

# Journal Pre-proof

Evaluation of the n-alkane technique for estimating the individual intake of dairy cows consuming diets containing herbage and a partial mixed ration

M.M. Wright (Conceptualization) (Methodology) (Investigation) (Writing - original draft) (Visualization) (Project administration), M.J. Auldish (Conceptualization) (Methodology) (Supervision) (Writing - review and editing), E. Kennedy (Conceptualization) (Methodology) (Resources) (Supervision) (Writing - review and editing), F.R. Dunshea (Conceptualization) (Methodology) (Supervision) (Writing - review and editing), N. Galvin (Resources), M.C. Hannah (Formal analysis) (Validation), W.J. Wales (Conceptualization) (Methodology) (Supervision) (Writing - review and editing)



PII: S0377-8401(20)30428-4

DOI: <https://doi.org/10.1016/j.anifeedsci.2020.114524>

Reference: ANIFEE 114524

To appear in: *Animal Feed Science and Technology*

Received Date: 5 November 2019

Revised Date: 5 April 2020

Accepted Date: 21 April 2020

Please cite this article as: { doi: <https://doi.org/>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier.

**Evaluation of the n-alkane technique for estimating the individual intake of dairy cows consuming diets containing herbage and a partial mixed ration.**

**M. M. Wright <sup>\*†</sup>, M. J. Auldist <sup>\*</sup>, E. Kennedy <sup>‡</sup>, F. R. Dunshea <sup>†</sup>, N. Galvin<sup>‡</sup>, M.C. Hannah <sup>\*</sup>, and W. J. Wales <sup>\*</sup>**

<sup>\*</sup> Agriculture Victoria, Department of Jobs, Precincts and Regions, Ellinbank, Victoria 3821, Australia.

<sup>†</sup> Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC 3010, Australia.

<sup>‡</sup> Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland.

Corresponding author: marlie.wright@agriculture.vic.gov.au

**Highlights:**

- The n-alkane method accurately measured intake of cows fed both herbage and a partial mixed ration
- Discrepancies between estimated and measured intakes were 5.1% or less
- Accuracy in estimating herbage DMI was slightly better when less herbage was fed

**Highlights:**

- The n-alkane method accurately measured intake of cows fed both herbage and a partial mixed ration
- Discrepancies between estimated and measured intakes were 5.1% or less
- Accuracy in estimating herbage DMI was slightly better when less herbage was fed

**ABSTRACT**

Estimation of dry matter intake (DMI) using the n-alkane technique was evaluated in lactating dairy cows fed fresh herbage and a partial mixed ration (PMR). Four dietary treatments were investigated in a 2×2 factorial experiment using 16 Holstein-Friesian dairy cows. Dietary

treatments were combinations of low and high amounts of fresh herbage (8 or 14 kg of DM/cow per day) and PMR supplement (6 or 12 kg of DM/cow per day). The pre-experimental period was 14 days followed by a 10-day experimental period. Cows were housed in individual metabolism stalls to allow for accurate measurement of DMI and total fecal output. Fecal n-alkane recovery rates were calculated to determine the most accurate corrections for incomplete fecal n-alkane recovery. The n-alkane technique accurately estimated DMI when corrected for incomplete fecal recovery using both published recovery rates and recovery rates calculated in this experiment. The most accurate application of recovery rates was with those calculated for each combination of dietary treatments, compared with using an average recovery rate. This research has important implications for the future use of the n-alkane technique, especially in PMR feeding systems. The discrepancy between estimated (when treatment recovery rates were applied) and measured herbage DMI increased with the amount of herbage offered but was not affected by amount of PMR. It was also found that the recovery rates of all natural n-alkanes increased as the amount of herbage increased. This research demonstrates that the n-alkane technique can be used to accurately estimate individual cow intake when fresh herbage and PMR are offered separately, evidenced by strong Lin's concordance estimates.

**Keywords:** dry matter intake; n-alkane; herbage; partial mixed ration; dairy cows.

## INTRODUCTION

Researchers and farmers alike continue to be challenged by the need to accurately measure individual dry matter intake (DMI) of livestock in pasture-based production systems. In dairy cows, energy intake often limits milk production in high producing cows. Energy intake is defined as the product of feed DMI and the metabolizable energy (ME) concentration of that feed (Allen, 2000). Consequently, the estimation of DMI is important in assessing the efficiency of the conversion of DM into milk in dairy cows (Vazquez and Smith, 2000). Knowledge of pasture DMI is also essential for formulating supplementary rations that complement the nutrients received from pasture. Estimating DMI in grazing systems is problematic as herbage intake varies substantially at both a herd and an individual cow level (Smit et al., 2005, Wright et al., 2016, Wright, 2017). A number of techniques have been developed including the use of indigestible markers, sward difference measurements, reversed feeding standards, water intake methods, feeding behavior measurements and, more recently, the development of motion sensors (Langlands and Donald, 1978, Dillon, 1993, Mayes and Dove, 2000, Dove, 2010, Dutta et al., 2014). However, many of these techniques disrupt normal grazing behavior and cannot be used when cows are consuming heterogeneous herbage swards.

An indigestible marker method called the n-alkane technique has become a common method used in research for estimating individual intake in cattle. It is commonly used because of the presence of various n-alkanes in plant species, its ease of analysis, ability to determine diet selection in grazing animals and its accuracy when estimating intake (Mayes et al., 1986, Dove, 1993, Wright et al., 2019). Alkanes are saturated aliphatic hydrocarbons, present in various chain lengths ( $C_{21}$  to  $C_{37}$ ) in the cuticular wax component of plant species, and are mostly indigestible to ruminant species (Mayes et al., 1986). The n-alkane technique can be used to estimate DMI and diet composition from the concentrations of n-alkanes of ingested feed and feces excreted, and is unique in that it estimates digestibility in individual animals, allowing for a true estimation of individual DMI (Dove and Mayes, 1991, Dove and Mayes, 1996, Dove, 2010). Alkanes found in plant species are predominantly odd-chain length and can be used, in combination, with orally dosed synthetic even-chain length alkane(s) to obtain estimates of individual intakes (Dove and Mayes, 1996). If using a single natural n-alkane and a synthetic n-alkane that is similar in carbon-chain length then a correction for incomplete fecal recovery is not required. However, when using the n-alkane technique to estimate diet composition using the differences in the concentrations of n-alkanes in various plant species, the various n-alkanes must be corrected for incomplete fecal recovery. Generally, n-alkane recovery rates increase curvilinearly with increasing n-alkane chain length and may be affected by the composition of the diet (Dove and Mayes, 2005, Dove et al., 2010).

It has been demonstrated that estimates of DMI using the n-alkane technique are most accurate when groups of animals are consuming herbage monocultures. Dove and Mayes (1996) reviewed nine scientific articles evaluating the n-alkane technique in both cattle and sheep and reported that the average discrepancies between known and estimated herbage DMI, at a group level, ranged from -2.60% to 2.57%. More recently, the n-alkane technique was validated in a herbage-only feeding system, and it was reported that the n-alkane technique accurately estimated herbage DMI and that its accuracy was not influenced by changes in herbage nutritive characteristics induced by changes pre-harvested herbage mass and season (Wright et al., 2019). Despite the success of the n-alkane technique in homogenous herbage swards, there is negligible research evaluating the use of this technique for the determination of DMI of grazing dairy cows offered more complex diets, where herbage is supplemented with large amounts of forage and grain/concentrates. To date the n-alkane technique has been used to estimate DMI of mixed grains and forage in sheep (Valiente et al., 2003). In dairy cows, the n-alkane technique has been used to estimate forage DMI in grazing systems, where individual supplement intakes were known or estimated using other techniques (Malossini et al., 1996, McEvoy et al., 2008, O'Neill et al., 2012). Bani et al.

(2014) estimated individual DMI of dairy cows consuming total mixed rations using the n-alkane technique, however no fresh herbage was consumed in that study.

In temperate regions of the world, including Australia and New Zealand, dairying is primarily pasture-based due to its low to medium cost (Jacobs, 2014). Traditionally, pasture-based systems involve feeding cereal grain or concentrates in the parlor during milking however, an increasing number of dairy producers are increasing the amount of supplements offered to their cows, and as a result are transitioning to other supplementary feeding strategies (Auld et al., 2019). One such strategy is feeding a partial mixed ration (PMR), where a mixed ration, is fed on a feed pad between periods of grazing. The PMR feeding system is used by approximately 12% of dairy producers in Australia, with this number likely to increase (Wales and Kolver, 2017). Implementing a PMR feeding system can result in energy-corrected milk (ECM) production increases of between 2 to 5 kg/cow per day compared with offering equivalent amounts of energy as grain in the dairy and conserved forage in the paddock (Auld et al., 2019). While the benefits of this feeding system have been demonstrated, the ability to measure individual cow DMI of fresh herbage and PMR has not been researched nor has the accuracy and precision of the n-alkane technique in this feeding system. As pasture-based dairying systems shift towards feeding more supplements, there is a requirement for a method to accurately estimate individual DMI of both fresh herbage and large amounts of supplements.

The objectives of this experiment were as follows: (1) to determine the accuracy and precision of the n-alkane technique for estimating individual cow DMI of fresh herbage and PMR; and (2) to determine the accuracy and precision of the technique at high and low amounts of fresh herbage and PMR.

## **MATERIALS AND METHODS**

The experiment was conducted at the Department of Jobs, Precincts and Regions (DJPR) Ellinbank Centre, Victoria, Australia (latitude 38°14'S, longitude 145°56'E). All procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013). Approval to proceed was obtained from the DJPR Agricultural Research and Extension Animal Ethics Committee.

### ***Cows and Design***

This experiment used 16 Holstein-Friesian dairy cows. All cows were between 4 and 7 years old ( $6 \pm 1.0$  years; mean  $\pm$  s.d.), had an average 7-day milk yield of  $36.5 \pm 3.41$  kg/cow.day,

and had an average bodyweight of  $646 \pm 46.3$  kg/cow. Four cows were allocated to each of four dietary treatments, with groups balanced for DIM, age, bodyweight, and 7-day average milk production immediately prior to the commencement of the experiment according to the method of Baird (1994). The four treatments were investigated using a  $2 \times 2$  factorial design. The dietary treatments were 8 or 14 kg of DM/cow per day of freshly cut herbage plus 6 or 12 kg of DM/cow per day of PMR.

The experiment was conducted over 24 days, consisting of a 14-day pre-experimental period, during which cows were gradually adapted to their diet, a 7-day period in day stalls, where individual intake was monitored, and a 10-day experimental period in which cows were housed in individual metabolism stalls, restrained with a headstall and fitted with fecal harnesses and urine separators. During the experimental period cows were milked *in situ* at ~0630 and 1500 h each day.

### ***Diets***

The PMR was fed following milking and contained crushed wheat grain (38%, DM basis), maize grain (18%), lucerne hay (22%) and canola meal (22%). The PMR was mixed and chopped in a feed wagon (model K160: Richard Keenan and Co. Ltd., Co. Carlow, Ireland) and water was added such that the DM concentration of the ration was approximately 50%. Each cow was offered perennial ryegrass (*Lolium perenne* L.) dominant herbage cut to approximately 4 cm above ground level twice per day following the PMR. Cows had access to fresh water *ad libitum*. Cows were also offered 500 g/cow per day of vitamin and mineral pellets in two equal amounts on top of the PMR at each feeding (Ridley custom concentrate pellets, Ridley Agriproducts, Victoria, Australia). The pellets contained Monensin (360 mg/cow per day) and Tylosin (200 mg/cow per day). Sodium bicarbonate (80 g/cow per day), ground limestone (150 g/cow per day) and magnesium oxide (30 g/cow per day) were mixed into the ration prior to feeding.

### ***Feed and fecal samples***

Samples of herbage and PMR offered and refused were collected at each feeding, and split into subsamples for determination of DM, nutritive characteristics, and n-alkane concentrations. Subsamples were oven-dried to a constant weight at 105°C for a minimum of 24 h to determine DM concentration. Measured individual cow feed intake was calculated as the difference between amount of ration and herbage DM offered and refused. Amounts of ration and herbage DM offered and refused were manually weighed using scales.

Subsamples used for nutritive characteristics were stored at -20°C until the completion of the experiment. These samples were then freeze-dried, ground through a 0.5-mm sieve, thoroughly mixed, and analyzed. Samples were composited across the final 5 days once freeze dried and ground. Feed components were analyzed for crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), starch, and ash by NIR spectroscopy using a Foss scanning monochromator (NIR XDS; Foss Pacific, Mulgrave, Vic., Australia) with a wavelength range of 400–2500 nm. The monochromator was calibrated using standards analyzed using standard reference methods described by the Australian Fodder Industry Association (AFIA, 2009). Nutritive characteristics of the herbage and PMR offered are presented in Table 1. Estimated ME was calculated using the following formula (National Research Council, 2001):

$$\text{ME (MJ/kg DM)} = (1.01 \times (0.04409 \times \text{TDN}) - 0.45) \times 4.184$$

where TDN is total digestible nutrient (%).

**[Table 1 here]**

#### *Estimation of dry matter intake by alkanes*

For each cow, DMI was estimated using the n-alkane technique (Dove and Mayes, 2006). Cows were dosed with paper pellets (Carl Roth GmbH and Co.KG, Karlsruhe, Germany) containing 378 mg per pellet of dotriacontane (C<sub>32</sub>) twice daily (following the morning and afternoon milkings). Cows were dosed for 10 consecutive days of the experimental period. Total fecal output of each cow was weighed twice a day. Following weighing, feces were mixed and representative samples for each cow were collected for fecal DM determination, where samples were dried at 105°C for a minimum of 24 h to a constant weight. Fecal samples were collected twice daily during the last 5 days and split into two subsamples for analysis of concentration of DM and n-alkanes. Fecal samples for analysis of alkane concentrations were stored at -20°C, then at the conclusion of the experiment samples were defrosted, oven dried at 40°C for 48 h, and composited over p.m. and a.m. (allowing for a 12h difference between feed and fecal samples) within cow per day from day 6 to 10. Total fecal output was weighed twice a day to allow for calculations of fecal recovery rates. Herbage and PMR samples representative of the feed offered to each cow were collected daily from day 6 to 10. The n-alkane concentrations heptacosane (C<sub>27</sub>-alkane), octacosane (C<sub>28</sub>-alkane), nonacosane (C<sub>29</sub>-alkane), triacontane (C<sub>30</sub>-alkane), hentriacontane (C<sub>31</sub>-alkane), C<sub>32</sub> alkane, tritriacontane (C<sub>33</sub>-alkane) and pentatriacontane (C<sub>35</sub>-alkane) of the herbage, PMR, and feces were analyzed using gas chromatography according to the method of Mayes et al. (1986), by means of a modification of Dillon (1993) using a 1.6 m × 4 mm glass column. The determination of ingested proportion of ryegrass and PMR were estimated from the concentrations of n-alkanes C<sub>27</sub>-C<sub>35</sub> (excluding C<sub>34</sub>) in feces, herbage, PMR, and the daily



dose rate of C<sub>32</sub>. A least-squares optimization procedure was used (SOLVER, Microsoft Excel) to determine the cow DMI of herbage and mixed ration. This method minimized the sum of the squared discrepancies for each n-alkane (C<sub>27</sub>-C<sub>35</sub>; excluding C<sub>34</sub>) between the actual fecal n-alkane concentrations (corrected for incomplete recovery) and calculated concentrations, as described by Mayes et al. (1994). Incomplete fecal recoveries were used to adjust the actual n-alkane concentrations in the feces DM using three methods. The first method applied recovery rates published by Dillon (1993) which were 0.618 (C<sub>27</sub>), 0.686 (C<sub>28</sub>), 0.722 (C<sub>29</sub>), 0.769 (C<sub>30</sub>), 0.777 (C<sub>31</sub>), 0.844 (C<sub>32</sub>), 0.844 (C<sub>33</sub>) and 0.891 (C<sub>35</sub>). The two calculated sets of recovery rates were (i) recovery rates determined by averaging calculated recovery rates over all cows within this experiment, termed 'average recovery rates'; and (ii) recovery rates averaged across cows within each dietary treatment combination, termed 'treatment recovery rates'. It should be noted that the calculation of recovery rates during the evaluation of the n-alkane technique could be considered circular, in that measured DMI and fecal output were used in the determination of recovery rates. Hence, the current research also incorporated experimentally independent fecal corrections made with previously reported recovery rates from Dillon (1993).

### *Statistical Analyses*

Fecal n-alkane concentrations, herbage and PMR n-alkane concentrations, and n-alkane recovery rates were analyzed by ANOVA with factorial treatment structure of amount of herbage offered by amount of PMR offered, with a nested blocking structure as cow split for day. Average daily DMI were calculated using n-alkane fecal recovery correction methods with different recovery rates (Dillon (1993), average recovery rates and treatment recovery rates) and the measured DMI, for each of the 16 cows. These data were used to calculate Lin's concordance correlation coefficients (Lawrence, 1989) between the four methods. Lin's concordance is the product of a bias factor, for which a value of 1 indicates no bias, and a Pearson correlation coefficient that measures linear relationship. The same data were used to examine factors associated with discrepancies between the n-alkane estimate and the measured DMI. The discrepancies, n-alkane estimated intake minus the measured intake (for each of herbage, PMR and total), were calculated and analyzed using ANOVA with factorial treatment structure of amount of herbage by amount of PMR, with no blocking structure (cow being the unit). This model was designed to test associations between the discrepancy and the amount of herbage and/or the amount of PMR. The ANOVA analyses were performed in GenStat software for Windows (Genstat release 18; VSN International Ltd., Hemel Hempstead, UK). The mean discrepancy was tested for inequality with zero (bias) by *t*-test with the *t*-statistic being the ratio of mean to SEM supplied by the ANOVA for each treatment

combination. Percentage mean discrepancies for each estimation method were calculated relative to the measured mean.

Calculated recovery rates were determined using the following equation:

$$\text{Fecal recovery rates} = (F_{n\text{-alkane}} \times \text{FDM output}) / (\text{PMR}_{n\text{-alkane}} \times \text{PMRDMI}) + (H_{n\text{-alkane}} \times \text{HDMI})$$

where  $F$ ,  $MR$  and  $H_{n\text{-alkane}}$  represent the concentrations (mg/kg DM) of n-alkanes in the feces (F), partial mixed ration (PMR) and herbage (H). Fecal DM (FDM) output and partial mixed ration DMI (PMRDMI) and herbage DMI (HDMI) inputted as kg DM/ cow per day. The equation above was equally applied to determine all n-alkane recovery rates, however when used for the dosed n-alkane ( $C_{32}$ ), the dose rate was added to the denominator.

## RESULTS

### *Accuracy and precision of the n-alkane technique*

Lin's concordance correlation coefficients contain measures of accuracy, with a bias correction factor, and precision was determined using Pearson correlation coefficient. The comparison of measured total DMI and estimated total DMI using the recovery rate corrections of Dillon (1993) resulted in a Lin's concordance correlation coefficient of 0.96 (bias factor 0.99). When comparing measured and estimated total DMI with the average recovery rate correction, Lin's concordance correlation coefficient was 0.97 (bias factor 1.00). For estimates of intake with the n-alkane technique using treatment recovery rates, when compared with measured intakes, Lin's correlation coefficient of 0.98 (bias factor 1.00). The Lin's concordance correlation coefficient between the two calculated fecal concentration corrections (average recovery rates and treatment recovery rates) was 0.97 (bias factor 0.99). Figure 1 represents the relationships between estimated DMI (using fecal corrections made either with treatment recovery rates or recovery rates reported by Dillon (1993)), at the cow level. Treatment recovery rates were more accurate than average recovery rates and hence only treatment recovery rates are displayed in Figure 1.

### **[Insert Figure 1]**

For several treatment combinations the n-alkane estimates of DMI (using recovery rates from Dillon (1993), average recovery rates and treatment recovery rates) were significantly different from measured DMI, indicating systematic biases under some diet combinations, Table 2. The discrepancy between measured and estimated PMR DMI and total DMI, using the recovery rates of Dillon (1993), were influenced by the amount of herbage and PMR ( $P < 0.05$ ). However, the discrepancy between estimated and measured herbage DMI, using the recovery rates from Dillon (1993), was not influenced by the amount of herbage or mixed ration ( $P > 0.05$ ). Using average recovery rates, the discrepancy in mixed ration DMI and total DMI depended significantly on the amount of mixed ration, and on the amount of herbage offered ( $P < 0.05$ ). For treatment recovery rates, the discrepancy of estimated herbage DMI depended on the amount of herbage that was offered ( $P < 0.05$ ) but not the amount of mixed ration ( $P > 0.05$ ). The amount of herbage or mixed ration did not influence the discrepancy between estimates using treatment recovery rates and measured intakes. The discrepancies between measured and estimated DMI indicate systematic biases, the median of the biases was 5.1%. The maximum bias for corrections using recovery rates from Dillon (1993) was 23% for mixed ration DMI when cows were fed the high amount of herbage and mixed ration diet. The maximum bias for DMI when average recovery rates were applied when a low amount of mixed ration and high amount of herbage was offered was 22% for mixed ration DMI. The maximum bias when treatment recovery rates were used was 7% for

herbage DMI when the high amount of both herbage and mixed ration were fed. When corrections were made with Dillon (1993) recovery rates, residual standard deviations for the discrepancy between estimated and measured DMI were 0.44 kg DM/cow per day for herbage DMI, 0.37 kg DM/cow per day for mixed ration DMI, and 0.77 kg DM/cow per day for total DMI (or expressed as coefficients of variation (CV), 3.9%, 4.1%, and 3.7%, respectively). The residual standard deviations for the discrepancy between estimated and measured DMI for both methods where recovery rates were calculated in this experiment, were 0.45 kg DM/cow per day for herbage DMI, 0.38 kg DM/cow per day for mixed ration DMI, and 0.79 kg DM/cow per day for total DMI (or expressed as CV of 4.1%, 4.2%, and 3.9%, respectively).

### ***Dry matter intake***

The mean measured DMI for herbage, mixed ration, and total DMI are presented in Table 2. Cows consumed more total DM when offered more feed from both the herbage and the mixed ration components of the diet. Increasing the amount of herbage offered from 8 to 14 kg DM/cow per day increased herbage intake by 6.6 kg DM/ cow per day. Increasing the amount of mixed ration offered resulted in a 6.1 kg DM/cow per day increase in mixed ration DMI. Measured DMI and discrepancies between estimated and measured DMI classified by diet treatments, are also presented in Table 2.

**[Insert Table 2 here]**

### ***Herbage and mixed ration n-alkanes***

Samples of the herbage and mixed ration were analyzed to determine if the n-alkane profile of these feeds differed (Table 3). Concentrations of all n-alkanes from C<sub>27</sub> to C<sub>35</sub>, excluding C<sub>28</sub> and C<sub>30</sub> were each significantly higher ( $P < 0.001$ ) in the herbage compared with the mixed ration.

**[Insert Table 3 here]**

### ***Fecal n-alkane recovery rates***

The amount of herbage offered had a significant effect ( $P < 0.05$ ) on the recovery rates of n-alkanes (C<sub>27</sub>-C<sub>35</sub> excluding C<sub>34</sub>) not including dosed alkane, C<sub>32</sub>. The recovery rates of these n-alkanes increased ( $P < 0.05$ ) with increasing amount of herbage offered (Table 4). There were no significant differences in fecal n-alkane recovery rates when the amount of mixed ration was altered ( $P > 0.05$ ). There were no significant interactions ( $P > 0.05$ ) between the effects of the amounts of herbage and mixed ration on fecal n-alkane recovery rates.

**[Insert Table 4]**

***Feces n-alkanes***

The concentrations of n-alkanes (C<sub>27</sub>-C<sub>35</sub> excluding C<sub>34</sub>) in feces, excluding dosed C<sub>32</sub>, were significantly higher ( $P < 0.05$ ) when the amount of herbage increased (Table 5). Dosed C<sub>32</sub> had a higher fecal concentration when the lower amount of herbage was offered ( $P < 0.05$ ). The fecal concentrations of all n-alkanes (C<sub>27</sub>-C<sub>35</sub> excluding C<sub>34</sub>) were significantly decreased ( $P < 0.05$ ) when the amount of mixed ration increased. There was an interaction between the effects of the amounts of herbage and mixed ration for n-alkanes, C<sub>29</sub>, C<sub>32</sub> and C<sub>33</sub> ( $P = 0.042, 0.038$  and  $0.013$ , respectively).

**[Insert Table 5 here]**

## DISCUSSION

The n-alkane technique accurately and precisely estimated total DMI in dairy cows fed fresh herbage in combination with a mixed ration. This is the first research that has reported the DMI of fresh herbage diets and mixed ration in dairy cows, where estimation has occurred without the application of artificial n-alkanes or beeswax mixtures onto the concentrate component of the diet. This finding, that the n-alkane technique can be accurately applied to a mixed ration feeding system in dairy cows, was evidenced by strong Lin's concordance estimates (see Figure 1 for the relationship between estimated, with different incomplete fecal corrections, and measured total DMI).

While there was a strong relationship between estimates and measured total intakes, some of the discrepancies between estimated and measured DMI were significant for herbage and mixed ration DMI. For all recovery rates used, several mean discrepancies were significantly different from zero, indicating systematic biases under some treatment combinations. While these discrepancies were significantly different to zero, biologically they were inconsequential. For example, using treatment recovery rates, the discrepancy between combined estimates of herbage and measured herbage DMI was a 5.3% underestimation of measured herbage DMI (or 3.0% of total DMI), and the mean discrepancy between the combined estimates of mixed ration and measured intakes was a 4.1% overestimation of measured ration DMI (or 1.8% of total DMI). This is a negligible difference, considering this equates to an underestimation of herbage ME intake of 7.2 MJ/cow per day and an overestimation of ration ME intake of 4.2 MJ/cow per day. Other studies have reported similar discrepancies with the n-alkane technique when estimating herbage DMI. For example, Piasentier et al. (1995) and Dove and Mayes (1996) reported a discrepancy of ~3% between measured and estimated herbage intakes. It is also important to consider that the measured DMI are not without error as they rely on representative subsampling from a heterogeneous mixture to determine moisture concentration.

The precision with which diet composition can be estimated improves with divergence in n-alkane concentrations between the feeds (Brosh et al., 2003). There were several differences in the n-alkane concentrations of the herbage offered compared to the mixed ration offered. Most n-alkane concentrations (excluding C<sub>28</sub> and C<sub>30</sub>) were higher in the herbage compared with the ration. Higher concentrations of the odd-chain n-alkanes in the herbage are a result of high cuticular wax on the external surface of pasture. The current research demonstrated that many of the n-alkanes in the mixed ration were low in concentration (less than 50 mg/kg DM) and previous reports suggest that the determination of diet composition using n-alkanes of low concentrations are more prone to measurement errors (Brosh et al., 2003). However, coefficients of variation (CV) presented in the results section illustrate constant CV, demonstrating relative precision is independent of n-alkane concentration. The data from Valiente et al. (2003) suggested that the notion of a minimum n-alkane

concentration should be investigated further. The inclusion of lucerne hay in the mixed ration was important for the alkane profile of the mixed ration, as the other components, including cereal grains and canola meal, have low n-alkane concentrations. Without the inclusion of lucerne hay, the alkane profile of the mixed ration would have been different, resulting in a different outcome.

When n-alkanes are used to estimate herbage DMI only, the double n-alkane procedure can be used, and this does not require corrections for incomplete recovery (Dove, 2010). However, when using n-alkanes as diet composition markers, corrections for incomplete fecal recovery are required in order to reduce bias of diet composition towards plant species with higher concentrations of longer chain length n-alkanes (Ferreira et al., 2009). This research investigated the application of two different calculations for correcting for incomplete fecal recovery of n-alkanes; (i) average recovery rates, and (ii) treatment recovery rates. The largest discrepancy between estimates and measured intakes occurred when the average recovery rate correction was applied. The findings of the current experiment are comparable with the findings of Ferreira et al. (2009) that the most accurate correction was one based on the mean fecal recovery data of each dietary treatment, and that the most accurate corrections were made using recoveries obtained under similar conditions (for example, diet composition). This is further supported by previous experiments demonstrating that estimates of diet composition are most accurate when individual cow recovery rates within a dietary treatment are used and declines when recovery rate means are used (Brosh et al., 2003, Elwert et al., 2004, Charmley and Dove, 2007, Ferreira et al., 2007).

In practice, however, care must be taken using treatment recovery rates because if one set of recovery rates are used for all cows in one treatment, and another set for all cows in another treatment, treatment effects could be confounded. In this context, treatment effects could not be accurately separated to determine whether effects were due to the treatments or the set of recovery rates used for that treatment. Therefore, independent recovery rates for each experimental unit (which may be a group or individual cow) within each treatment should be used. Increases in the accuracy of intake estimates have been shown when incomplete recoveries are corrected with recovery rates calculated on a per cow basis (Brosh et al., 2003). However, in reality, the application of recovery rates per cow is not practical and the application of recovery rates for all animals will result in only a small error in estimates of diet composition as it is the relative recovery that is important (Dove and Mayes, 1996, Elwert, 2004). The investigation of the most accurate application of recovery rates for intake estimations was investigated as it will enable the use of reported recovery rates in future mixed ration research when similar diets are fed. The current research also incorporated experimentally independent fecal corrections made with previously reported recovery rates from Dillon (1993).

The amount of herbage and mixed ration offered to cows in the PMR feeding system influenced a number of discrepancies between estimated and measured DMI. The amount of herbage offered influenced the discrepancy between estimated and measured mixed ration and total DMI (using Dillon 1993 and average recovery rates) and herbage DMI (using treatment recovery rates). The discrepancies between estimated and measured DMI with different amounts of herbage offered is a result of differences in the recovery rates of n-alkanes. Interestingly, the recovery rates of all the n-alkanes ( $C_{27}$ - $C_{35}$  excluding  $C_{34}$ ), apart from the dosed  $C_{32}$ , were affected by the amount of herbage offered, with recovery rates increasing as the amount of herbage offered increased. This contrasts with the findings of Dillon (1993), who reported no effect of feeding level on the fecal recovery rates of n-alkanes. The difference in the recovery rates of n-alkanes, when altering the amount of herbage offered, emphasizes the importance of determining the recovery rates for different feeds and feeding levels.

## CONCLUSIONS

In conclusion, this research is the first to validate the use of the n-alkane technique for estimating intake of both herbage and PMR in dairy cows consuming mixed diets containing a combination of concentrate, conserved forage, and fresh herbage. While there were some discrepancies between estimated and measured intakes that were significantly different from zero, indicating systematic biases under some diets, these were on average ~ 5% of individual diet component intakes, and are therefore acceptable for use in research. The most accurate application of fecal recovery rates, for future use of this technique in field-based mixed ration research, was using recovery rates averaged across treatments. Discrepancies between estimates (using the recovery rates of Dillon (1993) and average recovery rates) and measurements of both PMR DMI and total DMI were influenced by the amount of both herbage and PMR offered. The discrepancy between estimated (with treatment recovery rates) and measured herbage DMI increased as the amount of herbage offered increased but was not affected by amount of mixed ration. The relationship between recovery rates and the amount of herbage offered demonstrates that the recovery rates of all-natural n-alkanes increased as the amount of herbage increased.

The authors are grateful to G. Morris, D. Mapleson, A. McDonald, L. Dorling, T. Phillips, T. Hookey, S. Zeiro, D. Wilson, and Department of Jobs, Precincts and Regions (DJPR), Ellinbank farm staff for assistance with cow feeding and husbandry. The authors are also grateful for the n-alkane analysis by Teagasc Moorepark, Ireland. This research was funded by DJPR, Victoria, and Dairy Australia (Melbourne, Australia).

## AUTHOR STATEMENT



**M. M. Wright:** Conceptualization; Methodology; Investigation; Writing - Original Draft; Visualization; and Project administration.

**M. J. Auld:** Conceptualization; Methodology; Supervision; and Writing - Review & Editing.

**E. Kennedy:** Conceptualization; Methodology; Resources; Supervision; and Writing - Review & Editing.

**F. R. Dunshea:** Conceptualization; Methodology; Supervision; and Writing - Review & Editing.

**N. Galvin:** Resources.

**M.C. Hannah:** Formal analysis; and Validation.

**W. J. Wales:** Conceptualization; Methodology; Supervision; and Writing - Review & Editing.

Conflict of interest: none

**REFERENCES**

- AFIA. 2009. AFIA- laboratory methods manual. Australian Fodder Industry Association, Melbourne.
- Allen, M. S. 2000. Effects of Diet on Short-Term Regulation of Feed Intake by Lactating Dairy Cattle. *J. Dairy Sci.* 83(7):1598-1624.
- Auldish, M. J., M. M. Wright, L. C. Marett, M. C. Hannah, E. Kennedy, J. L. Jacobs, and W. J. Wales. 2019. Milk production of cows grazing pasture supplemented by a partial mixed ration with or without canola meal. *Anim Prod Sci.* 59(4):778-786.
- Baird, D. B. 1994. The design of experiments with covariates. University of Otago, New Zealand.
- Bani, P., F. P. Cappelli, A. Minuti, V. Ficuciello, V. Lopreiato, P. C. Garnsworthy, and E. Trevisi. 2014. Estimation of dry matter intake by n-alkanes in dairy cows fed TMR: Effect of dosing technique and faecal collection time. *Anim Prod Sci.* 54(10):1747-1751.
- Brosh, A., Z. Henkin, S. J. Rothman, Y. Aharoni, A. Orlov, and A. Arieli. 2003. Effects of faecal n-alkane recovery in estimates of diet composition. *J. Agric. Sci.* 140(1):93-100.
- Charmley, E. and H. Dove. 2007. Using plant wax markers to estimate diet composition and intakes of mixed forages in sheep by feeding a known amount of alkane-labelled supplement. *Aust J. Agric. Res* 58(12):1215-1225.
- Dillon, P. G. 1993. The use of n-alkanes as markers to determine herbage intake, botanical composition of available or consumed herbage and in studies of digesta kinetics. The National University of Ireland, Ireland.
- Dove, H. 1993. Advances in the Estimation of Intake and Diet Selection in the Grazing Animal Recent Advances in Animal Nutrition in Australia. University of New England, Armidale, N.S.W., Australia.
- Dove, H. 2010. Assessment of intake and diet composition of grazing livestock. Pages 31-56 in Proc. 4th grazing livestock nutrition conference.
- Dove, H. and R. Mayes. 1991. The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. *Aust J. Agric. Res.* 42(6):913-952.
- Dove, H. and R. W. Mayes. 1996. Plant wax components: a new approach to estimating intake and diet composition in herbivores. *J. Nut.* 126, 13-26.
- Dove, H. and R. W. Mayes. 2005. Using n-alkanes and other plant wax components to estimate intake, digestibility and diet composition of grazing/browsing sheep and goats. *Small Ruminant Res.* 59(2-3):123-139.
- Dove, H. and R. W. Mayes. 2006. Protocol for the analysis of n-alkanes and other plant-wax compounds and for their use as markers for quantifying the nutrient supply of large mammalian herbivores. *Nature protocols* 1(4):1680-1697.

- Dove, H., S. McLennan, and D. Poppi. 2010. Application of nutrient requirement schemes to grazing animals. Pages 133-149 in Proc. Proceedings of the 4th grazing livestock nutrition conference, Estes Park, CO, USA'. (Eds BW Hess, T Delcurto, JPG Bowman, RC Waterman).
- Dutta, R., D. Smith, R. Rawnsley, G. Bishop-Hurley, and J. Hills. 2014. Cattle behaviour classification using 3-axis collar sensor and multi-classifier pattern recognition. Pages 1272-1275 in Proc. Instrumentation and Measurement Technology. IEEE International.
- Elwert, C. 2004. Studies on the use of alkanes to estimate diet composition, intake, and digestibility in sheep. Vol. PhD. Martin Luther University, Halle-Wittenberg, Germany.
- Elwert, C., H. Kluth, and M. Rodehutschord. 2004. Effect of variable intake of alfalfa and wheat on faecal alkane recoveries and estimates of roughage intake in sheep. *J. Agric. Sci.* 142(2):213-223.
- Ferreira, L. M. M., R. Celaya, U. García, M. A. M. Rodrigues, and K. Osoro. 2009. Differences between domestic herbivores species in alkane faecal recoveries and the accuracy of subsequent estimates of diet composition. *Ani. Feed Sci. Technol.* 151(1-2):128-142.
- Ferreira, L. M. M., U. Garcia, M. A. M. Rodrigues, R. Celaya, A. Dias-da-Silva, and K. Osoro. 2007. The application of the n-alkane technique for estimating the composition of diets consumed by equines and cattle feeding on upland vegetation communities. *Anim. Feed Sci. Technol.* 138(1):47-60.
- Jacobs, J. L. 2014. Challenges in ration formulation in pasture-based milk production systems. *Anim. Prod. Sci.* 54(9):1130-1140.
- Langlands, J. and G. Donald. 1978. The nutrition of ruminants grazing native and improved pastures. II. Responses of grazing cattle to molasses and urea supplementation. *Aust J. Agric. Res* 29(4):875-883.
- Lawrence, I. K. L. 1989. A Concordance Correlation Coefficient to Evaluate Reproducibility. *Biometrics* 45(1):255-268.
- Malossini, F., S. Bovolenta, E. Piasentier, C. Piras, and F. Martillotti. 1996. Comparison of n-alkanes and chromium oxide methods for estimating herbage intake by grazing dairy cows. *Anim. Feed Sci. Technol.* 61(1-4):155-165.
- Mayes, R. W., N. A. Beresford, C. S. Lamb, C. L. Barnett, B. J. Howard, B. E. V. Jones, O. Eriksson, K. Hove, Ø. Pedersen, and B. W. Staines. 1994. Novel approaches to the estimation of intake and bioavailability of radiocaesium in ruminants grazing forested areas. *Sci. Total Environ.* 157:289-300.
- Mayes, R. W. and H. Dove. 2000. Measurement of dietary nutrient intake in free-ranging mammalian herbivores. *Nutr. Res. Rev.* 13(1):107-138.
- Mayes, R. W., C. S. Lamb, and P. M. Colgrove. 1986. The use of dosed and herbage n-alkanes as markers for the determination of herbage intake. *J. Agric. Sci.* 10:161-170.

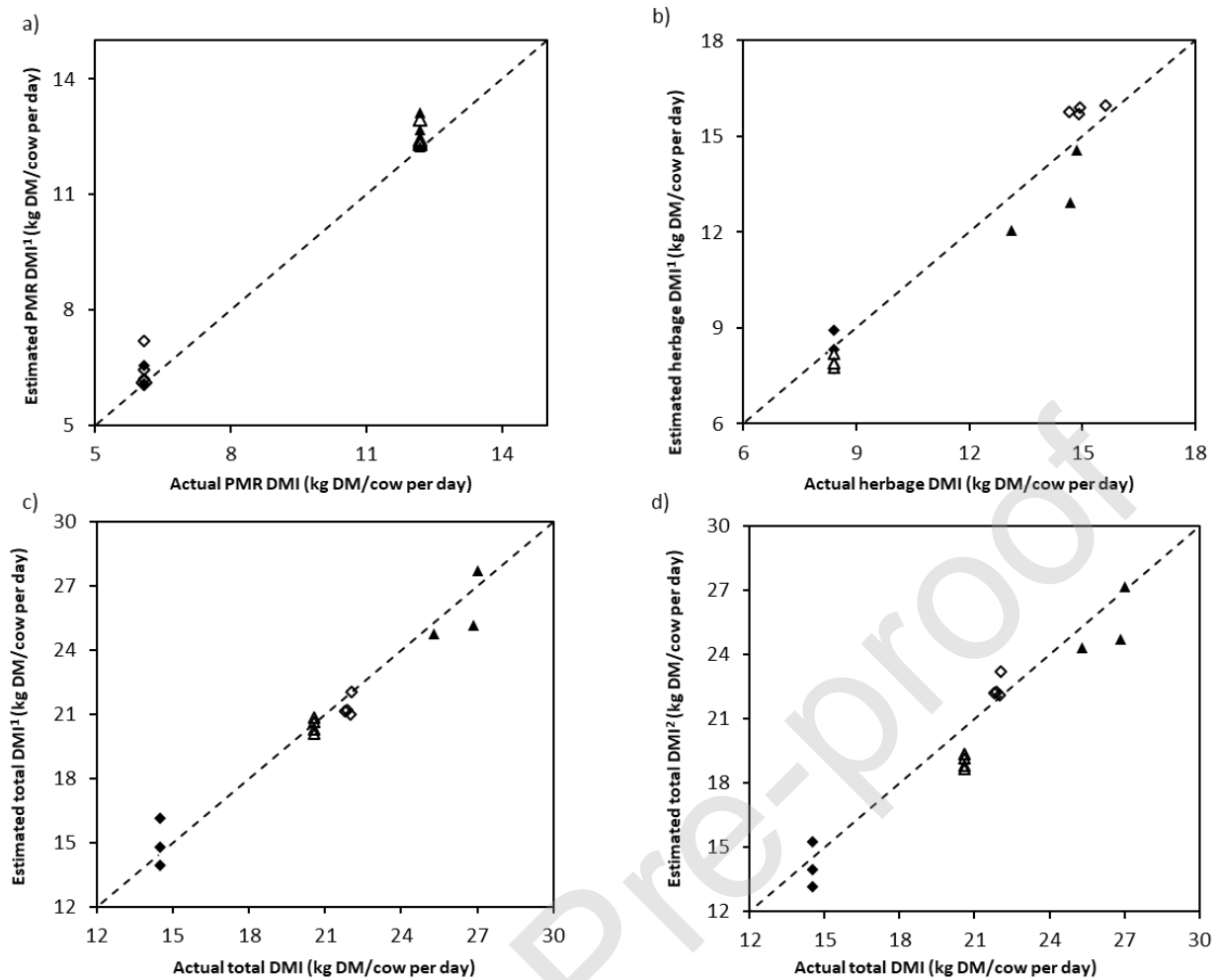
- McEvoy, M., E. Kennedy, J. P. Murphy, T. M. Boland, L. Delaby, and M. O'Donovan. 2008. The Effect of Herbage Allowance and Concentrate Supplementation on Milk Production Performance and Dry Matter Intake of Spring-Calving Dairy Cows in Early Lactation. *J. Dairy Sci.* 91(3):1258-1269.
- National Health and Medical Research Council. 2013. Australian code for the care and use of animals for scientific purposes 8th ed. Australian Government, Canberra.
- National Research Council. 2001. Nutrient requirements of dairy cattle. 7 ed. National Academy Press, Washington, DC.
- O'Neill, B. F., M. H. Deighton, B. M. O'Loughlin, N. Galvin, M. O'Donovan, and E. Lewis. 2012. The effects of supplementing grazing dairy cows with partial mixed ration on enteric methane emissions and milk production during mid to late lactation. *J Dairy Sci.* 95(11):6582-6590.
- Piasentier, E., S. Bovolenta, F. Malossini, and P. Susmel. 1995. Comparison of n-alkanes or chromium oxide methods for estimation of herbage intake by sheep. *Small Ruminant Res.* 18(1):27-32.
- Smit, H. J., H. Z. Taweel, B. M. Tas, S. Tamminga, and A. Elgersma. 2005. Comparison of Techniques for Estimating Herbage Intake of Grazing Dairy Cows. *J. Dairy Sci.* 88(5):1827-1836.
- Valiente, O. L., P. Delgado, A. d. Vega, and J. A. Guada. 2003. Validation of the n-alkane technique to estimate intake, digestibility, and diet composition in sheep consuming mixed grain:roughage diets. *Aust J. Agric. Res.* 54(7):693-702.
- Vazquez, O. P. and T. R. Smith. 2000. Factors Affecting Pasture Intake and Total Dry Matter Intake in Grazing Dairy Cows. *J. Dairy Sci.* 83(10):2301-2309.
- Wales, W. J. and E. S. Kolver. 2017. Challenges of feeding dairy cows in Australia and New Zealand. *Anim. Prod. Sci.* 57(7):1366-1383.
- Wright, M. M. 2017. Quantifying the individual intake of grazing dairy cows offered mixed rations. Vol. PhD thesis. The University of Melbourne, Melbourne, Australia.
- Wright, M. M., M. J. Auldist, E. Kennedy, F. R. Dunshea, M. Hannah, and W. J. Wales. 2016. Variation in feeding behavior and milk production among dairy cows when supplemented with 2 amounts of mixed ration in combination with 2 amounts of pasture. *J. Dairy Sci.* 99:6507-6518.
- Wright, M. M., E. Lewis, B. Garry, N. Galvin, F. R. Dunshea, M. C. Hannah, M. J. Auldist, W. J. Wales, P. Dillon, and E. Kennedy. 2019. Evaluation of the n-alkane technique for estimating the herbage dry matter intake of dairy cows offered herbage harvested at two different stages of growth in summer and autumn. *Anim. Feed Sci. Technol.* 247:199-209.

**Table 1: Mean nutritive characteristics of PMR offered, and herbage offered and refused, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), starch, ash, and metabolizable energy (ME).**

	CP (g/kg DM)	NDF (g/kg DM)	ADF (g/kg DM)	Starch (g/kg DM)	Ash (g/kg DM)	ME (MJ/kg DM)
PMR offered <sup>1</sup>	207	231	139	292	70	11.7
Herbage offered <sup>1</sup>	186	515	297	16	91	11.6
Herbage refused <sup>1,2</sup>	185	476	278	37	111	10.6

<sup>1</sup>Partial mixed ration, herbage offered, and herbage refused values are from bulked samples collected during the measurement period. There were no ration refusals in this experiment.

<sup>2</sup>Herbage refused is a mean value from individual cow and herbage refused samples.



**Figure 1: Individual cow dry matter intakes (kg DM/cow per day) (DMI) demonstrating the relationship between measured and estimated intakes using treatment average recovery rate corrections<sup>1</sup> for a) PMR DMI, b) herbage DMI, c) total DMI, and d) total DMI when estimated DMI was calculated using recovery rate corrections from Dillon (1993)<sup>2</sup>. Cows were fed different amounts of herbage and PMR: 8 kg DM/ cow per day of herbage and low PMR (◆); 8 kg DM/ cow per day of herbage and high PMR(△); 14 kg DM/ cow per day of herbage and low PMR (◇); and 14 kg DM/ cow per day of herbage and high PMR (▲).**

**Table 2: The difference between measured and estimated dry matter intakes (DMI) (kg of DM/cow per day) when cows were offered PMR and herbage, at different rates. Estimates of DMI using the n-alkane technique were calculated using three different methods of correcting for incomplete fecal n-alkane recovery (see superscripts). Estimated DMI (kg of DM/cow per day) are presented in brackets. The standard error of the mean (SEM) and *P*-values relate to the difference between estimated and measured (kg of DM/cow per day).**

Amount of herbage Amount of PMR	8 kg DM/ cow per day		14 kg DM/ cow per day		SEM	Amount of Herbage	<i>P</i> -value	
	Low	High	Low	High			Amount of PMR	Herbage × PMR
	<i>Herbage DMI</i>							
Measured DMI	8.4	8.4	15.8	14.2				
Estimated <sup>1</sup> DMI	-0.79* (7.6)	-0.94* (7.5)	-0.88* (14.9)	-1.58* (12.6)	0.222	0.131	0.084	0.246
Estimated <sup>2</sup> DMI	-0.44 (8.0)	-0.54* (7.9)	-0.20 (15.6)	-0.93* (13.3)	0.235	0.760	0.102	0.202
Estimated <sup>3</sup> DMI	-0.19 (8.2)	-0.46 (7.9)	-0.82* (15.0)	-1.00* (13.2)	0.235	0.030	0.356	0.859
	<i>PMR DMI</i>							
Measured DMI	6.1	12.2	6.1	12.2				
Estimated <sup>1</sup> DMI	0.18 (6.3)	-0.65* (11.6)	1.38* (7.5)	0.61* (12.8)	0.184	<0.001	0.001	0.884
Estimated <sup>2</sup> DMI	0.18 (6.3)	-0.57* (11.6)	1.32* (7.4)	0.67* (12.9)	0.185	<0.001	0.003	0.818
Estimated <sup>3</sup> DMI	0.40 (6.5)	0.34 (12.5)	0.24 (6.3)	0.53* (12.7)	0.188	0.945	0.554	0.370
	<i>Total DMI</i>							
Measured DMI	14.5	20.5	21.9	26.4				
Estimated <sup>1</sup> DMI	-0.62 (13.9)	-1.59* (18.9)	0.50 (22.4)	-0.97* (25.4)	0.383	0.044	0.009	0.535
Estimated <sup>2</sup> DMI	-0.27 (14.2)	-1.11* (19.4)	1.12* (23.0)	-0.27 (26.1)	0.396	0.017	0.017	0.502
Estimated <sup>3</sup> DMI	0.21 (14.7)	-0.12 (20.4)	-0.58 (21.3)	-0.47 (25.9)	0.398	0.177	0.784	0.594

<sup>1</sup>Estimated n-alkane intake was determined using the recovery rates presented in Dillon (1993).

<sup>2</sup>Estimated n-alkane intake was determined using an average of all recovery rates calculated in this experiment.

<sup>3</sup>Estimated n-alkane intake was determined using average recovery rates for each treatment calculated in this experiment.

\*Mean discrepancy was found to be significantly different to zero.

**Table 3: Mean herbage and PMR concentrations (mg/kg DM) of n-alkanes C<sub>27</sub>-C<sub>35</sub> (excluding C<sub>34</sub>). Data are means of samples collected daily during the 5-day measurement period.**

n-alkane	Herbage	PMR	SED <sup>1</sup>	P-value (Feed type)
C <sub>27</sub>	24.1	10.1	0.91	<0.001
C <sub>28</sub>	2.4	2.5	0.18	0.531
C <sub>29</sub>	112.1	63.8	3.75	<0.001
C <sub>30</sub>	6.0	5.8	0.28	0.477
C <sub>31</sub>	189.3	144.8	6.19	<0.001
C <sub>32</sub>	6.4	3.9	0.29	<0.001
C <sub>33</sub>	105.5	9.9	2.79	<0.001
C <sub>35</sub>	11.7	0.1	0.44	<0.001

<sup>1</sup>SED= standard error of the difference.



**Table 4: Mean fecal n-alkane recovery rate coefficients of alkanes C<sub>27</sub>-C<sub>35</sub> (excluding C<sub>34</sub>) when cows were offered different amounts of herbage and PMR.**

Amount of Herbage	8 kg DM/cow per day		14 kg DM/ cow per day		<i>P</i> -value				
	Low	High	Low	High	Average	SED <sup>1</sup>	Amount of Herbage	Amount of PMR	Herbage × PMR
n-alkane									
C <sub>27</sub>	0.59	0.60	0.69	0.69	0.64	0.195	<0.001	0.896	0.780
C <sub>28</sub>	0.75	0.75	0.93	0.87	0.82	0.214	<0.001	0.221	0.180
C <sub>29</sub>	0.70	0.70	0.80	0.79	0.74	0.200	<0.001	0.862	0.805
C <sub>30</sub>	1.01	0.96	1.20	1.14	1.07	0.268	<0.001	0.146	0.985
C <sub>31</sub>	0.80	0.80	0.90	0.89	0.85	0.210	0.002	0.896	0.869
C <sub>32</sub>	0.91	0.92	0.94	0.98	0.93	0.228	0.120	0.290	0.532
C <sub>33</sub>	0.87	0.90	0.95	0.96	0.92	0.219	0.013	0.463	0.867
C <sub>35</sub>	0.88	0.92	0.95	0.96	0.93	0.205	0.034	0.279	0.552

<sup>1</sup>SED= standard error of the difference.

**Table 5: Mean fecal concentrations (mg/kg DM) of alkanes C<sub>27</sub>-C<sub>35</sub> (excluding C<sub>34</sub>) when cows were offered different amounts of herbage and PMR.**

Amount of Herbage	8 kg DM/cow per day		14 kg DM/cow per day		SED <sup>1</sup>	<i>P</i> -value		
	Low	High	Low	High		Amount of Herbage	Amount of PMR	Herbage × PMR
n-alkane								
C <sub>27</sub>	43.4	34.7	56.0	41.3	1.93	<0.001	<0.001	0.050
C <sub>28</sub>	7.6	7.0	9.2	7.4	0.47	0.009	0.004	0.085
C <sub>29</sub>	259.2	217.4	315.6	243.7	9.27	<0.001	<0.001	0.042
C <sub>30</sub>	24.5	20.9	28.7	23.2	0.81	<0.001	<0.001	0.109
C <sub>31</sub>	558.4	486.7	642.5	516.9	17.64	<0.001	<0.001	0.054
C <sub>32</sub>	210.0	142.4	154.1	115.3	8.67	<0.001	<0.001	0.038
C <sub>33</sub>	226.7	157.5	296.8	194.9	7.80	<0.001	<0.001	0.013
C <sub>35</sub>	22.9	15.2	30.2	19.0	2.23	0.005	<0.001	0.294

<sup>1</sup>SED= standard error of the difference