season did not affect the accuracy of the n-alkane technique. However, herbage mass and season effects may have been masked by sizable between sward variation, at the same pre-harvest herbage mass and in the same season. Nevertheless, there were differences in the fecal recovery rates of a number of n-alkanes between summer and autumn, suggesting that the effect of season (stage of growth) requires further investigation prior to the application of previously published fecal recovery rates in the determination of diet selection. Overall, the n-alkane technique provided a good estimate of DMI when cows were fed *ad libitum* herbage, with concordance estimates demonstrating accuracy but slight imprecision. Estimates based on the C_{33}/C_{32} pair provided a more precise estimate of herbage DMI compared with using C_{31}/C_{32}. This experiment was conducted when herbage-only was

provided and therefore there is a requirement for future research to evaluate the n-alkane technique in more complicated feeding systems where supplementary feeds are offered.

Declarations of interest: none

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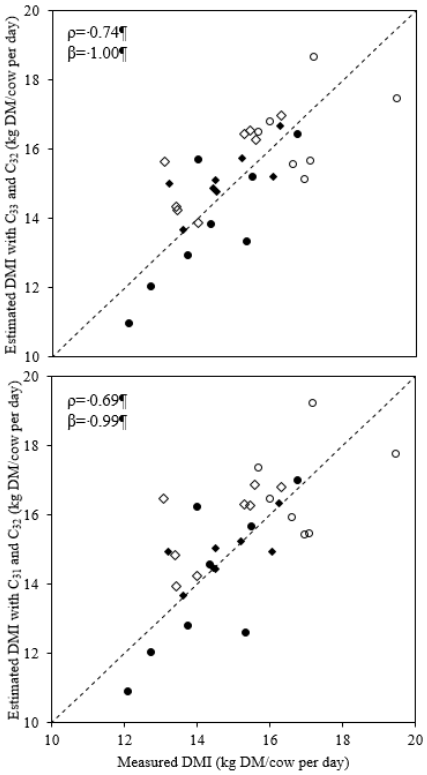

Figure 1: Measured and estimated dry matter intakes (DMI) using the n-alkane technique with herbage alkane C_{33} and dosed alkane C_{32}, and herbage alkane C_{31} and dosed alkane C_{32}, when cows were offered different pre-harvested herbage mass treatments in summer and autumn. Low herbage mass in summer (◊); high herbage mass in summer (♦); low herbage mass in autumn (○); and high herbage mass in autumn (●). The β represents the bias correction factor and ρ represents the Pearson correlation coefficient.

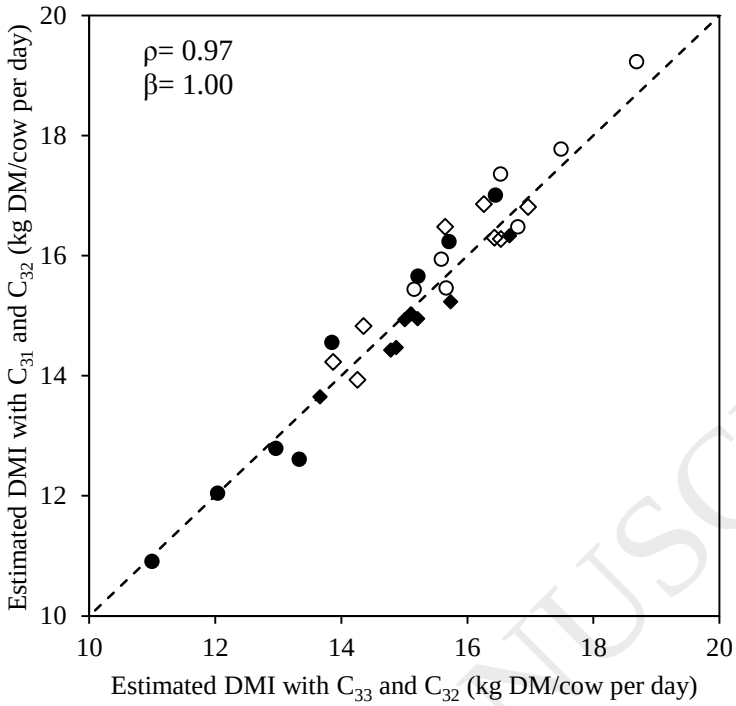

Figure 2: Estimated dry matter intakes (DMI) using the n-alkane technique with herbage alkane C_{33} and dosed alkane C_{32}, and herbage alkane C_{31} and dosed alkane C_{32}, when cows were offered different pre-harvested herbage mass treatments in summer and autumn. Low herbage mass in summer (◊); high herbage mass in summer (♦); low herbage mass in autumn (○); and high herbage mass in autumn (●).

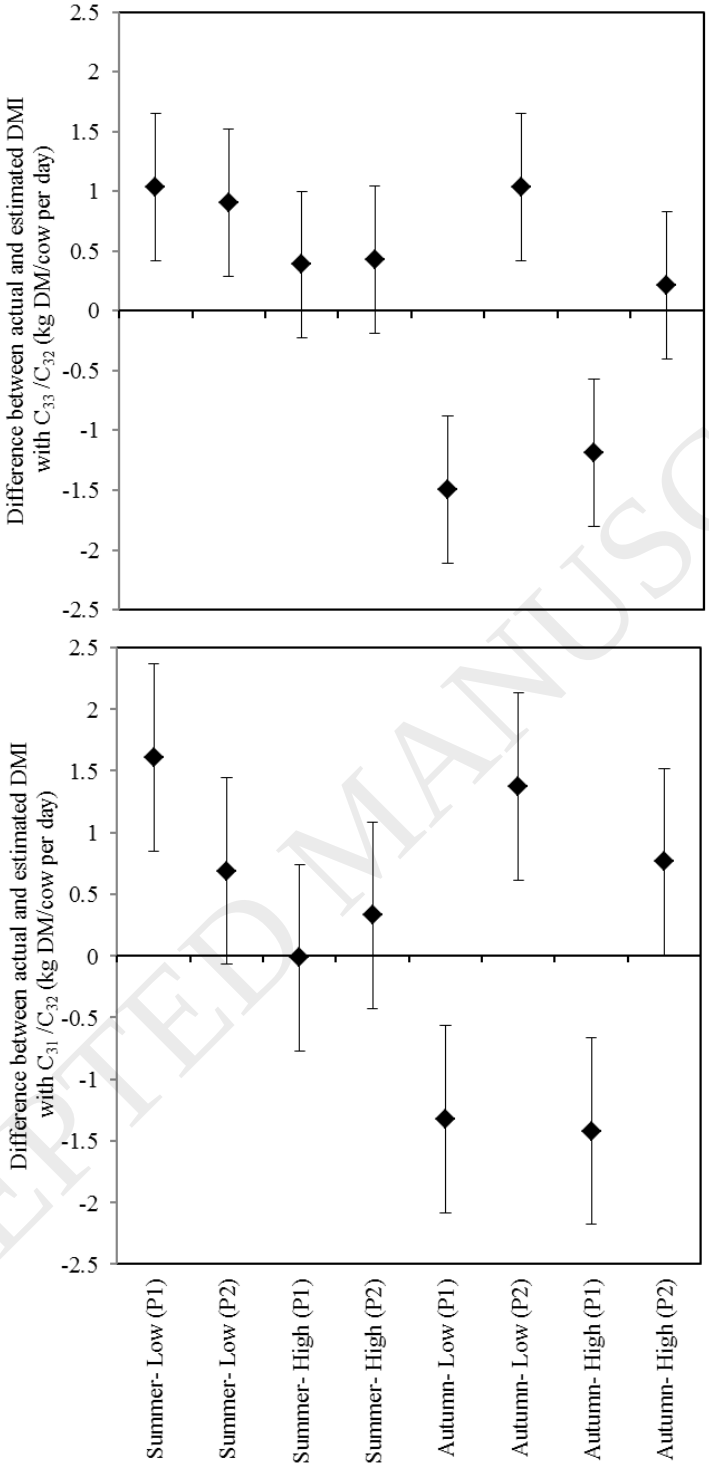

Figure 3: Differences in dry matter intake (DMI) between estimated DMI with two n-alkane pairs and measured DMI, when cows were offered different pre-harvested herbage mass treatments in summer and autumn. Low and high represent the herbage mass treatments

during two periods (period 1; P1, and period 2; P2; summer and autumn, respectively). Error bars represent 95% confidence intervals.

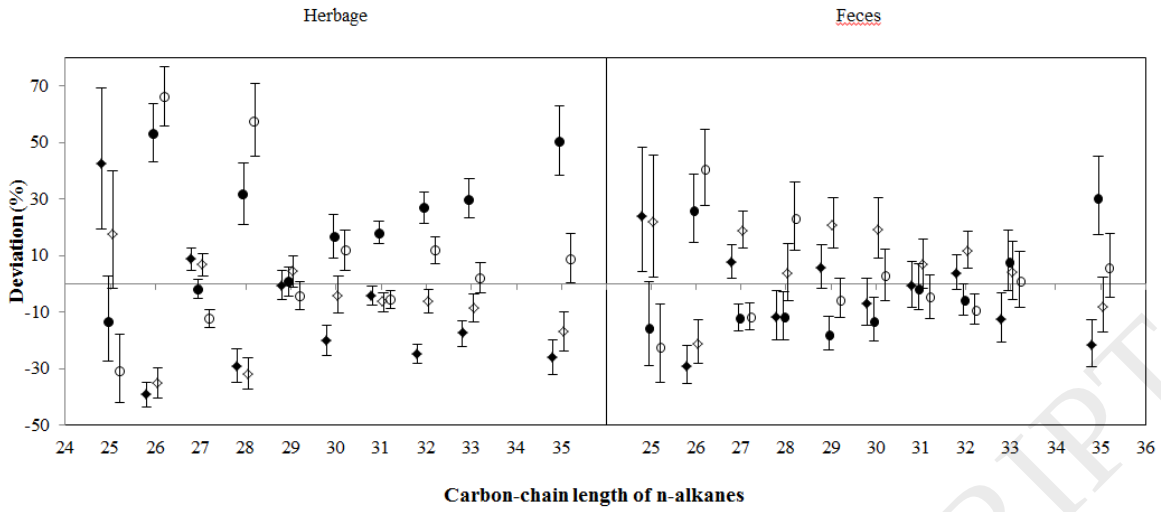

Figure 4: Deviation from grand geometric mean (%) of various n-alkane (C_{25}-C_{35}) concentrations in herbage offered and feces, when cows were offered low and high pre-harvested herbage mass treatments, in summer and autumn. Low pre-harvested herbage mass in summer (◊); high pre-harvested herbage mass in summer (♦); low pre-harvested herbage mass in autumn (○); and high pre-harvested herbage mass in autumn (●). Points at various chain lengths have been separated horizontally to ensure that overlapping data points are visible. Error bars represent least significant differences, 5% level of significance.

Table 1: Pre-harvest herbage mass[1], pre-cutting sward height[1], and nutritive characteristics[2] of herbage, in summer and autumn, when cows were offered two pre-harvested herbage masses (measured above 4 cm).

Pre-harvest herbage mass treatment	Pre-harvest herbage mass (kg DM/ha)	Pre-cutting sward height (cm)	Ash (g/kg DM)	Neutral detergent fiber (g/kg DM)	Acid detergent fiber (g/kg DM)	Crude Protein (g/kg DM)
Summer						
Low	1800	14.4	78	494	266	180
High	4110	22.1	84	516	312	162
Autumn						
Low	1740	13.9	76	437	264	173
High	4330	24.3	84	454	301	162

[1] Data are means of 6 consecutive days for each season and period.

[2] Data are from one composite sample per treatment per period.

Table 2: Measured and estimated dry matter intakes (DMI) (kg DM/cow per day) in summer and autumn, when cows were offered two pre-harvested herbage masses. Estimated DMI determined with the n-alkane technique using either alkane pair C_{33} and C_{32}, or C_{31} and C_{32}.

[1]SED= standard error of the difference.

	Summer		Autumn			SED[1]			*P*-value	
	Low herbage mass	High herbage mass	Low herbage mass	High herbage mass		Within Season	Between Season	Season	Herbage mass	Season × Herbage mass
Measured DMI	14.6	14.7	16.9	14.3		0.23	0.60	0.194	0.042	0.026
Estimated DMI with C_{33} and C_{32}	15.5	15.1	16.7	13.8		1.27	1.31	0.929	0.148	0.247
Estimated DMI with C_{31} and C_{32}	15.7	14.9	16.9	14.0		1.48	1.50	0.892	0.147	0.368

Table 3: Fecal recovery rate coefficients of n-alkanes (C_{25}-C_{35}), when cows were offered low and high pre-harvest herbage masses, in summer and autumn.

	Summer		Autumn					*P*-value		
n-alkane	Low herbage mass	High herbage mass	Low herbage mass	High herbage mass		SED[1]		Season	Herbage mass	Season × Herbage mass
C_{25}	0.66	0.62	0.84	0.82		0.039		0.004	0.386	0.809
C_{26}	0.62	0.88	0.72	0.73		0.089		0.721	0.104	0.138
C_{27}	0.85	0.84	0.95	0.93		0.052		0.062	0.762	0.793
C_{28}	1.10	1.02	0.71	0.67		0.059		0.001	0.209	0.585
C_{29}	0.77	0.79	0.82	0.75		0.041		0.864	0.432	0.167
C_{30}	0.76	0.80	0.71	0.64		0.048		0.042	0.702	0.160
C_{31}	0.84	0.86	0.93	0.84		0.042		0.265	0.303	0.164
C_{32}	0.80	0.86	0.90	0.88		0.071		0.310	0.729	0.425
C_{33}	0.83	0.87	0.92	0.83		0.036		0.355	0.400	0.074
C_{35}	0.83	0.88	0.93	0.87		0.028		0.086	0.920	0.040

[1]SED= Average standard error of the difference. The within and between season SEDs were very similar.

Table 4: Mesan daily yields of milk (kg/cow per day) and energy corrected milk (ECM) (kg/cow per day), and concentrations (%) and yields (kg/cow per day) of milk protein and fat, for cows fed different pre-harvested herbage masses during summer and autumn[1].

	Summer		Autumn			*P*-value	
	Low herbage mass	High herbage mass	Low herbage mass	High herbage mass	SED[2]	Season	Herbage mass
Milk yield	26.5	25.8	19.9	14.9	2.84	0.008	0.221
ECM	31.2	27.8	21.2	15.5	3.94	0.015	0.174
Fat concentration	5.74	5.03	4.54	4.41	0.421	0.047	0.235
Protein concentration	3.05	2.98	3.32	3.21	0.149	0.077	0.388
Fat yield	1.53	1.30	0.90	0.65	0.242	0.021	0.239
Protein yield	0.80	0.77	0.66	0.48	0.101	0.033	0.200

[1]No interactions were significant and therefore are not presented.

[2] SED= Average standard error of the difference. The within and between season SEDs were very similar.