Utilization of the n-Alkane Marker Technique to Estimate Dry Matter Intake and Digestibility in Grazing Dairy Cows

A Thesis Submitted to

The Faculty of Graduate Studies

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by

Heather Michelle Froebe

In partial fulfilment of requirements for the degree of

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Department of Animal Science

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FACULTY OF GRADUATE STUDIES

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UTILIZATION OF THE N-ALKANE MARKER TECHNIQUE TO ESTIMATE DRY MATTER INTAKE AND DIGESTIBILITY IN GRAZING DAIRY COWS

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Studies were conducted to evaluate the potential use of the n-alkane marker technique as a tool to improve the nutrition of grazing animals. A grazing experiment consisting of two completely randomized trials was conducted to compare the n-alkane marker technique to the well-accepted Cr_2O_3 marker technique for estimating dry matter intake and digestibility. Each trial had 15 primaparous Holstein lactating cows that were randomly allocated to one of three dietary treatment groups, with concentrate fed at 20% (L), 35% (M) or 50% (H) of pre-trial total dry matter intake (DMI). Cows grazed a primarily orchardgrass (*Dactylis glomerata* L.) pasture and were moved to a new section of pasture daily. Indigestible faecal markers, Cr_2O_3 and n-alkane (C_{32} and C_{36}) controlled-release capsules were placed into the cow's reticulo-rumen by oral administration. There was a trend ($P < 0.10$) for pasture forage DMI to decrease with increasing concentrate levels when intake was estimated using the C_{31}:C_{32} ratio. Pasture forage DMI estimations were similar across dietary treatments using the Cr_2O_3 marker technique. Using the Cr_2O_3 marker, total DMD estimates were significantly different, with H having the highest and L having the lowest. No differences were observed for total diet DMD estimates when using the n-alkane technique. Three Jersey steers were fed freshly cut forage from the same pasture to establish in vivo n-alkane recovery rates. The mean n-alkane recovery rates were high, averaging 97.8 ± 1.7, 97.4 ± 2.4 and 90.5% ± 1.4 for C_{29}, C_{31} and C_{33}, respectively. An in vitro method to determine n-alkane recovery rates was tested and results were compared with the actual in vivo n-alkane recovery rates. Samples underwent a modified in vitro DM digestibility (IVDMD) procedure using bovine rumen

fluid and incubation times of 48 and 72 h for mixed forage samples and 48 h for individual plant species. The n-alkane recovery rates were 20.6 to 37.5 percentage units lower ($P > 0.05$) for in vitro vs. in vivo due to a major loss of n-alkanes in the filtration step. There is potential to use the n-alkane marker technique to estimate DMI and DMD for certain forage species, but commercial application of this method would require a rapid estimate of n-alkane recovery rates. The IVDMD procedure used in this experiment did not produce good estimates of n-alkane recovery rates.

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ABBREVIATIONS

ADF	acid detergent fibre
BW	body weight
Ca	calcium
CP	crude protein
d	day
DM	dry matter
DMD	dry matter digestibility
DMI	dry matter intake
g	gram
h	hour
ha	hectare
IVDMD	in vitro dry matter digestibility
kg	kilogram
km	kilometre
L	litre
m	metre
mg	milligram
min	minute
NDF	neutral detergent fibre
P	phosphorus
TMR	total mixed ration
°C	degree Celsius

INTRODUCTION

Dairy producers today are looking for ways to decrease costs and increase efficiency of production. With increased feed and other farm expenses, pasture is being re-examined as a method to reduce the costs of production. Pasture can be the cheapest source of nutrients for lactating dairy cows and can contribute to the competitiveness of milk production, preservation of the rural landscape and projection of a good image of dairy production. Also, a pasture season may provide many animal welfare benefits, such as clearing-up joint problems and developing muscle tone.

Profitable milk production from pasture depends on many factors, including the amount of pasture forage available, the rate of intake of forage and concentrate, and the nutritional composition of the forage and concentrate. The major constraint to grazing lactating dairy cows in Manitoba is the quota system, as herd milk production must remain constant on a year-round basis. This is a challenge for Manitoba dairy producers because it is very difficult to estimate the nutrient intake of grazing cows and, therefore, difficult to provide an adequate supplementary feeding program that will ensure a constant and high level of milk production.

Full exploitation of grazing requires the development of grazing systems designed to maximize daily forage intake per cow and improve the efficiency of nutrient use through supplementary feeding. Concentrates are generally provided to grazing dairy cows to increase total energy intake and animal performance above that which can be produced from pasture alone. However, a supplementary feeding program must be continuously adjusted, as the amount and quality of pasture forage available varies throughout the grazing season. Continuous adjustment is only possible if individual animal forage intake can be estimated on a regular basis. The use of an indigestible marker, such as the n-alkane marker technique, may

provide the ability to monitor individual animal intake behaviour on pasture in response to management strategies.

The n-alkane marker technique uses a combination of an internal and external marker to estimate intake (Mayes et al. 1986; Dove and Mayes 1991), digestibility (Unal and Garnsworthy 1999) and botanical composition (Hameleers and Mayes 1998) of the diet. The n-alkanes are simple straight-chain hydrocarbons that are present in the cuticular wax of plants (Tulloch 1976) and are principally indigestible. Naturally occurring n-alkanes found in most pasture species contain odd-numbered carbon chains in the range of C_{25} (pentacosane) to C_{35} (pentatriacontane). Shorter chain length n-alkanes can be detected but are usually present in much smaller quantities. Even-chain n-alkanes are present in very low concentrations (Mayes et al. 1986). Since plant n-alkanes have odd-numbered chain lengths, the n-alkanes with even-chain lengths may be fed as external markers. The most commonly dosed n-alkanes are dotriacontane (C_{32}) and hexatriacontane (C_{36}).

Individual n-alkanes differ in concentration for each forage plant species, resulting in each species having a unique n-alkane profile, with hentriacontane (C_{31}) and tritriacontane (C_{33}) usually as the major components (Dove and Mayes 1991). The extraction, purification and quantitative separation of the n-alkanes by gas chromatography is a relatively simple procedure and involves less work than other techniques, like the chromium technique (Marais 2000).

A major advantage of the n-alkane marker technique is that it accommodates differences in diet digestibility between individual animals, rather than relying on a single in vitro estimate of digestibility used by the chromium technique. The technique is well-suited to sheep consuming both pasture forage and supplement because the technique accounts for any

interaction between pasture forage and supplement in the digestive tract. These interactions are commonly referred to as the "associative effect" (Dove et al. 2000).

The first experiment was conducted to evaluate the n-alkane marker technique to estimate dry matter intake (DMI) and dry matter digestibility (DMD) of individual lactating grazing dairy cows consuming three levels of a barley-based concentrate. Level of concentrate was used to determine milk production, DMI, DMD and grazing time responses. A second animal experiment was conducted to determine recovery rates for the individual n-alkanes for the pasture forage from the first experiment. These recovery rates were used as correction factors in the DMD calculations in the first experiment. A third experiment was undertaken to develop a laboratory method to estimate in vivo recovery rates of the individual n-alkanes from the pasture forage.

LITERATURE REVIEW

Introduction

Dairy producers today are looking for ways to decrease costs and increase efficiency of production. With increased feed and other farm expenses, pasture is being re-examined as a method to reduce the costs of production. Grazing is the cheapest source of nutrients for dairy cows and contributes to the competitiveness of milk production, preserves the rural landscape and projects a good image of dairy production. Also, a pasture season may provide many animal welfare benefits, such as clearing-up joint problems and developing muscle tone.

Profitable milk production from pasture depends on many factors, including the amount of pasture forage available, the intake of the forage, the nutritional quality of the forage and the supplemental feed provided. The major constraint to grazing lactating dairy cows in Manitoba is the quota system, as milk production must remain constant on a year-round basis. This is a challenge for Manitoba dairy producers because it is very difficult to estimate the nutrient intake of grazing cows and, therefore, difficult to provide an adequate supplementary feeding program that will ensure a constant level of milk production.

Full exploitation of grazing requires the development of grazing systems designed to maximize daily forage intake per cow and improve the efficiency of nutrient use through supplementary feeding. Concentrates are generally provided to grazing dairy cows to increase total energy intake and animal performance above that which can be produced from pasture alone. However, a supplementary feeding program must be continuously adjusted, as the amount and quality of pasture forage available varies throughout the grazing season. Continuous adjustment is only possible if individual animal forage intake can be estimated on

a regular basis. The use of an indigestible marker, such as the n-alkane marker technique, may provide the ability to monitor individual animal intake behaviour on pasture in response to management strategies.

Pasture Characteristics Affecting Productivity of Grazing Dairy Cows

Pasture Forage Quality

Animal productivity on pasture is directly related to pasture forage quality, which is the palatability, nutrient concentration and digestibility of the forage. Forage quality in any given pasture is a function of three separate but related factors; the kind of plants present, their stage of maturity and the time of year. The proportion of legumes to grasses is important, as legumes have a higher digestibility and higher protein level than grasses at a similar physiological stage (Sheaffer et al. 1998). The stage of maturity at which the forage will be grazed has a major influence on the quality of the forage. Forage crops generally decline in nutritive value as they enter the reproductive stage, usually with an increase in acid detergent fibre and neutral detergent fibre concentrations and a decrease in crude protein concentration (Sheaffer et al. 1998). Quality changes with maturity are related to increased stem lignification and an increased proportion of stem vs. leaves on the plants. Leaves of both grasses and legumes contain a much greater concentration of digestible nutrients than do stems. Additionally, the proportion of leaves declines over time, if a pasture is not managed to maintain vegetative growth (Albrecht and Hall 1995). The ratio of live:dead plant material will also affect forage quality. When pastures are not grazed down low enough or grazed unevenly in a rotationally grazing system, dead plant material is leftover for the next grazing period, resulting in lower quality. Many factors affect pasture quality, which will in turn affect

animal productivity. Most dairy cow grazing studies do not list pasture forage quality, listing only the plant species included in the pasture. This makes it very difficult to compare production results relative to forage quality.

Pasture Productivity

The quantity of pasture forage available to the grazing animal depends on plant species included in the pasture and the stage of maturity these plants are managed for. Results from Guelph, Canada show forage yields ranging from 2016 to 3911 kg dry matter (DM) ha^{-1} for orchardgrass (*Dactylis glomerata* L.), 2177 to 6593 kg DM ha^{-1} for timothy (*Phleum pratense* L.) and 2960 to 5861 kg DM ha^{-1} for smooth bromegrass (*Bromus inermis* Leyss.) from early vegetative state to heading (Christie and McElroy 1995). In the USA tall fescue (*Festuca arundinacea* Schreb.) yields ranged from 3000 to 10,000 kg DM ha^{-1} depending on location and management practices (Sleper and Buckner 1995).

As pasture forage yield increases, pasture forage quality decreases at any given grazing period. Pasture forage should be grazed at optimal yield and quality. If the yield is too low, pasture forage DM intake (DMI) will decrease. However, if yield and/or pasture height is too high, pasture forage DMI will also decrease. Therefore, grazing animals should graze the pasture when yield is at optimum levels and the number of grazing animals should be matched to the amount of available pasture forage to ensure optimum pasture forage use and DMI.

There is little published literature regarding individual animal performance or animal output per unit of pasture for high production dairy cows. Recent research has compared milk production from a confined, TMR feeding situation to a grazing situation and has examined

supplementation type and level of supplementation on DMI (Arriaga-Jordan and Holmes 1986; Jones-Endsley et al. 1997).

Animal Factors Influencing DM Intake, Digestibility, Behaviour, Pasture Forage Selection and Milk Production

Factors Affecting Pasture Forage DMI

Animal physiological factors have been identified as one of the many factor that influence DMI. Over the short-term, DMI is controlled by a combination of plant structural factors that influence rate of ingestion and the effect of the masticated forage on gut fill (Forbes 1988). In dairy cows fed forages, physical limitation of the digestive tract has been proposed as the main constraint to obtaining higher DMI (Chilibroste et al. 2000; Waldo 1986). In the grazing animal, DMI is usually dominated by the effects of plant cell wall material in the digestive tract, especially the rate at which digesta particles can leave the rumen (Dove 1996b). Forage particles will only leave the rumen when reduced in particle size, so cell wall material will contribute to a slower particle passage rate and, therefore, a lower DMI. In a grazing situation, sward height may limit DMI if it is too high or too low (Forbes 1988). Marshall et al. (1998) found an optimal sward height of to be between 12 and 15 cm for a mixed grass and legume pasture. High pasture forage cell wall content and sward height that is too low or too high will limit DMI.

Animals eat principally to supply their tissues with the nutrients required to fuel the physiological processes of maintenance, growth (including fat deposition in mature animals), reproduction, milk production and work. Therefore, consumption of a particular feed will increase with increased milk yield providing that intake is not constrained by some

characteristic of the feed (Beever et al. 2000). Over the long-term, DMI is controlled by the energy balance of the animal. Blood concentrations of fermentative end products have also been postulated to control DMI in the grazing ruminant (Chilibroste et al. 2000).

Lactating dairy cows cannot consume enough pasture forage DM to meet the nutrient requirements of higher milk production (Reis and Combs 2000b). Therefore, concentrates are generally provided to grazing dairy cows to increase total energy intake and animal performance above that which can be produced from pasture forage alone. A group of Holstein cows grazing high quality pasture forage consisting of perennial ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.) and mixed grasses consumed on average 19 kg DM d^{-1} and produced 29.6 kg milk d^{-1} (Kolver and Muller 1998). Pasture forage availability did not limit total DMI, as pregrazing and postgrazing pasture forage biomass was 3081 and 1597 kg ha^{-1}, respectively. Based on NRC estimates, grazing cows in this study would only be able to support milk production at 28.8 kg d^{-1} with a small loss in body condition score. The cows in this study had significant mobilization of energy reserves indicating that supplemental energy may be required to achieve milk production greater than 30 kg d^{-1} in intensive grazing systems with similar pasture forage quality (Kolver and Muller 1998).

A major challenge to utilizing pasture efficiently is adjusting the supplementary feeding program to the amount and quality of forage that is available and consumed during the grazing season. It is difficult to estimate the nutrient intake of grazing cows because of their selection preferences on pasture. This makes supplementation for high producing dairy cows difficult, but maintaining adequate nutrient intake is essential for high milk production (NRC 2001).

Supplementation programs for high producing dairy cows on pasture are difficult due to the variability of responses on forage DMI. In one study, lactating Holstein cows grazing an alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), orchardgrass and smooth bromegrass pasture (approximately 50% legumes and 50% grasses), receiving 0, 5, or 10 kg d^{-1} of a corn-based concentrate had an average increase of 0.6 kg total DMI and a decrease of 0.6 kg of pasture forage DMI for each kg of concentrate consumed (Reis and Combs 2000b). Berzaghi et al. (1996) found pasture forage DMI to decrease 0.59 kg for each kg of concentrate consumed by Holstein cows grazing a tall fescue, white clover, Kentucky bluegrass and orchardgrass pasture while receiving 0 or 6.4 kg of corn-based concentrate. Pulido and Leaver (2001) found pasture forage DMI to decrease 1.12 kg for each kg of concentrate consumed by lactating Holstein Friesian cows grazing perennial ryegrass and receiving 0 and 6 kg d^{-1} of a barley-based concentrate. These studies found that energy-based concentrates decrease pasture forage DMI by 0.6 to 1.1 kg DM kg^{-1} concentrate consumed. However, no difference in forage DMI was found when Jones-Endsley et al. (1997) used a protein supplement. The protein supplement was offered at 6.4 or 9.6 kg DM d^{-1} to cows grazing an alfalfa and orchard grass pasture.

Meijs and Hoekstra (1984) compared a low (16 kg organic matter (OM) cow^{-1} d^{-1}) and a high (24 kg OM cow^{-1} d^{-1}) pasture forage biomass for Dutch Friesian cows grazing perennial ryegrass, receiving either a low (0.8 kg cow^{-1} d^{-1}), medium (3.2 kg cow^{-1} d^{-1}) or high level (5.6 kg cow^{-1} d^{-1}) of concentrate. Daily pasture forage OM intake (OMI) per cow at the low pasture allowance did not differ for the three levels of concentrate, averaging 10.9, 10.6 and 10.4 kg. At the high pasture allowance, daily pasture forage OMI was higher and was influenced by supplementation, averaging 14.8, 13.6 and 12.4 kg, respectively for the low,

medium and high concentrate levels. The daily DMI of lactating Jersey and Friesian cows grazing pastures dominated by paspalum (*Paspalum dilatatum* L.) increased by 1.11 kg DM cow^{-1} for each additional t DM ha^{-1} of pasture forage biomass (Stockdale 1985). These research studies all showed that pasture forage intake increased as pasture forage biomass increased. However, the majority of these studies were conducted on lower producing cows.

Grazing management strategies also affect pasture forage intake, as demonstrated by Kitessa and Nicol (2001), who found higher OMI ($P < 0.05$) for Hereford X Angus yearling heifers rotationally grazing a perennial ryegrass and white clover pasture than those continuously grazing a similar pasture. Hirschfeld et al. (1996) also found higher OMI ($P < 0.05$) for crossbred steers rotationally grazing a blue grama (*Bouteloua gracilis* (H.B.K.) Lag. ex Steud.) and Kentucky bluegrass (*Poa pratensis* L.) pasture than those continuously grazing a similar pasture. These two research studies indicated that higher pasture forage intake can be achieved with rotational grazing in comparison to continuous grazing.

Effect of Supplementation on Forage DM Digestibility

Researchers are interested in determining DM digestibility (DMD) for individual animals and how DMD is affected by supplementation. Total diet DMD was 61.4% for lactating Holstein cows rotationally grazing an alfalfa, red clover, orchardgrass and smooth bromegrass pasture (approximately 50% legumes and 50% grasses), and receiving 9 kg d^{-1} of a corn-based concentrate (Reis and Combs 2000a). In a second study, cows on that same pasture receiving 5 kg of a corn-based concentrate had 7.6% higher total diet DMD over non-supplemented cows, while those receiving 10 kg concentrate were 10.8% higher than the group receiving 5 kg concentrate, as estimated by the Ytterbium marker technique (Reis and Combs 2000b).

This grazing study suggested that total diet DMD increases when energy-based concentrates are added to the diet of grazing dairy cows.

Gekara et al. (2001) found that as supplementation increased, pasture forage DMD decreased ($P < 0.05$) for lactating Angus beef cows. The cows grazed a Kentucky bluegrass, orchardgrass, white clover and red clover pasture, and received either no supplement or a corn-based concentrate at low (29% of total DMI) or high (56% of total DMI) levels. The pasture forage DMD was 72.6%, 69.9% and 63.2% for no supplement, low and high, respectively, as estimated by the Ytterbium marker technique. As energy-based concentrates increase, pasture forage DMD decreases.

Grazing Behaviour

The day of the grazing animal is split into alternating periods of grazing, ruminating and resting. Jersey X Friesian lactating cows strip-grazing pasture consisting of paspalum, ryegrass and white clover were found to spend 40% of their time grazing (9.6 h), 27% ruminating (6.5 h), 23% resting (5.5 h) and 10% at the dairy (Stockdale and King 1983). The duration and distribution of these periods may be influenced by supplemental feeding, sward conditions, grazing management and weather (Arnold 1981).

Grazing is not exclusively confined to the daylight hours in any part of the season. Grazing lactating dairy cows have two main grazing bouts: one in the morning and one in the evening. This pattern of grazing may be due to a short-term fasting effect during milking (Rook et al. 1994). Phillips and Leaver (1986) studied the grazing behaviour of lactating cows from April 15 until September 30 and 54% of grazing was between the afternoon and morning milking and 46% between morning and afternoon milking. There is a tendency for

high milk producers to have the longest periods of grazing between dusk and dawn (Stobbs 1970). Stobbs (1970) found that the distribution of grazing during the day followed a definite pattern that did not vary widely from cow to cow. The grazing patterns of the group are usually similar and the main periods of grazing are strongly influenced by the time of sunrise and sunset (Arnold 1981). These grazing patterns are altered with adverse weather conditions, such as wind, rain and heat (Arnold 1985). High rainfall resulted in a reduction of 0.8 h in total grazing time due to the discomfort of grazing in wet weather (Hinch et al. 1982). In moderately wet weather, grazing activity will be changed to a different time of the day (Ruckebusch and Bueno 1978).

Concentrate supplementation reduces grazing time. Stobbs (1970) found that all unsupplemented lactating Jersey cows had a longer mean daily grazing time (9.90 h) than those fed 4 kg of hammer-milled sorghum (8.47 h) when grazing a pure stand of a tropical legume, *Phaseolus atropureus* (DC). All unsupplemented cows had a longer grazing time during the hours of darkness (Stobbs 1970). This has also been shown in high producing (averaging 32.3 kg milk d^{-1}) lactating Friesian cows grazing perennial ryegrass and white clover receiving either 1 kg or 6 kg of a barley-based concentrate. The cows receiving 6 kg of concentrate grazed 0.82 h less than those receiving only 1 kg of concentrate (Arriaga-Jordan and Holmes 1986). These studies clearly show that grazing animals receiving concentrate spend less time grazing.

There is a trend towards longer total grazing time with rotational grazing compared to continuous grazing. Walker and Heitschmidt (1989) compared Angus X Hereford crossbred cows continuous grazing, rotational grazing on 14 paddocks (RG-14) and rotational grazing on 42 paddocks (RG-42). The total grazing times were 9.55, 9.85 and 10.88 h per cow per

day for CG, RG-14 and RG-42, respectively. Phillips and Denne (1988) found that lactating Friesian dairy cows continuously grazing perennial ryegrass had a total grazing time of 9.27 ± 2.22 h per cow per day. Holstein Friesian dairy cows continuously grazing similar pasture had a higher total grazing time of 10.53 ± 0.34 h (Gibb et al. 1998). These studies did not include any supplementation. Charolais cows, with similar DMI, had grazing times of 7.97, 7.63 and 6.60 h when grazing orchardgrass with sward heights of 7.3, 10.3 and 20.5 cm (Ferrer Cazcarra et al. 1995).

In summary, a number of factors influence time spent grazing. Time spent grazing is higher with rotational grazing versus continuous grazing and decreases with increasing sward height. Energy-based concentrate supplementation on pasture decreases grazing times. Increasing grazing time is the main response mechanism exhibited by cows coping with either changes in their physiological status (Chilibroste et al. 1997) or with restrictive sward conditions (Gibb et al. 1997).

Grazing Cattle and Plant Selection

Cattle graze by using their tongues to gather the forage into the mouth before biting and tearing it off, unless the plant is too short (Arnold 1985). They move their muzzles in a horizontal plane when grazing all but very long forage, in which case they will either sever the tops of the plants or plunge their muzzle into the mass and tear off a mouthful (Voisin 1988).

Grazing animals have the opportunity to change the composition of their diet by selecting different plant parts or plant species. These different parts or species may differ in nutritional values, therefore the diet composition is as important as the intake. Herbivores do exert choice in consuming plant species and plant parts from the available forage. Studies have

shown that cattle consume pasture forage of higher nutritional quality than that of the total pasture forage available (Coleman and Barth 1973; Schlegel et al. 2000). Coleman and Barth (1973) examined grazing seasons (May-October) for three years. Steers either grazed a tall fescue and Korean lespedeza (*Lespedeza stipulacea* (Maxim.) Makino) pasture or an orchardgrass and ladino clover (*Trifolium repens* L.) pasture. The steers selected a diet that was more digestible with higher forage CP and lower forage ADF concentrations, throughout the grazing season.

Diet selection is a complex behavioural act that is influenced by several factors. Physiological condition, degree of hunger, topography, other animals, and present and past grazing experience can influence what and how much is consumed (Kreuger et al. 1974). Diet selection involves a hierarchy of decisions by the grazing animal relative to the spatial assemblage of plants across a landscape that includes plant community, patch, feeding station and plant (Senft et al. 1987). These animals can vary both the quantity and quality of ingested forage because the botanical composition of the consumed forage, and thus nutrient composition, differs from the pasture plant species profile (Dove and Mayes 1996). Due to the variability in diet selection, studies on grazing herbivore nutrition have been hampered by difficulties in the estimation of nutrient intake.

Effect of Supplementation on Milk Production

The effect of concentrate supplementation on milk production by grazing cattle has been widely studied. There have been a variety of responses to supplementation. Hoden et al. (1991) reported an increase of 0.6 kg milk kg^{-1} of concentrate, when Holstein and Normandy lactating dairy cows were grazing a perennial ryegrass, rough meadow grass (*Poa trivialis* L.)

and fine bent (*Agrostis tenuis* Sibth.) pasture, while receiving 3.7 kg of a beet pulp, maize and wheat concentrate. Responses of up to 1.9 kg milk kg^{-1} of concentrate have been reported, when there was a severe grass restriction of 25 g DM kg BW^{-1} in a strip-grazing situation and concentrate was offered at 2 kg DM cow^{-1} d^{-1} (Le Du and Newberry 1982). Holstein cows grazing tall fescue, white clover, Kentucky bluegrass and orchardgrass and receiving 0 or 6.4 kg of a corn-based concentrate had milk production increase 0.65 kg milk kg^{-1} of concentrate (Berzaghi et al. 1996). Cows that consumed only the alfalfa, red clover, orchardgrass and smooth bromegrass pasture (approximately 50% legumes and 50% grasses) produced 18.7 and 28.3% less milk, respectively, than did cows supplemented with 5 kg and 10 kg of a ground dry shelled corn concentrate (Reis and Combs 2000b). This is a milk production response of 1.0 and 0.86 kg milk kg^{-1} concentrate for 5 kg and 10 kg supplementation regimes, respectively. Concentrate supplementation increases milk production, however, the size of the response varies from an increase of 0.6 to 1.9 kg milk kg^{-1} of concentrate. Response is thought to be influenced by the quantity and quality of grass available, the cow's requirement for nutrients and the type of concentrate offered (O'Brien et al. 1999). However, many of these studies did not describe the pasture forage quantity or quality and, therefore, support for O'Brien et al.'s hypothesis is not available.

Estimation Methods

Estimating DMI of Pastured Cattle

The daily DMI for an animal is a critical measurement needed in order to formulate a balanced diet to achieve a particular animal response. Many techniques have been used to estimate

DMI, but the most precise techniques have either a large labour component or interfere with normal grazing behaviour.

Estimating the forage DMI of grazing animals is difficult and has been estimated from the amount of forage available before and after grazing (Walters and Evans 1979), from measurements of bite rate, bite size and feeding time (Forbes and Hodgson 1985) and from short-term changes in live weight (Penning and Hooper 1985).

Due to the difficulty of making direct determinations of DMI of grazing animals, a number of indirect methods have evolved. These methods are based on measuring faecal excretion and the digestibility of the respective forage. Total faecal collection is measured by the use of harness and collections bags and is labourious, inconvenient and may disrupt normal grazing behaviour. To avoid this, faecal output is often estimated from the dilution of an indigestible marker given to the test animals. This marker is assumed to be excreted in a specific known pattern and is quantitatively recovered in the faeces.

The easiest way to estimate DMI is to calculate the difference in amount of pasture forage DM available before and after grazing. The reduction of available pasture forage DM observed in a paddock due to grazing is divided by the product of the number of animals and days grazed. However, this technique only gives an estimate for the group of animals and the grazing period must be short (1 to 3 d) or cages must be used in the pasture to estimate the growth rate of the pasture for the period in question. There is potential error due to trampling loss.

Individual animal DMI are desired and can be obtained from the product of biting rate, bite size and grazing time. Biting rate is measured with a chewing meter (Luginbuhl et al. 1987) and grazing time is measured with a vibracorder (Stobbs 1970). The major limiting

factor is that oesophageal-fistulated animals are required for bite size determination and this is not practical for a lactating cow, as it will interfere with production. Other problems are the size of bite can vary greatly, the technique has a short observation period and the observation methods may disturb the natural behaviour of animals.

Concentrate intake can be measured if it is fed individually to cattle in the barn. Lab analysis testing of the concentrate, combined with known intake will provide the nutrient intake that the animal is receiving from it. Concentrate intake also can be estimated by labelling the concentrate with a marker and, depending on the type of marker, monitoring its concentration in the faeces, blood plasma or body H_2O (Mayes and Dove 2000). Monitoring concentrate intake is most accurate if it is fed individually to cattle.

Monitoring DMD on Pasture

Pasture forage DMD is usually estimated by obtaining clipped forage samples and conducting in vitro DMD analyses (Tilley and Terry 1963). Samples are incubated in rumen fluid in the lab and the amount of sample left after incubation is compared to the starting amount of sample. The rumen fluid is typically from one animal, so the animal-to-animal variation in DMD is not represented in this method. Obtaining samples that represent the forage selected and consumed by the animal is not easy. The two basic approaches in obtaining a representative diet sample are the manual collection of forage samples by the experimenter or the use of surgically altered (rumen or oesophageal cannula) animals (Le Du and Penning 1982). However, individual animals may also select a diet different than the oesophageal-fistulated animal selected (Dove and Mayes 1991). In vitro DMD is a good method for comparing forages or pasture types, not animal responses. The best sampling technique to use

to obtain representative diet samples for IVDMD is the oesophageal-fistulated animal, but it is not practical to use with lactating animals.

In vivo DMD is another digestibility technique that can be used. It measures digestibility using the animal and takes into account both animal and plant factors. Feed consumed and faeces excreted must be accurately measured for this calculation. The in vivo DMD method is good for comparing feeds, but does not give individual animal results.

These digestibility estimates are then used on all the test animals, regardless of differences there may be in individual levels of intake or supplementation, or class of animal. Neither technique gives individual animal results.

Monitoring Behaviour on Pasture

Most observations on grazing bout length are conducted visually over short periods of one to a few days. Grazing behaviour observations are usually made every 15 min (Stockdale and King 1983) and are based on the assumption that the behaviour remains the same until the next observation. It is difficult to make these observations at night, so infrared equipment has even been used for night observation (Castle et al. 1950). Visual observations of grazing behaviour is labour intensive, limited by daylight and may disturb the animal's normal grazing patterns.

Continuous recording of animal behaviour is possible with the use of a vibracorder (Stobbs 1970). Vibracorders are recorders that were developed for logging truck operating times. The vibracorder uses a vibrating pendulum, the movement of which is recorded by a stylus onto a circular wax chart driven by a clockwork motor. The pendulum responds to movements of the animal's head or body, but the unit is mounted in such a way that it is only

activated when the animal is in the head-down position (and presumed grazing) position. A visual assessment of these wax charts determines grazing time. Vibracorders provide a simple way of measuring grazing time and are likely to be particularly valuable where labour is scarce and small differences in grazing behaviour are to be measured (Ruckebusch and Bueno 1978). Vibracorders are expensive to purchase and maintain and must be sealed in wet weather. Vibracorders can overestimate grazing time by including short periods of activity as grazing time (Coleman et al. 1989).

Monitoring Species Selection

Grazing animals have the opportunity to alter the composition of their diet in mixed species pasture by selecting different plant species, under most conditions. Plant species vary in nutritive value, so the composition of the consumed pasture forage may be as important as pasture forage DMI. It is difficult to establish the species composition of both the sward and the pasture forage consumed by the grazing animals.

Sward composition can be estimated by visual scoring systems and physical separation of hand-harvested pasture samples (Cook and Stubbendieck 1986), but these techniques are labourious and do not provide an estimate of the species composition of the consumed forage. Analysis of rumen contents can estimate species composition, but this involves sacrifice of the animal (Smith and Shandruk 1979). Researchers have also used faecal analysis for evaluating forage consumed, but accuracy is a problem because forage species passed in the faeces are often not proportional to those consumed (Smith and Shandruk 1979).

Microscopic and hand-separation methods have been used to estimate the botanical composition of extrusa from oesophageal-fistulated animals (Dove 1996a), but these are

tedious and difficult. Oesophageal-fistulated animals graze similar pastures as test animals and then are held without food for a period of time. These animals are taken to the test pasture and allowed to graze for approximately 20 min, after which a sample is collected from the bag attached to the fistula. Due to the short grazing period that is sampled, the diet selected may be different than that of the test animals that may be grazing the area for days or even weeks. The diet selected by the oesophageal-fistulated animals may differ from that of the test animals; due to the animals being surgically prepared, handled and managed differently or being in a different physiological state (Mayes and Dove 2000). Oesophageal-fistulating lactating dairy cows also is not practical because of its severe interference in the production of lactating cows (Malossini et al. 1994).

Use of Markers to Estimate DM Intake and Digestibility

Markers are used to estimate DM intake and digestibility. Ideal markers should: be inert with no toxic effects; be neither metabolised nor absorbed in the gastro-intestinal tract; have no appreciable bulk; mix intimately with and remain uniformly distributed in the digesta; not have any influence on the microflora of the gastro-intestinal tract; not have influence on gastro-intestinal secretions, digestion, absorption or normal motility; and have physicochemical properties, readily discernible throughout the gastro-intestinal tract, which allow ready, precise quantitative measurement (Kotb and Luckey 1972).

Markers are classified as internal or external markers. An internal marker is a chosen substance that forms an integral part of the forage or feed consumed by the animal. Examples of internal markers include indigestible acid detergent fibre (IADF), indigestible lignin and odd-chain n-alkanes. External markers are indigestible substances which are either added to

the diet or administered orally or intraruminally to the animal. Examples of external markers include chromium sesquioxide (Cr_2O_3) and even-chain n-alkanes.

Marker procedures use one of two types of dosing. The marker can be administered as a pulse-dose, where the animal receives one dose of the marker that is followed by frequent faecal collections in order to characterize the pulse in marker concentration found in the faeces. The characteristics of the excretion curve make it possible to estimate passage rate, faecal output and forage intake, with a digestibility estimate (Owens and Hanson 1992). The other type of dosing involves administration of the marker at a constant or frequent rate for a period of days to reach steady state conditions (Owens and Hanson 1992). An adaptation period of 6 and 8 days for sheep and cattle, respectively, is required before the collection period. The oral administration of markers to animals is often carried out using once or twice daily dosing with marker-impregnated paper pellets (Sibbald et al. 2000), gelatin capsules containing marker suspended on cellulose powder (Dove and Mayes 1991; Vulich et al. 1991), or as an aqueous suspension of marker-impregnated grass particles (Marais et al. 1996). In some instances, a marker can be sprayed onto the forage (Ciavarella et al. 2000). To reduce the labour required for daily or more frequent dosing, an intraruminal controlled-release capsule (CRC) has been developed (Captec, Nufarm, Auckland, New Zealand).

The CRC was developed to overcome the difficulties with once or twice daily dosing, as well as the diurnal variation in output of the marker. This substantially reduces the disturbance to the animals and allows the use of a larger number of animals (Dove and Mayes 1996). It is recommended that data collection occur between d 8 and d 14 after dosing (Berry et al. 2000). Laby et al. (1984) found a single, uniform marker release rate for the Cr-CRC with grazing and penned sheep, independent of diet. Adams et al. (1991) had estimates of daily

faecal DM output from the Cr-CRC within 1% of total faecal collection results for steers grazing irrigated tall wheatgrass (*Elytrigia pontica* [Podp.] Holub). The n-alkane-CRC was found to be an accurate method for estimating forage DMI of Brown Swiss lactating cows consuming a known amount of forage with (10.4 kg DM d^{-1}) or without concentrate (Berry et al. 2000).

Markers can be used to estimate DMI, using the following equations (Marais 2000):

Faecal DM output (kg d^{-1}) = M_d (g d^{-1})/M_f (g kg^{-1} DM)

DM intake (kg d^{-1}) = faecal output (kg DM d^{-1})*[100/(100 - %IVDMD)]

where M_f and M_d are marker concentrations (mg kg^{-1} DM) in faeces and diet respectively.

Markers can be used to estimate digestibility, using the following equation (Unal and Garnsworthy 1999):

Digestibility (%) = [M_f (g kg^{-1} DM) – M_d (g d^{-1})]/M_f (g kg^{-1} DM)

where values for M_f are corrected for incomplete recovery of markers. Values for M_d are calculated from the marker concentration of individual components and the proportion of these components in the diet actually consumed.

These equations are based on the assumption that the marker is completely inert and not absorbed and that marker concentration has reached a steady state in the digestive tract. Furthermore, if an external marker is used, feed intake should be known in order to establish feed marker contribution.

Many markers have been evaluated and used in DM intake and digestion studies. Acid-insoluble ash is an internal marker for estimating digestibility in ruminants by using the ash fraction in feed that was insoluble in boiling HCl. However, contamination of feed and faeces samples with dust or soil could lead to erroneous results (Marais 2000). Indigestible acid-

detergent fibre (IADF) and lignin are common internal markers that are used to estimate forage digestibility in cattle (Sandberg et al. 2000). The usefulness of IADF as an internal marker has been limited by a lack of standardized analytical procedures, as lignin has a low and inconsistent recovery in the faeces (Marais 2000).

Attempts have been made to use markers to determine plant selection by grazing animals. Playne et al. (1978) used forage calcium content to distinguish legume from grass in oesophageal samples collected by grazing cattle. Naturally occurring ^{13}C and ^{12}C isotopes have been used to estimate the proportions of C_3 and C_4 plant species in the diet (Jones et al. 1979). However, these approaches do not allow the separation of mixtures to the level of individual plant species.

Chromium sesquioxide (Cr_2O_3), an insoluble metal oxide, is one of the most commonly used markers to estimate faecal DM output. Faecal DM output is required to calculate DMI when digestibility estimates are available, usually from the in vitro DM digestibility technique (IVDMD). The main disadvantage of this approach is that the IVDMD technique does not account for changes in digestibility of a forage due to the level of intake, supplementary feeding or parasite burden of the animal (Marais 2000). A source of error with the chromium technique is the unknown recovery of the marker and the adoption of a single value for the pasture forage digestibility, which, in effect, could vary between animals. Therefore, if concentrates are used, it is necessary to know the relevant proportion of faecal DM output that can be attributed to them (Malossini et al. 1996). This method only provides group estimates, as it cannot account for digestibility differences of individual animals.

The above mentioned marker techniques all have limitations. However, researchers believe that a relatively new marker technique may be able to estimate DM intake, digestibility

and diet selection for individual animals (Dove and Mayes 1996). This technique is known as the n-alkane marker technique.

The n-Alkane Marker Technique

What are n-Alkanes?

The n-alkane technique is a marker technique that uses a combination of internal and external markers (Mayes et al. 1986) to estimate intake (Dove and Mayes 1991), digestibility (Unal and Garnsworthy 1999) and botanical composition (Hameleers and Mayes 1998) of the diet. The n-alkanes are simple straight-chain hydrocarbons that are present in the cuticular wax of plants (Tulloch 1976) and are principally indigestible. Cuticular wax is the waxy layer that covers the plant cuticle, and the waxy layer varies in thickness on different parts of the plants (Tulloch 1976). These waxes are complex mixtures, of which common constituents are long-chain hydrocarbons, alcohols, ketones, fatty and hydroxy-fatty acids and esters (Martin and Juniper 1970). The plant cuticle is a noncellular protective membrane covering the outer layer of tissue of higher plants, which is a barrier to water vapour diffusion, is moderately permeable to water and performs a number of other functions in plants. It protects the plants from injuries due to wind and physical abrasions, frost, radiation, insects and pathogenic fungi (Srivastava and Kumar 1995).

Concentration of n-Alkanes

The occurrence of n-alkanes in forage plant species has been reasonably well-documented (Tulloch 1976), but their potential as markers was not considered until the early-1980s (Mayes et al. 1986). Naturally occurring n-alkanes found in most forage species contain odd-

numbered carbon chains in the range of C_{25} (pentacosane) to C_{35} (pentatriacontane). Shorter chain length n-alkanes can be detected but are usually present in much smaller quantities. Even-chain n-alkanes are present in very low concentrations (Mayes et al. 1986). Since plant n-alkanes have odd-numbered chain lengths, the n-alkanes with even-chain lengths may be used as external markers. The most commonly used n-alkanes for dosing are dotriacontane (C_{32}) and hexatriacontane (C_{36}).

Individual n-alkanes in plant species differ in concentration, resulting in each plant species having a unique n-alkane profile, with nonacosane (C_{29}), hentriacontane (C_{31}) and tritriacontane (C_{33}) usually as the major components (Dove and Mayes 1991). Examples of some plant n-alkane profiles can be found in Table 1. For example, C_{31} was the highest n-alkane concentration (356.4 mg kg^{-1} DM) in alfalfa, while C_{29} was the highest n-alkane concentration (72.4 mg kg^{-1} DM) in birdsfoot trefoil. It has been suggested that the odd-chain n-alkane concentrations should be at least 50 mg kg^{-1} DM for accurate estimations (Sandberg et al. 2000). It is not known if this threshold value is justifiable, as there is no explanation on how this threshold value was established. Laredo et al. (1991) concluded that for some tropical forages, C_{33} was not present in sufficient quantity for intake to be estimated using C_{32}:C_{33} ratios.

Analysis of n-Alkanes

Extraction of n-alkanes from plant or faecal samples involves saponification in alcoholic potassium hydroxide and extraction with non-polar solvents such as heptane (Marais 2000). The extracted n-alkanes are purified by means of a silica gel column, then separated and

Table 1. The concentrations of n-alkanes in pasture forages from Manitoba[z]

	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{36}
	mg kg^{-1} DM										
Orchardgrass	2.0	12.3	2.4	16.3	2.8	23.4	2.3	22.5	3.1	9.3	5.2
Smooth bromegrass	1.1	9.9	4.5	28.9	1.7	34.5	2.1	76.3	2.6	7.7	5.3
Tall fescue	2.4	4.9	3.2	12.3	5.2	75.8	9.9	239.6	7.2	76.5	5.2
Timothy	2.1	18.7	9.4	47.5	4.5	34.2	2.1	15.6	2.4	4.6	5.3
Alfalfa	1.1	5.4	1.3	25.3	7.6	127.7	14.6	356.4	12.4	19.2	5.2
Birdsfoot trefoil	3.2	17.6	4.7	36.1	10.6	72.4	5.4	38.2	4.5	23.0	5.3

[z]Boadi et al. 2002.

quantified by means of capillary gas chromatography (Dove and Mayes 1991). Gas chromatography analysis allows plant (odd) and dosed (even) markers to be determined simultaneously, limiting analytical error and bias (Mayes and Dove 2000). The extraction, purification and quantitative separation of the n-alkanes by gas chromatography are relatively simple procedures and involve less work than other techniques, like the chromium technique (Marais 2000).

Recovery of n-Alkanes

The recovery of the n-alkane marker following passage through an animal's digestive tract is incomplete, however recovery can be estimated by dosing grazing animals with known mixtures of even-chain n-alkanes. Then the relative concentrations of n-alkanes in a faecal sample are compared with the amounts dosed or the even-chain n-alkane output is measured by total faecal collection. Due to the low concentration of even-chain alkanes in the forage, odd-chain n-alkanes can be calculated by interpolation. The most reliable estimates for n-alkane recovery require the use of a separate group of housed animals with known n-alkane intake and for which total faecal collections have been made (Marais 2000).

Mayes et al. (1986) found that faecal recovery of odd-chain n-alkanes from perennial ryegrass increased with increasing C-chain length in sheep, from 71% for C_{27} to 95% for C_{35}. Faecal recovery of C_{31} for freshly harvested meadow forages was 87.2% and was reduced to 70.5% when the same forage was fed as hay to steers (Sandberg et al. 2000). Faecal recovery of C_{31} was lower (78.1%) for alfalfa hay than grass hay (90.9%) in cattle (Ohajuruka and

Palmquist 1991). Faecal recovery rates of n-alkanes may be lower for cattle than sheep and recovery rates vary across forages.

Mayes et al. (1986) found that faecal recoveries of n-alkanes were not significantly affected by diet or feeding level in sheep when they were fed different levels of perennial ryegrass and barley-based concentrate. Ohajuruka and Palmquist (1991) found no differences in faecal recovery of C_{31} due to either fat level or forage type x fat level, when feeding nonlactating Holstein cows alfalfa hay or grass hay with or without 500 g d^{-1} of calcium soap.

An increase in faecal recovery of n-alkanes with increasing carbon chain length has been reported in sheep (Mayes et al. 1986). Dove and Mayes (1996) and Dove and Olivan (1998), on the other hand suggest that faecal recovery of n-alkanes is related to carbon chain length in a curvilinear fashion in sheep. Unal and Garnsworthy (1999) found there was not this increase in faecal recovery of n-alkanes as carbon chain length increased in cattle. However, the study only examined n-alkane recovery rates from C_{32} to C_{36}, whereas most other studies had examined n-alkane recovery rates from C_{27} to C_{36}. Moshtaghi Nia and Wittenberg (2002) examined n-alkane recovery rates from steers consuming hay with and without grain. They found that n-alkane recovery rates for C_{25} to C_{36} across all diets increased with increasing carbon chain length, in a curvilinear fashion. These studies demonstrate that recovery rates of n-alkanes increase as carbon chain length increases.

Mayes et al. (1988) dosed C_{28}, C_{32} and C_{36} to determine the site of hydrocarbon loss in the gastrointestinal tract. They concluded that little disappearance of pasture forage n-alkanes occurred in the fore-stomachs and that absorption occurred in the small intestine in sheep. In contrast, Ohajuruka and Palmquist (1991) estimated that 15% of a ruminally infused synthetic n-alkane marker disappeared in the rumen in cattle. The dose site of C_{32} did influence ($P <$

0.09) C_{32} recovery in the faeces. Recovery was lower with ruminal than with duodenal dosing, when dosed with 300 mg of C_{32} every 12 h (Ohajuruka and Palmquist 1991). Loss of n-alkanes occurs in the rumen in cattle, but in sheep the loss occurs in the small intestine.

Lactating Brown Swiss cows fed a forage mixture containing 51% grass silage, 39% maize silage and 10% hay on a DM basis with or without concentrate (50% of total DMI; 10.4 kg DM) had mean recovery rates of 85 and 87% for C_{33} and C_{32}, respectively, whereas C_{31} recovery was lower, averaging 76% (Berry et al. 2000). They found that C_{31} recovery was too low in comparison with C_{32} to estimate intake correctly and did not have any theories on why the C_{31} n-alkane recovery was too low. Even though the concentrations of C_{33} in the forage and concentrate (44 and 2 mg kg^{-1}) were low, it was more important that C_{33} and C_{32} had similar recovery rates. However, Piasentier et al. (1995) concluded that the opposite was true. Sheep consuming pasture forage only, pasture forage plus maize (26% of DMI) and pasture forage plus maize plus gluten meal (13% of DMI each) diets had faecal C_{31} recovery rates (86.7%) that were closer to C_{32} (89.6%) than C_{33} (85.1%). These studies do not report why the recovery rates were different. More work is required in cattle to obtain further estimates of n-alkane recovery, as cattle seem to be more variable than sheep.

Odd-chain n-alkanes are associated predominately with the particulate matter in digesta and even-chain n-alkanes are predominately associated with the liquid phase (Marais 2000). The presence of concentrate in the diet did not affect the faecal concentrations of dosed n-alkanes (C_{28} and C_{32}), but significantly reduced the concentrations of all other n-alkanes in sheep faeces (Mayes et al. 1986). Increasing the amount of forage significantly decreased the faecal concentrations of all n-alkanes except for C_{35}, when perennial ryegrass was fed to wether lambs at 500 to 900 g DM d^{-1} (Mayes et al. 1986).

Estimation of DMI Using the n-Alkane Marker Technique

Application of the n-alkane marker technique for estimation of DMI offers a number of advantages over other techniques. The technique estimates individual animal DMI, which is the case for all marker techniques, but is an advantage over pasture clipping techniques. It also can be used in situations where grazing animals are receiving supplements.

Mayes et al. (1986) found that the mean pasture forage DMI estimated using C_{33} and C_{32} was identical to the actual pasture forage DMI of sheep eating perennial ryegrass. Unal and Garnsworthy (1999) fed housed Holstein Friesian cows either a known amount of hay or silage diet and DMI estimates using the C_{33}:C_{32} ratio for intake were similar to actual DMI.

Lactating Brown Swiss cows that consumed a known amount of a forage mixture with or without a known amount of concentrate (mean 10.4 kg DM d^{-1}), had DMI estimates using C_{33}:C_{32} ratio that were similar to actual DMI. The high level of concentrate supplementation did not bias the estimation for forage intake (Berry et al. 2000).

Researchers have compared forage intake estimations from the n-alkane and chromium marker techniques (Piasentier et al. 1995; Malossini et al. 1996; Dove et al. 2000). The forage DMI estimates were consistently higher with the chromium technique, when using sheep and cattle. However, these studies did not compare the estimations with actual intake, therefore, it is not known which marker technique estimated forage intake most accurately. Moshtaghi and Wittenberg (2002) studied steers consuming meadow brome and/or alfalfa hay with or without barley grain. They found that the chromium technique consistently overestimated DMI and n-alkanes underestimated DMI when compared to actual DMI.

Estimation of DMD Using the n-Alkane Marker Technique

The n-alkane marker technique offers potential advantages over other methods for determining digestibility in grazing ruminants. This technique allows for digestibility estimates in vivo, rather than using in vitro estimates as with other marker techniques. The n-alkane marker technique accommodates differences in diet digestibility between individual animals, rather than relying on a single in vitro estimate of digestibility. The n-alkane technique is better suited for high production animals, as the level of intake may be much greater than that used to establish the calibrations upon which the in vitro procedure is based. This technique is also better suited to animals consuming both forage and supplement, where there may be an interaction between forage and supplement digestibilities (Dove et al. 2000). This is advantageous over the chromium marker technique, which uses in vitro digestibilities and intake estimates to calculate total diet DMD because the latter assumes that digestibilities are additive when in fact they are not.

The accuracy of the DMD estimates depends on the degree of variation in recovery. Errors in digestibility estimates may be larger in cattle than in sheep due to an apparent lower and more erratic recovery of n-alkanes (Dove and Mayes 1991). At first, C_{35} was used as the marker for the digestibility estimates because of its high recovery rate. However, a disadvantage of C_{35} as a marker is the relatively low C_{35} content of many forages (Dove and Mayes 1996). Any of the natural n-alkanes present in sufficient concentrations may be used as a marker provided its recovery is known. However, the highest carbon chain length is often used because it has the highest faecal recovery rate.

Unal and Garnsworthy (1999) compared digestibility using the total faecal collection technique and the n-alkane technique. Dry matter digestibility values were very similar when

housed Holstein Friesian dairy cows were fed either hay or silage. Moshtaghi Nia and Wittenberg (2002) found that digestibility estimates using the n-alkane marker technique were lower ($P < 0.05$) than actual digestibility using the total faecal collection method. Dry matter digestibility estimates have been accurate with the n-alkane marker technique, even with supplementation, as long as recovery rates are known. However, more research should be done because the results have not consistently produced accurate digestibility estimates with the n-alkane marker technique.

Estimation of Diet Composition Using the n-Alkane Marker Technique

Differences in n-alkane pattern can be used to estimate the proportions of different plant species in pasture forage mixtures (Dove 1992). The n-alkane technique is equally applicable for the estimation of diet composition from extrusa samples using oesophageal-fistulated animals or from faecal samples (Dove et al. 1999). Due to the incomplete recovery of n-alkanes, the n-alkane concentrations must be corrected for differences in recovery, when using digesta or faecal samples, in order to prevent a bias towards dietary components with a predominance of longer chain n-alkanes (Dove and Mayes 1996; Dove et al. 2000). Dove and Moore (1995) developed a computer program entitled, "EatWhat", which uses a non-negative least-squares procedure to search for the best fit for species composition of the mixture in question.

The greater the differences between species in n-alkane composition, the greater the sensitivity of the estimation of plant species selected. For greatest sensitivity, the total n-alkane contents of the component species should be similar, but their patterns markedly different. If one species has generally low levels of n-alkanes compared with the others in a

forage mixture, estimates for species preferences have low sensitivity because the contribution of n-alkanes from the low n-alkane species will tend to be overwhelmed by those from the other species (Dove and Mayes 1991).

In order to validate diet composition estimates, known plant mixtures were analysed to establish the relative proportions of grass and legume using the n-alkane marker technique (Dove 1992). Hameleers and Mayes (1998) found that the n-alkane marker technique accurately predicted the ratio of white clover and perennial ryegrass consumed by Holstein Friesian cows. The n-alkane marker technique can estimate diet composition with simple pasture mixtures containing two species, however no information is available relative to the ability to estimate diet composition for typical complex pasture mixtures.

The Use of n-Alkanes in Nonruminant Animals

There is an increased interest in horse nutrition and outdoor-rearing of pigs, so there is a need to estimate intake, digestibility and diet composition in nonruminants (Dove and Mayes 1996). Studies have been done in pigs fed various mixtures of pelleted dried grass and cereal concentrates (Dove and Mayes 1996) and horses fed tall fescue/alfalfa mixed hay or orchardgrass/alfalfa mixed hay (Ordakowski et al. 2001). These studies showed good agreement with actual intakes and digestibilities. The main difference found in nonruminant animals is that the faecal recovery of dosed and dietary n-alkanes did not increase with increasing carbon chain length (Mayes and Dove 2000). Very few studies have been done using the n-alkane marker technique with nonruminant animals, but it appears that the n-alkane technique may work with nonruminant animals.

Further Research Required for the n-Alkane Marker Technique

Further research is required to validate the n-alkane marker technique for use in cattle. The technique has been proven to work in sheep, but results from the cattle studies are more variable. In particular, a better understanding of where n-alkane loss occurs in cattle is needed. Recovery rates of even and odd-chain n-alkanes need to be compared, as they are supposed to be similar for intake estimation, but they associate with different phases in the digesta (Dove and Mayes 1991). Total faecal collection needs to be compared with faecal grab sampling in cattle to see if recovery rates are similar with both sampling methods, for both even and odd-chain n-alkanes. Further research is required in the estimations of diet composition with cattle. The n-alkane marker technique needs to be tested with a diet that is more representative of a typical pasture, in other words, a diet that consists of more than two plant species. Cattle results have been variable with the n-alkane marker technique and further validation is required.

Summary

Implementing a grazing program can decrease costs and increase efficiency of production on dairy farms. Concentrate supplementation is required for lactating dairy cows to increase total energy intake and animal performance above that which the pasture can provide. Continuous adjustment of the supplementary feeding program is required to compensate for the variation in quantity and quality of the pasture forage throughout the grazing season if constant milk production is to be maintained. The n-alkane marker technique may provide the ability to monitor individual animal intake behaviour on pasture in response to management strategies.

Each animal is unique when it comes to DMI on pasture, response to supplementation, the diet they select and even their digestibilities. It is very important to validate the n-alkane method, as it should be able to provide insight into individual intake behaviour. With the ability to estimate these parameters on an individual basis, it will allow supplementation programs to be designed for individual animals.

HYPOTHESES AND OBJECTIVES

Hypotheses

As concentrate supplementation increases, pasture forage dry matter intake (DMI) and grazing time should decrease. The n-alkane marker technique can estimate DM intake and digestibility for individual grazing animals and, therefore, should give better results than the chromium marker technique. However, n-alkanes are not fully recovered so recovery rates must be estimated. The loss of n-alkanes is thought to occur in the rumen in cattle, so by using in vitro DM digestibility (IVDMD) to simulate the rumen environment, recovery rates could be calculated in the lab. If pasture forage samples are analysed both before and after IVDMD for n-alkane concentration, calculation of recovery rates should be possible.

Objectives

The general objectives of the thesis research were 1) to compare the n-alkane marker technique to the well-accepted Cr_2O_3 marker technique using grazing dairy cows fed three levels of concentrate, 2) to determine n-alkane recovery rates for a typical Canadian grass pasture, and 3) to develop a lab technique for estimation of in vivo n-alkane recovery rates.

MANUSCRIPT I

Use of the n-Alkane Marker Technique to Measure Dry Matter Intake and Digestibility of Grazing Dairy Cows

ABSTRACT

A grazing experiment consisting of two completely randomized trials was conducted to compare the n-alkane marker technique to the well-accepted Cr_2O_3 marker technique. Each trial had 15 primaparous Holstein lactating cows that were balanced according to milk production (mean ± SD; 33.1 ± 5.0 kg d^{-1}), stage of lactation (168 ± 89 days in milk) and body weight (552 ± 49 kg), and randomly allocated to one of three dietary treatment groups. The three dietary treatment groups consisted of concentrate supplement fed at 20% (L), 35% (M) and 50% (H) of pre-trial total dry matter intake (DMI). The concentrate was fed in equal portions at each milking (AM and PM). Cows grazed a primarily orchardgrass (*Dactylis glomerata* L.) pasture and were moved to a new section of pasture daily. Indigestible faecal markers, Cr_2O_3 and n-alkane (C_{32} and C_{36}) controlled-release capsules were placed into the cow's reticulo-rumen by oral administration. Faecal grab samples were collected twice daily at milking for a 7-d period, starting on the seventh day post administration. There was a trend ($P < 0.10$) for pasture forage DMI to decrease with increasing concentrate levels when intake was estimated using the C_{31}:C_{32} ratio. Using the C_{33}:C_{32} ratio for intake estimation, the H group had significantly lower pasture forage DMI ($P < 0.05$) than the L group. Pasture forage DMI estimations were lower for the L group, similar for the M group, and higher for the H group when using Cr_2O_3 vs. the n-alkane marker technique. Total diet DMD estimates were significantly higher in trial 1 than in trial 2 when using the Cr_2O_3 technique. Treatment groups were significantly different, with the H group having the highest total diet DMD estimates and the L group having the lowest. However, no differences between dietary treatments were observed when using the n-alkane technique for total diet DMD estimates. The marker techniques had significantly different DMD estimates for the M and H treatments. Three

Jersey steers were fed freshly cut forage from the same pasture to establish in vivo n-alkane recovery rates. The mean n-alkane recovery rates were high, averaging 97.8 ± 1.7, 97.4 ± 2.4 and 90.5% ± 1.4 for C_{29}, C_{31} and C_{33}, respectively. Recovery rates of n-alkanes for typical Canadian grass pasture were similar among animals and increased with increasing carbon-chain length, except for C_{33}. The n-alkane marker technique produced different DMI and DMD estimates than the Cr_2O_3 marker technique.

Abbreviations: IVDMD, in vitro dry matter digestibility

Key words: n-alkanes, intake, digestibility, grazing, dairy, supplementation

INTRODUCTION

Dairy producers are re-examining pasture as a method to decrease costs and increase efficiency, due to the rise in feed and other farm expenses. The major constraint to grazing lactating dairy cows in Canada is the quota system, as milk production must remain constant on a year-round basis. This is a challenge for dairy producers because it is difficult to estimate the nutrient intake of grazing cows and, therefore, difficult to provide an adequate supplementary feeding program that will ensure a constant level of milk production. However, if individual animal forage intakes can be estimated on a regular basis, concentrate supplementation can be adjusted according to pasture quantity and quality in order to maintain constant milk production.

The n-alkane marker technique has been principally developed for estimation of dry matter intake (DMI) and dry matter digestibility (DMD) of individual grazing ruminant animals (Dove and Mayes 1991). Odd-chain n-alkanes that are naturally present in plant cuticular waxes are used together with dosed even-chain n-alkanes as markers for estimating DMI and DMD from diet ingredient and faecal n-alkane concentrations (Mayes et al. 1986). Most of the work with n-alkanes has been conducted with sheep. There has been less work with n-alkanes and cattle and the results with cattle have not been conclusive. Recovery of the n-alkanes are incomplete, so recovery rates must be determined (Dove and Mayes 1996).

The objectives of this research were to compare the n-alkane marker technique to the well-accepted Cr_2O_3 marker technique using grazing dairy cows fed three levels of concentrate. A second objective was to determine n-alkane recovery rates from the digestive tract of lactating dairy cows for a typical Canadian grass pasture.

MATERIALS AND METHODS

Grazing Experiment

Experimental Design

Fifteen primaparous Holstein lactating cows were selected from the University of Manitoba Dairy Research Unit, located at the Glenlea Research Station (Glenlea, Manitoba) for each of the two completely randomized trials in the year 2000. Lactating cows in each trial were balanced according to milk production (33.1 ± 5.0 kg d^{-1}, mean ± SD), stage of lactation (168 ± 89 days in milk) and body weight (552 ± 49 kg), and randomly allocated to one of three dietary treatment groups. Trial 1 started June 3 on pasture initial growth and trial 2 started July 27 on pasture regrowth. Each trial started with a five-day period during which pre-trial DMI was determined while cows were fed a total mixed ration (TMR), containing alfalfa silage, corn silage and a barley-based concentrate. Thereafter, cows were adjusted to pasture by gradually decreasing the TMR offered and increasing the time spent on pasture, for five days in trial 1 and four days in trial 2. Cows were assigned their dietary treatments and allowed a 19 or 17-d adaptation prior to the seven-day sampling period, for trials 1 and 2, respectively.

Pasture

The pasture, established in 1998, contained primarily orchardgrass (*Dactylis glomerata* L.) with small amounts of timothy (*Phleum pratense* L.), smooth bromegrass (*Bromus inermis* Leyss.), tall fescue (*Festuca arundinacea* Schreb.) and birdsfoot trefoil (*Lotus corniculatus* L.). The pasture was fertilized on May 5, 2000 at a rate of 77 kg ha^{-1} of nitrogen applied as

urea (46-0-0). Grazed pastures were 0.30 to 0.63 km away from the dairy barn. Fresh water was provided in a 256-L Rubbermaid tank that was moved as cattle were moved. The pasture was divided into eight 0.43-ha paddocks, and then further subdivided with temporary electric polywire. The first third of the paddock was offered on the first day, two thirds of the paddock on the second day, and the whole paddock was offered on the third day, allowing cows access to fresh pasture every 24 h. Cows from the three treatment groups were managed as one group on pasture.

Animal Management and Sampling Regime

The cows were on pasture continuously, except when brought into the barn for milking and concentrate feeding, from 0400 to 0600 h and from 1530 to 1730 h. Concentrate was fed at 20% (L), 35% (M) and 50% (H) of total DMI, determined pre-trial, with a bypass protein supplement (Table 2) supplying 1.6 kg DM d^{-1} and a barley-based concentrate (Table 3) making up the remainder. Cows were fed the alloted concentrate in equal portions at the a.m. and p.m. milkings, in individual tie-stalls. Cobalt-iodized salt blocks and water were available in each tie-stall.

Samples of concentrate and orts were collected and dried daily. Weighbacks of orts were recorded daily, to calculate concentrate intake. Concentrate samples were analysed for dry matter (DM), crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF), calcium, phosphorus, in vitro DM digestibility (IVDMD) and n-alkane concentration.

Table 2. Ingredient and nutrient composition of the bypass protein supplement fed to grazing dairy cows

	Bypass protein supplement (% as fed)	
Ingredient		
Distillers dried grain	69.7	
Soybean meal	3.0	
Fish meal	14.0	
Beet molasses	3.0	
Niacinamide (98% niacin)	0.3	
Limestone	5.0	
Sodium bicarbonate	5.0	
Chemical analysis (% DM)	*Trial 1*	*Trial 2*
CP	32.1	31.1
ADF	11.0	11.8
NDF	30.3	29.7
IVDMD	98.3	96.6
Ca	2.72	2.73
P	0.33	0.31

Table 3. Ingredient and nutrient composition of the barley-based concentrate fed to grazing dairy cows

	Barley-based concentrate (% as fed)	
Ingredient		
Steam-rolled barley	47.2	
Steam-rolled corn	11.0	
Vegetable oil	1.0	
Tallow	1.0	
Soybean meal	5.5	
Canola meal	7.0	
Wheat shorts	12.0	
Corn distillers grain	2.0	
Blood meal	1.8	
Limestone	0.7	
Wheat	6.0	
Barley	2.5	
Dynamate®[z]	0.8	
Salt	0.6	
Dicalcium phospate	0.7	
Mineral/vitamin premix[y]	0.2	
Chemical analysis (% DM)	*Trial 1*	*Trial 2*
CP	17.3	16.9
ADF	8.1	9.2
NDF	19.2	18.6
IVDMD	90.2	88.2
Ca	0.87	0.91
P	0.28	0.23

[z]Dynamate® (Eastern Mineral Inc., Henderson, NC) composition not less than 18% K, 11% Mg and 22% S.

[y]Mineral/vitamin premix contains Ca 0.8%, P 0.7%, K 0.8%, S 0.3%, I 1.4 mg kg^{-1}, Mn 100 mg kg^{-1}, Zn 100 mg kg^{-1}, Co 0.3 mg kg^{-1}, Se 0.45 mg kg^{-1}, vitamin A 15,000 IU kg^{-1}, vitamin D 2,000 IU kg^{-1}, vitamin E 75 IU kg^{-1}.

Indigestible markers, chromic oxide (Cr_2O_3) and n-alkane in controlled-release capsules (CRC, Captec Ltd., Auckland, N.Z.), were placed into the cow's reticulo-rumen by oral administration seven days before the sampling period began. The n-alkane capsules delivered C_{32} at 361.5 or 317.2 mg d^{-1} and C_{36} at 365.1 or 335.4 mg d^{-1}, while the chromic oxide capsules delivered Cr_2O_3 at 1.43 g d^{-1}. Different daily dose rates represent different batches of capsules.

Faecal grab samples were taken at each milking during the sampling period, composited by day for 7 days and stored at -20°C until analysed for DM, chromium and n-alkane concentration.

Milk yields were recorded at each milking during the 7-d sampling period. Milk samples were taken for a 48-h period beginning on the first day of the sampling period and again on the fourth day of the sampling period. Milk samples were preserved with Broad Spectrum Microtabs II (6 mg 2-Bromo-2-Nitropropane-1, 3-Diol and 0.3 mg Pimaricin, Systems Plus, New Hamburg, ON) and stored at 4°C, until analysed for milk fat, protein and solids-non-fat (SNF).

Ten 0.1-m^2 quadrats of the first section of each paddock were selected on a random basis for clipping to a height of 2 cm immediately prior to placement of cows onto the first section of the paddock and immediately after they were moved to the next section. These samples were used to determine paddock forage DM yield, forage quality, botanical composition, forage CP, ADF, NDF, calcium, phosphorus, IVDMD and n-alkane concentration. Half of the pre-grazing samples were separated by hand, according to plant species, to determine botanical composition. Samples of each plant species were also collected from the same area to obtain n-alkane profiles for each species.

Grazing time was recorded using vibracorders (The Servis Recorder Co., Marion, Ohio, USA), strapped around the cows' necks. The clocks were worn for one 24-h period for each cow, during the sampling period. In trial 1, seven cows were successfully monitored and in trial 2, eight cows were monitored. The time charts were then interpreted as described by Stobbs (1970). Two cows were visually monitored for two hours to verify the results from the vibracorders.

Body weights were recorded after the morning milking on the last two days of the pre-trial period and the last two days of the sampling period. Body condition score was measured by a trained observer on the last day of the pre-trial period and the last day of the sampling period, using a scale of 1 = thin to 5 = obese (Wildman et al. 1982). The care and handling of the cows used in this experiment conformed to the guidelines established by the Canadian Council on Animal Care (1993).

n-Alkane Recovery Experiment

Three Jersey steers grazed the same pasture described in the grazing experiment for a 15-d period prior to being placed in metabolism crates for three days for adaptation, followed by five days of total faecal collection. During the time that the steers were held in the metabolism crates, freshly cut forage from the same pasture was offered on an ad libitum basis, twice a day, at 0830 and 1600 h.

Feed offered was recorded at each feeding and weighbacks of orts were recorded daily. Feed and orts samples were taken daily, during the sampling period, and stored at -20°C until analysed for DM, CP, ADF, NDF, IVDMD, calcium, phosphorus and n-alkane concentration.

Faeces was collected, weighed and thoroughly mixed at 0830 and 1600 h. A 10% sub-

sample was removed and stored at -20°C until analysed for DM and n-alkane concentration.

The care and handling of the steers used in this experiment conformed to the guidelines established by the Canadian Council on Animal Care (1993).

Chemical Analyses

Concentrate, individual plant species samples, pasture forage and faecal samples were freeze-dried. Quadrats 1-5 and quadrats 6-10 were composited, for both pre- and post-grazing pasture forage samples. Orts were dried at 60°C for 48 h in a forced-air oven. Dried samples were ground through a 1-mm screen (Tecator Cylotec 1093 sample mill). Crude protein was analysed by the Kjeldahl method (Method No. 984.13; Association of Official Analytical Chemists (AOAC) 1990) using a Tecator 1030 analyser. Acid detergent fibre (Komarek et al. 1993) and NDF (Komarek et al. 1994) were analysed using the ANKOM Fibre Analyser #F200 (Fairport, NY). Calcium and phosphorus were determined after dry ashing at 550°C for 12 h. Calcium concentration was determined (Method No. 968.08; AOAC 1990) using flame atomic absorption spectroscopy (AA/AE spectrophotometric model 551; Instruments Laboratory Inc., Willmington, MA) and phosphorus concentration was determined colorimetrically (Method No. 965.17; AOAC 1990). In vitro DM digestibility of the feed samples was determined as described by Tilley and Terry (1963). Chromium was determined by flame atomic absorption spectroscopy as described by Williams et al. (1962).

Analysis for n-alkane concentration was performed using a modification of the method by Dove (1992). A 1-g sample of feed, plant species or faeces was placed into a 50-mL thick-walled screw-top Pyrex tube (OD 150 x 25 mm) with a Teflon-lined cap together with 15 mL

of 1.5 M ethanolic KOH and 0.5 mg of tetratriacontane (C_{34} n-alkane, Sigma catalogue No. T-4883) as an internal standard. The tube was sealed with a cap fitted with a Teflon insert (10-mil, 22-mm diameter, Chromatographic Specialities Inc. catalogue No. C661022) and the contents were then well-mixed and incubated overnight in an oven at 90°C. After the sample cooled, 8 mL of heptane was added and mixed well after which 5 mL of deionised water was added, the sample was centrifuged at 500 rpm for 5 min and placed in a water bath at 60°C for 5-10 min before separation of the aqueous and solvent layers. The top (solvent) layer was transferred by Pasteur pipette to a previously acid-washed glass scintillation vial. Separation was carried out twice, adding 5 mL of heptane to the original sample each time. The extracted n-alkane fractions were purified using a silica gel column. The extract was evaporated to dryness by placing the vials in a water bath maintained at 65°C, using compressed air to hasten the process. The samples were then re-dissolved in 2 mL heptane and applied to the top of a small column consisting of a 5-mL pipette tip which had the tip plugged with a small amount of silane-treated glass wool (Supelco 2-0411). The column was filled with a saturated heptane solution of silica gel (Sigma S-2509) with a bed volume of 5 mL. A further four 2-mL aliquots of heptane were added separately to the vial and applied to the column. The purified extract was evaporated to dryness and re-dissolved in 0.8 mL of n-undecane.

A 2-μL sample was injected onto a 30-m Supelco SPB-5 bonded, poly (5% diphenyl:95% dimethylsiloxane) capillary column with 0.32-mm id and film thickness of 0.25 μm installed in a gas chromatograph. A Varian gas chromatograph Model 3400, with flame ionisation detector and 8100 auto sampler was used. The instrument was fitted with a Varian 1078 Split/Splitless injector. The flow rate of the helium carrier gas was adjusted to 3.25 mL min^{-1} with a split ratio of 5:1, air at 300 mL min^{-1} and hydrogen at 30 mL min^{-1}. The column oven

temperature was 200°C for 1 min, then increased 10°C min^{-1} to 300°C and held for 5 min at 300°C. Injector and detector temperatures were 270°C and 300°C, respectively. The peak areas were measured using Varian star chromatography software (version 4.5) and expressed as mg kg^{-1} DM. Identification of the individual n-alkane peaks and calculations were made using a known standard mixture (C_{24} 25.5, C_{25} 25.6, C_{26} 25.5, C_{27} 25.3, C_{28} 24.8, C_{29} 25.4, C_{30} 25.9, C_{32} 25.3, C_{34} 25.6, C_{36} 25.0 mg 50 ml^{-1}, Sigma Chemical Co., St. Louis, MO), while peak areas were converted to n-alkane concentration by reference to the internal standard (C_{34}).

Milk samples were analysed by an accredited lab (#125) ISOGuide25, for fat, protein and SNF (Milk-O-Scan 303AB, Foss Electric, Hillerød, Denmark).

Calculations

Chromium was used to calculate faecal output and DMI, using the following equations:

Faecal DM output (kg d^{-1}) = Cr release rate (g d^{-1})/Cr concentration in faeces (g kg^{-1}DM)

DMI (kg d^{-1}) = faecal output (kg DM d^{-1})*[100/(100 - %IVDMD)]

Herbage DMI was calculated daily using the ratio of one natural dietary odd-chain n-alkane, n (C_{31} or C_{33}), and a dosed even-chain n-alkane, d (C_{32}), using the following equation (Mayes et al. 1986):

Herbage DMI (kg DM d^{-1}) = $[(F_n/F_d)*(D_d+I_c*C_d)-I_c*C_n]/[H_n-(F_n/F_d)*H_d]$

where H_n, C_n and F_n are the concentrations (mg kg^{-1} DM) of the n-alkane (C_{31} or C_{33}) in pasture, concentrate and faeces; H_d, C_d and F_d are the concentrations (mg kg^{-1} DM) of the C_{32} n-alkane in pasture, concentrate and faeces; I_c is the intake of the concentrate (kg DM d^{-1}) and D_d is the daily release (mg d^{-1}) of C_{32}, the dosed even-chain n-alkane.

Diet digestibility was calculated from n-alkane marker concentrations using the equation (Unal and Garnsworthy 1999):

Digestibility (%) = $[F_n - [(H_n*I_h)+(C_n*I_c)]/F_n]$

where H_n, C_n and F_n are the concentrations (mg kg^{-1} DM) of the n (C_{31} or C_{33}) n-alkane in pasture, concentrate and faeces; and I_h and I_c are intake values (kg DM d^{-1}) for herbage and concentrate. Values for F_n were corrected for incomplete recovery of markers using recovery rates determined in the n-alkane recovery experiment.

Recovery rates of individual n-alkanes were determined as a proportion of ingested n-alkanes excreted in the faeces, as follows:

Recovery rate (%) = $[(H_n*I_h)/(F_n*O_f)]$

where H_n and F_n are the concentrations (mg kg^{-1} DM) of n-alkane in pasture and faeces; I_h is the herbage DMI (kg DM d^{-1}); and O_f is faecal output (kg DM d^{-1}).

Statistics

Grazing Experiment

The experiment was conducted as a completely randomized design with two replications. Data for one cow assigned to the 35% concentrate treatment was removed from trial 1 due to cow illness. Effect of treatment, trial, and their interaction effect on DM intake, digestibility, grazing time and milk composition were analysed using the following model:

$$Y_{ijkl} = \mu + P_i + T_j + PT_{ij} + C_k(PT_{ij}) + D_l + DT_{lj} + e_{ijkl}$$

where, Y_{ijkl} = observation, μ = overall mean, P_i = trial (i = 1, 2), T_j = treatment (j = 1-3), PT_{ij} = trial by treatment interaction effect, $C_k(PT_{ij})$ = error term with C_k = cow (k = number of cows in trial), D_l = day (l = 1-7), DT_{lj} = day by treatment interaction effect, e_{ijkl} = overall

error term, analysed by the General Linear Model (SAS Institute, Inc. 1999).

Comparison of marker techniques for forage DMI, total DMI (%BW) and total diet DMD estimates were anaylsed using the following model:

$Y_{ijkl} = \mu + M_i + MT_{ij} + MP_{ik} + TP_{jk} + MTP_{ijk} + C_l(MTP_{ijk}) + e_{ijkl}$

where, Y_{ijk} = observation, μ = overall mean, M_i = technique (i = 1-3), T_j = treatment (j = 1-3), P_k = trial (k = 1, 2), MT_{ij} = technique by treatment interaction effect, MP_{ik} = technique by trial interaction effect, TP_{jk} = trial by treatment interaction effect, MTP_{ik} = technique by treatment by trial interaction effect, $C_l(MTP_{ijk})$ = error term with C_k = cow (k = number of cows in trial), e_{ijkl} = overall error term, analysed by the General Linear Model (SAS Institute, Inc. 1999).

Statistical differences among the treatment means were tested using the Duncan's multiple range test when treatment differences were observed ($P < 0.05$).

n-Alkane Recovery Experiment

Means for n-alkane recovery rates were calculated for each steer. Overall mean, standard deviation and coefficient of variation were calculated.

RESULTS AND DISCUSSION

Grazing Experiment

Precipitation from May to September was 581.7 mm, which is well above the average of 282.8 mm. The mean daily temperature for May to September was 16.3°C, which is similar to the average of 16.5°C (Environment Canada, Glenlea Station, MB). The weather was rainy

during the sampling period in trial 1 with low sunshine (< 4.0 h) on d 1 and 2 of the sampling period and high rainfall on d 1 and 6 (23.2 and 12.8 mm, respectively). This weather pattern continued in the sampling period in trial 2 with low sunshine (< 4.0 h) on d 5 and high rainfall on d 1 and 6 (20.6 and 10.0 mm, respectively).

Initial milk yields were 33.5 ± 5.36 (mean ± SD), 33.0 ± 6.29 and 34.8 ± 6.40 kg d^{-1} in trial 1 and 33.3 ± 3.16, 32.5 ± 3.82 and 33.2 ± 3.57 kg d^{-1} in trial 2 for L, M and H groups, respectively. Initial body weights were 569 ± 52.9, 573 ± 26.6 and 592 ± 52.1 kg in trial 1 and 549 ± 44.1, 563 ± 34.4 and 538 ± 52.5 kg in trial 2 for L, M and H groups, respectively. Initial body condition scores were 3.1 ± 0.34, 3.2 ± 0.27 and 3.2 ± 0.29 in trial 1 and 3.3 ± 0.35, 3.0 ± 0.16 and 3.3 ± 0.29 in trial 2 for L, M and H groups, respectively.

Pasture forage biomass was higher in trial 2 than trial 1 (Table 4) and both trials had adequate pasture forage biomass available. The range of ideal pasture forage biomass was 1.7 to 4.6 t DM ha^{-1} (Popp et al. 1997) and trial 1 was within this range and trial 2 was above this range. This optimum range was found to produce liveweight gains of 107 to 462 kg ha^{-1} in beef cattle. Popp et al. (1997) did not identify the maximum pasture forage biomass before intake is adversely affected. The same plant species were present in both trials, but smooth bromegrass increased by 22.7 percentage units on a DM basis, while orchardgrass decreased by 27.4 percentage units on a DM basis from trial 1 to trial 2. The DM content of the pasture forage was 16.7% and 21.0% in trial 1 and 2, respectively. Crude protein and IVDMD were lower in trial 2 than in trial 1, while ADF and NDF were higher due to an increase in dead material in the pasture from 7.4% to 22.8% on a DM basis from trial 1 to trial 2. The increased percentage of dead material was directly related to excessive rains during the growing season. Pasture forage samples were clipped at a 2 cm height which was lower than

Table 4. Forage botanical composition, biomass and quality in paddocks pre- and post-grazing during the time of data collection in trial 1 and 2

	Trial 1				Trial 2			
	Pre (n=3)	SEM	Post (n=3)	SEM	Pre (n=2)	SEM	Post (n=2)	SEM
Species composition (% , DM basis)								
Orchardgrass	78.0	2.68			50.6	7.48		
Smooth Bromegrass	9.5	2.10			32.2	8.03		
Timothy	4.7	1.25			5.9	4.56		
Tall Fescue	3.2	0.84			9.9	5.38		
Birdsfoot Trefoil	0.8	0.45			2.7	0.85		
Weeds	3.8	0.69			0.3	0.21		
Biomass (t DM ha^{-1})	2.3	0.09	1.3	0.12	6.4	0.50	6.1	0.33
Chemical analysis								
DM (%)	16.7	0.45	11.7	0.72	21.0	1.19	23.3	0.58
CP (% DM)	14.2	0.77	12.3	0.96	10.0	0.55	9.2	0.65
ADF (%DM)	34.2	0.19	35.7	0.23	41.3	0.32	41.5	0.35
NDF (%DM)	58.2	0.69	58.6	0.63	61.6	0.83	61.3	0.69
IVDMD (%DM)	72.3	0.86	72.4	1.03	58.2	1.09	58.0	0.54
Ca (%DM)	0.27	0.004	0.26	0.012	0.33	0.005	0.32	0.019
P (%DM)	0.18	0.006	0.18	0.012	0.15	0.010	0.16	0.013

grazing level, so the forage quality that was actually consumed may have differed than from samples collected.

The C_{31} n-alkane was the highest individual n-alkane concentration in the pasture forage samples, which is in agreement with C_{31} or C_{33} being the major component in the grass n-alkane profiles (Dove and Mayes 1991). Trial 2 had a higher concentration of C_{31} (Table 5) than trial 1 because the pasture contained a higher percentage of smooth bromegrass in trial 2, which had a higher concentration of C_{31} (Appendix Table 14). All n-alkane concentrations in the pasture forage were below 50 mg kg^{-1} DM (Table 5), which is the minimum concentration suggested for accurate n-alkane estimations (Sandberg et al. 2000). However, Unal and Garnsworthy (1999) used grass hay with a C_{33} concentration of 38 mg kg^{-1} DM successfully with C_{33}:C_{32} DMI estimations similar to actual DMI. The barley-based concentrate and the bypass protein supplement had very low n-alkane concentrations ranging from 2.0 to 9.8, which has also been reported by Hameleers and Mayes (1998). The even-chain n-alkanes for concentrate and pasture forage were similar.

Pasture forage intake estimated using the difference method was 10.1 and 13.1 kg DM d^{-1} for trial 1 and 2, respectively. This is lower than the estimates from the marker techniques and is only a group estimate, which was affected by trampling. Forage DMI estimates were similar among marker techniques, however there was a method by treatment interaction (Table 6). There was a trend ($P < 0.10$) for pasture forage DMI to decrease with increasing concentrate levels when using faecal C_{31}:C_{32} ratios, but using the C_{33}:C_{32} ratios, the H treatment had significantly lower pasture forage DMI than the L treatment (Table 7). All treatments had similar total DMI, using C_{31}:C_{32} and C_{33}:C_{32} estimates. Estimated intakes increased when n-alkane pairs of longer chain lengths were used, which is in agreement with

Table 5. The n-alkane concentrations for pasture forage, barley-based concentrate and bypass protein supplement in trial 1 and 2

	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{36}
	mg kg^{-1} DM										
Trial 1											
Pasture forage[z]	0.4	8.2	0.6	9.1	1.2	18.0	2.1	30.5	2.8	13.0	5.9
Barley-based concentrate[y]	0.0	2.6	0.0	2.9	0.0	6.7	1.2	5.8	2.5	2.0	5.7
Bypass protein supplement[x]	0.8	1.2	0.9	2.4	0.8	2.9	1.0	1.9	2.2	1.5	5.9
Trial 2											
Pasture forage[z]	1.4	6.6	1.5	9.1	2.1	20.7	2.9	45.6	3.0	10.7	5.7
Barley-based concentrate[y]	1.4	3.9	1.7	4.6	1.7	9.8	1.7	7.4	2.8	2.5	5.7
Bypass protein supplement[x]	1.8	2.3	1.2	2.9	1.2	5.1	1.7	3.5	2.7	1.8	5.9

[z] $n = 3$ for trial 1 and $n = 2$ for trial 2.
[y] $n = 2$.
[x] $n = 2$.

Table 6. Daily concentrate, forage and total DM intake and digestibility as estimated using the chromium and the n-alkane controlled-release capsule marker techniques

Method (n = 196)			Trial (P)		Treatment (T)				
Cr	C_{31}	C_{33}	1	2	L	M	H	M x T	P x T
Concentrate DMI (kg)			n = 98	n = 105	n = 70	n = 63	n = 70		
			6.1	6.4	3.8*a*	6.1*b*	8.8*c*		NS
Forage DMI (kg)			n = 266	n = 308	n = 189	n = 189	n = 196		
13.7	12.7	13.5	14.3	12.5	14.5	13.4	12.3	0.05	NS
Total DMI (%BW)									
3.6	3.4	3.6	3.6	3.5	3.3	3.6	3.7	NS	NS
Total Diet DMD (%)									
73.6*a*	70.3*b*	73.6*a*	74.4	70.8	72.2	72.0	73.5	0.01	NS

a,b For any experimental variable, M, P or T, least square means in the same row with different letters are different ($P < 0.05$) as determined by Duncan's multiple range test.

Table 7. Ranking of dietary treatments using the chromium and n-alkane controlled-release capsule marker techniques

	Treatment (T)[z]				
	L	M	H	SEM	P x T
Forage DMI (kg)					
Cr	13.4	13.3	14.2	0.69	NS
C_{31}	14.4	13.1	11.2	1.05	NS
C_{33}	15.6*a*	13.8*ab*	11.7*b*	1.01	NS
Digestibility (%)					
Cr	72.0*a*	73.2*b*	75.5*c*	0.27	0.0003
C_{31}	70.6	69.8	71.0	0.77	NS
C_{33}	74.0	73.0	73.9	0.73	NS

a,b,c For any experimental variable, P or T, least square means in the same row with different letters are different ($P < 0.05$) as determined by Duncan's multiple range test.

[z]n=63, except for H treatment group using Cr estimates, where n=70.

results from Dove et al. (2000). Using the Cr/in vitro estimations, pasture forage DMI was similar between treatment groups, but the H treatment had significantly higher total DMI than the L treatment. Chromium/in vitro estimations for pasture forage DMI were lower for the L treatment, similar for the M treatment, and higher for the H treatment than n-alkane estimations using either ratio. Dove et al. (2000) used grazing ewes to compare estimates from the Cr/in vitro and n-alkane marker techniques. They found that estimates of forage DMI based on the Cr/in vitro and n-alkane procedures were not the same, and that the relationship between these estimates was not consistent. The relationship between estimates appeared to be associated with both the level of intake and the physiological state of the grazing animals. Other studies have found Cr/in vitro forage DMI estimates to be consistently higher than estimates from the n-alkane procedure (Malossini et al. 1996; Moshtaghi Nia and Wittenberg 2002), when examined in cattle consuming forage with and without supplemental feeding. The difference between Cr/in vitro and n-alkane estimations for forage and total DMI was greater in trial 1 than in trial 2.

The ratio of forage to concentrate offered to cattle can affect DMI (NRC 2001). The targeted forage to concentrate ratios were 4.0, 1.9 and 1.0 for the L, M and H treatments, respectively. The forage to concentrate ratio was significantly different for all treatment groups, using both C_{31}:C_{32} and C_{33}:C_{32} forage DMI estimates (Figures 1 and 2). Using the C_{31}:C_{32} ratio, the estimated forage to concentrate ratio was 3.6, 2.3 and 1.2 ± 0.25 for the L, M and H treatments, respectively. Estimates were higher using the C_{33}:C_{32} ratio, with estimated forage to concentrate ratios of 4.1, 2.5 and 1.3 ± 0.26 for the L, M and H treatments, respectively. The C_{33}:C_{32} ratio estimates for forage to concentrate ratio were

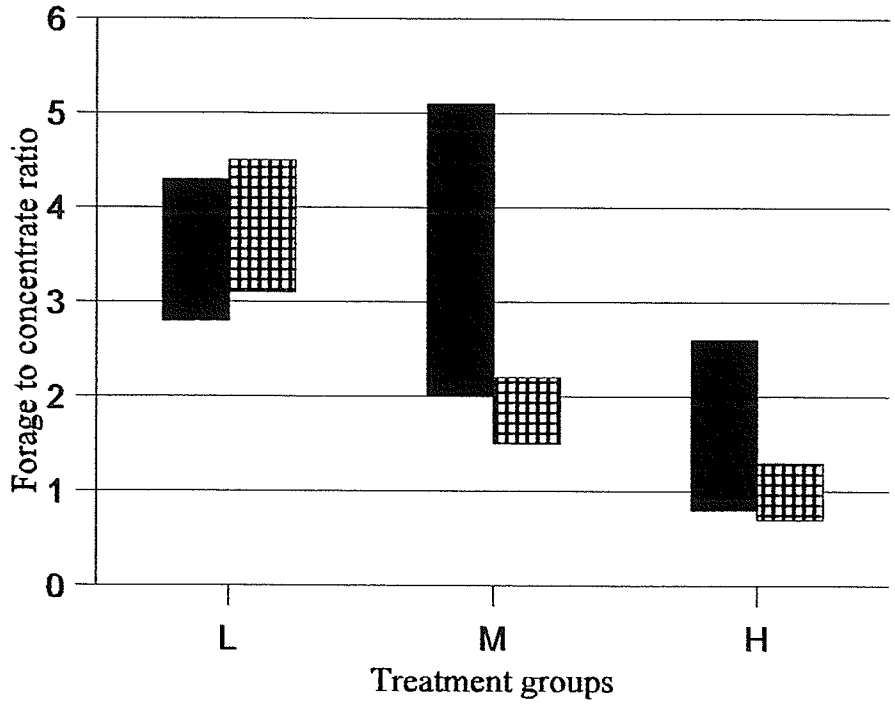

Fig. 1. The range of forage to concentrate ratios, using C_{31}:C_{32} forage intake estimates for grazing dairy cows fed 20% (L), 35% (M) or 50% (H) concentrate in trial 1 and trial 2.

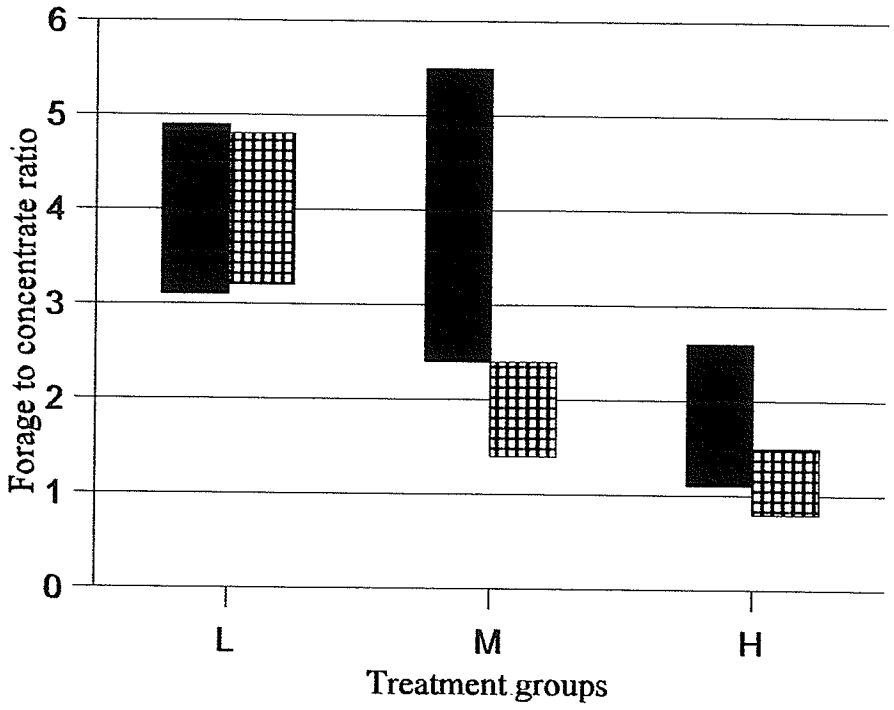

Fig. 2. The range of forage to concentrate ratios, using C_{33}:C_{32} forage intake estimates for grazing dairy cows fed 20% (L), 35% (M) or 50% (H) concentrate in trial 1 ■ and trial 2 ▦.

higher because the forage DMI estimates were higher with the C_{33}:C_{32} ratio. Trial 1 had more variability within treatments for forage to concentrate ratio than in trial 2. Forage to concentrate ratio estimates using the C_{31}:C_{32} ratio in trial 2 were the closest to the targeted ratios, with a difference of 0.1. As concentrate intake for treatments was similar for both trials, this indicates that grazing conditions of trial 1 appear to have resulted in more variation relative to forage DMI.

Total diet DMD estimates were significantly different among marker techniques (Table 6). All treatment groups were significantly different for total diet DMD, when using the Cr marker technique. The H treatment had the highest total diet DMD estimates and the L treatment had the lowest. Total diet DMD estimates were similar across dietary treatments when n-alkane marker technique was used. Reeves et al. (1996) also found that the total diet DMD (as determined by the n-alkane technique) did not change as concentrate levels increased when cows were fed kikuyu grass (*Pennisetum clandestinum* Hochst. ex Chiov.). They suggested that there was a decrease in forage DMD with increasing levels of concentrate. Agnew et al. (1996) and Mayne and Gordon (1984) studied cows consuming silage and also observed a decrease in forage DMD with increasing levels of concentrate. In both cases the decreased digestibility was attributed it to a reduction in cellulolytic activity of rumen microorganisms, leading to a decrease in cellulose digestibility. However, Jones-Endsley et al. (1997) suggested that the decrease in forage DMD is caused by an increased rate of passage of digesta due to the addition of concentrate to the diet. The total diet DMD estimates were influenced by marker technique, as the Cr/in vitro marker technique did not produce the same ranking of treatments as the n-alkane estimations. The difference between the estimates from the marker techniques was greater in trial 1 than in trial 2.

Time spent grazing from morning to evening milking and over a 24-h time frame was lower in trial 1 than trial 2 ($P < 0.05$) (Table 8). The lower grazing times in trial 1 could be attributed to higher pasture forage quality than in trial 2, as forage quality is one of the sward characteristics that can affect grazing time (Arnold 1981). Grazing times in trial 2 could be due to higher sward height and forage biomass, which takes longer to consume. Time spent grazing during the night was similar in both trials. Grazing times were similar across dietary treatments. This was not expected, as it has been shown in high producing (averaging 32.3 kg milk d^{-1}) lactating Friesian cows grazing perennial ryegrass and white clover that higher levels of concentrate supplementation will decrease grazing time (Arriaga-Jordan and Holmes 1986). Total time spent grazing was lower than expected, as the literature reports an average of 480 minutes (Voisin 1988). Less time spent grazing may have been due to the addition of concentrates and the rainy weather. Given the high pasture forage intake, as estimated by the Cr_2O_3 and n-alkane techniques, grazing time does not appear to be a limiting factor.

The L group had lower ($P < 0.05$) milk yield compared to the M and H groups (Table 9), which confirmed previous research results that increasing concentrate level significantly increases milk yield (Pulido and Leaver 2001). Milk fat concentrations decreased, while milk protein and SNF concentrations increased with increasing concentrate levels. Research has demonstrated that milk fat concentration decreases and milk protein and SNF concentrations increase significantly with increasing levels of concentrate up to 50% of DMI (Reis and Combs 2000b). However, the pasture forage in that study was 26% NDF on a DM basis and our pasture forage averaged 60% NDF on a DM basis. So, the pasture forage quality was possibly too low for a significant response in our study. Milk fat concentrations were higher

Table 8. Grazing times for lactating dairy cows receiving 20% (L), 35% (M) or 50% (H) concentrate

	Trial (P)			Treatment (T)				
Time (min)	1 (*n*=20)	2 (*n*=24)	SEM	L (*n*=15)	M (*n*=14)	H (*n*=15)	SEM	P x T
Day	94*a*	283*b*	19.1	182	184	200	23.0	NS
Night	168	185	16.5	177	167	186	19.9	NS
Total	261*a*	468*b*	29.5	359	350	385	35.6	NS

a,b For any experimental variable, P or T, least square means in the same row with different letters are different ($P < 0.05$) as determined by Duncan's multiple range test.

Table 9. Daily milk yield and composition from grazing primiparous cows receiving 20% (L), 35% (M) or 50% (H) concentrate

	Trial (P)			Treatment (T)				
	1 (*n*=98)	2 (*n*=105)	SEM	L (*n*=70)	M (*n*=63)	H (*n*=70)	SEM	P x T
Milk yield (kg)	28.5	24.1	1.26	21.8*a*	26.6*b*	30.5*b*	1.57	NS
Milk fat (%)	3.7	3.5	0.13	3.7	3.8	3.4	0.16	NS
Milk protein (%)	3.3*a*	3.1*b*	0.08	3.1	3.2	3.3	0.10	NS
Milk SNF (%)	9.0*a*	8.6*b*	0.10	8.6	8.8	8.9	0.12	NS

a,b For any experimental variable, P or T, least square means in the same row with different letters are different ($P < 0.05$) as determined by Duncan's multiple range test.

in trial 1 than in trial 2. Concentrations of milk protein and SNF were higher ($P < 0.05$) in trial 1 than in trial 2. This can be attributed to the lower pasture forage quality found in trial 2.

n-Alkane Recovery Experiment

Recoveries of natural n-alkanes increased with increasing carbon-chain length in a curvilinear fashion (Figure 3), except for C_{33}, similar to that described previously (Dove and Mayes 1996; Moshtaghi Nia and Wittenberg 2002). The n-akane recovery rates were 97.8 ± 1.7, 97.4 ± 2.4 and $90.5\% \pm 1.4$ for C_{29}, C_{31} and C_{33}, respectively. The low recovery rate for C_{33} may have been due to the low concentration of C_{33} in the pasture forage of 13.0 mg kg^{-1} DM in trial 1 and 10.7 mg kg^{-1} DM in trial 2, which was considerably lower than the concentration of C_{31} (30.5 mg kg^{-1} DM in trial 1 and 45.6 mg kg^{-1} DM in trial 2). The C_{33} concentrations were also much lower than the suggested minimum concentration of 50 mg kg^{-1} DM for accurate estimations (Sandberg et al. 2000). Therefore, the C_{31} n-alkane should be used for estimates because of the higher recovery rate and the higher concentration.

There was little animal-to-animal variation, as the coefficient of variation for the three steers was 6.0, 2.3, 1.7, 2.5, and 1.6 for C_{25}, C_{27}, C_{29}, C_{31} and C_{33}, respectively. The n-alkane recovery rates for the natural n-alkanes behave similarly among animals. Dove and Mayes (1996) noted that errors from animal-to-animal variation in faecal recovery of n-alkanes are likely to be small if relative recoveries are consistent between animals.

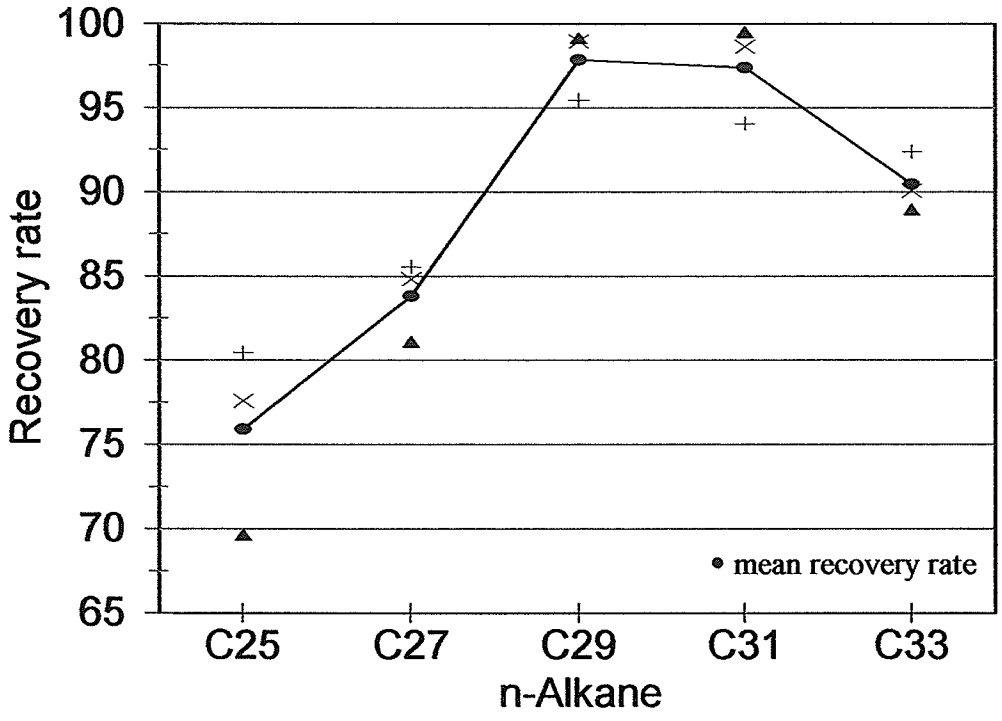

Fig. 3. The actual recovery rates and the mean recovery rate for odd-chain n-alkanes C_{25} to C_{33} from 3 steers fed the pasture forage mixture.

CONCLUSIONS

The n-alkane marker technique produced different DMI and DMD estimates than the Cr/in vitro technique. Pasture forage DMI estimations were similar across treatments using the Cr/in vitro marker technique, but decreased with increasing level of concentrate using the n-alkane marker technique. Total diet DMD estimations increased with increasing level of concentrate using the Cr/in vitro marker technique, but were similar across treatments using the n-alkane marker technique. Recovery rates of n-alkanes for a typical Canadian grass pasture were similar among animals and increased with increasing chain length, except for C_{33}. The n-alkane marker technique is advantageous because it is a useful tool in examining individual animal response in a pasture situation.

MANUSCRIPT II

An In Vitro Technique That Does Not Work to Estimate In Vivo Recovery Rates of n-Alkanes

ABSTRACT

An easier and faster method to determine n-alkane recovery rates needs to be developed to replace the labourious total faecal collection method. An in vitro method to determine n-alkane recovery rates was tested and results were compared with the actual in vivo n-alkane recovery rates reported in Manuscript I. The pasture forage samples that were used in the in vitro procedure were the same as that fed to the steers for the in vivo data. Plant species samples were collected from the same pasture and included orchardgrass (*Dactylis glomerata* L.), timothy (*Phleum pratense* L.), smooth bromegrass (*Bromus inermis* Leyss.), tall fescue (*Festuca arundinacea* Schreb.) and birdsfoot trefoil (*Lotus corniculatus* L.). An alfalfa (*Medicago sativa* L.) sample normally used as lab standard was also used because of its high n-alkane concentrations. Samples underwent a modified in vitro DM digestibility (IVDMD) procedure using bovine rumen fluid and incubation times of 0, 48 and 72 h for mixed forage samples, 0 and 48 h for alfalfa samples and 48 h for individual plant species to estimate n-alkane recovery rates. Mixed pasture samples incubated for 48 h had mean n-alkane recovery rates of 60.3, 69.7 and 69.9%, for C_{29}, C_{31} and C_{33}, respectively, while samples incubated for 72 h had n-alkane recovery rates of 70.3, 74.3 and 77.6% for C_{29}, C_{31} and C_{33}, respectively. Alfalfa samples had n-alkane recovery rates of 74.4, 81.5 and 81.8% for C_{29}, C_{31} and C_{33}, respectively at 48 h, which were higher than mixed pasture samples. Recovery rates of individual n-alkanes for individual plant species varied from 6.4 to 135.3%. Mixed pasture samples had higher CV values than the individual plant species. The n-alkane recovery rates for in vivo compared to the n-alkane recovery rates for in vitro were not equal ($P > 0.05$) for most samples for C_{29}, C_{31} and C_{33} incubated at 48 and 72 h, with in vitro n-alkane recovery rates of 20.6 to 37.5 percentage units lower than in vivo n-alkane recovery rates. The n-

alkane recovery rates for the mixed pasture forage and alfalfa samples at 0 h were between 17.7 and 24.8 percentage units lower than the samples incubated for 48 h, suggesting that there was major loss of n-alkanes in the filtration step. It was concluded that the modified IVDMD procedure did not produce similar n-alkane recovery rates to the in vivo values.

Abbreviations: IVDMD, in vitro dry matter digestibility; **CV**, coefficient of variation

Key words: n-alkanes, recovery rates, IVDMD

INTRODUCTION

The recovery of the n-alkane marker following passage through an animal's digestive tract is incomplete, however, recovery can be estimated by dosing grazing animals with known mixtures of even-chain n-alkanes and collecting total faecal production. The most reliable estimates for n-alkane recovery require use of a separate group of housed animals with known n-alkane intake, using the same diet ingredients as pastured test animals and for which total faecal collections have been made and analysed for n-alkane concentration (Marais 2000). Recovery rate determination can be quite cumbersome, as pasture forage has to be cut and brought to the animals. Total faecal collection also requires a great deal of work and a facility where this can be done.

Mayes et al. (1988) dosed sheep with C_{28}, C_{32} and C_{36} and discovered that n-alkane disappearance occurs in the small intestine of sheep. In contrast, Ohajuruka and Palmquist (1991) found that n-alkane recovery was lower with ruminal than with duodenal dosing, when cattle were dosed with 300 mg of C_{32} every 12 h. The dose site of C_{32} influenced C_{32} recovery in the faeces, with an estimated 15% of the ruminally infused synthetic n-alkane marker disappearing in the rumen. Disappearance of n-alkane occurs in the small intestine of sheep, while the disappearance of n-alkane occurs in the rumen in cattle (Ohajuruka and Palmquist 1991).

Application of the n-alkane marker technology to commercial production practices requires a fast and simple method to estimate in vivo n-alkane recovery rates. The Tilley and Terry (1963) in vitro DM digestibility has long been used for rapid estimation of feed constituent disappearance in the ruminant gastro intestinal tract. Therefore, if forage samples are subjected to this method and then the residue is analysed for n-alkane content, this should

be representative of the n-alkane disappearance that occurs in vivo, resulting in n-alkane recovery rate estimation. The pasture forage samples had low n-alkane concentration, so alfalfa was also tested because it had higher n-alkane concentration.

MATERIALS AND METHODS

Samples were analysed for n-alkane concentration to obtain initial n-alkane concentration for mixed pasture forage, alfalfa and individual plant species samples. Mixed pasture samples that were used in the in vitro method were the same as that fed to the steers in the n-alkane recovery experiment (pasture described in Manuscript I). Plant species samples were collected from the same pasture and included orchardgrass (*Dactylis glomerata* L.), timothy (*Phleum pratense* L.), smooth bromegrass (*Bromus inermis* Leyss.), tall fescue (*Festuca arundinacea* Schreb.) and birdsfoot trefoil (*Lotus corniculatus* L.). An alfalfa (*Medicago sativa* L.) sample normally used as lab standard was also used. The mixed pasture forage and individual plant species samples were freeze-dried and the alfalfa was dried at 60°C for 48 h in a forced-air oven. Samples were ground through a 1-mm screen (Tecator Cylotec 1093 sample mill) and analysed for n-alkane concentrations by gas chromatography (as described in Manuscript I) to obtain initial n-alkane concentrations for samples.

Samples were subjected to a modified Tilley and Terry (1963) in vitro DM digestibility (IVDMD) process. Samples were incubated for 0, 48 or 72 h in rumen fluid from a cannulated Jersey steer consuming timothy hay. Timothy hay was chosen because it had low n-alkane concentrations and this would keep the n-alkane concentrations in the rumen fluid low. The IVDMD technique also uses pepsin which reflects abomasal digestion.

Rumen fluid samples from 48 and 72 h, as well as samples from 0, 48 and 72 h incubation

were filtered through glass fibre filters with a pore size of 2.5 μm (4.25 cm diameter, Fisher Scientific, Pittsburgh, PA) and the residues of two tubes and accompanying glass fibre filters were composited and analysed for n-alkane concentrations. Before filtering there were 6 replicates of all samples, which translated into 3 replicates for n-alkane analysis when residues were combined. Rumen fluid samples were analysed for n-alkane content to determine the n-alkane contribution from the rumen fluid. The amount of n-alkane contributed by the rumen fluid was subtracted from the n-alkane content of the digested samples.

Experiment 1 tested mixed pasture forage samples at 48 and 72 h incubation. Experiment 2 was the same as experiment 1 with an additional incubation time of 0 h. Samples at 0 h were washed and filtered the same as the digested samples. Experiment 3 tested mixed pasture forage samples and the alfalfa sample at 0 and 48 h incubation and individual plant species samples at 48 h incubation.

Calculations

Recovery rates were calculated with the following equation, with alkane IN being the forage n-alkane concentration before digestion and alkane OUT being the residue of n-alkane concentration after filtration:

Alkane IN (mg) = sample weight (kg DM) * sample n-alkane concentration (mg kg^{-1} DM)

Alkane OUT (mg) = [post digestion residue weight (kg DM) * post digestion residue n-alkane concentration (mg kg^{-1} DM)] – [rumen fluid blank weight (kg DM) * rumen fluid n-alkane concentration (mg kg^{-1} DM)]

In vitro n-alkane recovery rate (%) = [alkane OUT (mg) / alkane IN (mg)] * 100

Statistics

Coefficients of variation were calculated to compare n-alkane recovery rates within an experiment and between experiments. A T-test was conducted with an assumption of non-equal variance for in vivo n-alkane recovery rates using three steers and the in vitro n-alkane recovery rates.

RESULTS AND DISCUSSION

Faecal recovery rates of C_{29}, C_{31} and C_{33} were examined as these were the major n-alkanes found in the n-alkane profile of the pasture forage. Mixed pasture forage n-alkane concentrations were all below 40 mg kg^{-1} DM (Table 10). Alfalfa samples had high n-alkane concentrations of 227.7 and 354.9 mg kg^{-1} DM for C_{29} and C_{31}, respectively. Individual plant species had n-alkane concentrations under 60 mg kg^{-1} DM, except for tall fescue, which had n-alkane concentrations of 156.4 and 164.6 mg kg^{-1} DM for C_{29} and C_{31}, respectively.

In experiment 1, mixed pasture forage samples incubated for 48 h had n-alkane recovery rates of 64.3, 70.3 and 71.6% for C_{29}, C_{31} and C_{33}, respectively (Table 11). Mixed pasture forage samples incubated for 72 h had n-alkane recovery rates of 80.2, 85.6 and 84.6% for C_{29}, C_{31} and C_{33}, respectively.

In experiment 2, mixed pasture forage samples incubated for 0 h had n-alkane recovery rates of 38.7, 42.0 and 49.0% for C_{29}, C_{31} and C_{33}, respectively. Mixed pasture forage samples incubated for 48 h had n-alkane recovery rates of 48.4, 60.7 and 66.4% for C_{29}, C_{31} and C_{33}, respectively. Mixed pasture forage samples incubated for 72 h had n-alkane recovery rates of 60.4, 63.1 and 70.5% for C_{29}, C_{31} and C_{33}, respectively.

Table 10. The n-alkane concentrations for mixed pasture, plant species and alfalfa standard samples

	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{36}
	mg kg^{-1} DM										
Mixed pasture	2.1	12.3	1.7	13.5	0.7	36.3	3.4	38.7	3.3	15.1	6.4
Orchardgrass	3.2	17.2	2.9	9.9	1.8	14.0	2.2	14.2	2.8	14.6	6.1
Smooth bromegrass	1.6	14.3	2.8	29.9	2.5	6.5	3.1	58.0	2.6	6.9	6.1
Timothy	5.4	29.5	8.1	47.9	8.2	45.9	5.5	8.3	5.2	4.6	6.3
Tall fescue	1.7	10.0	1.6	23.4	5.5	156.4	9.1	164.6	4.7	36.0	5.9
Birdsfoot trefoil	4.8	16.8	6.7	46.6	7.4	50.3	6.1	27.3	6.8	28.7	6.4
Alfalfa	0.7	5.1	1.2	26.9	7.7	227.7	6.0	354.9	11.3	25.3	6.0

Table 11. In vitro DM digestibility, recovery rates (mean ± SE) and coefficient of variation (CV) for n-alkanes from forages subjected to 0, 48 or 72 h in vitro digestion in three experiments

	IVDMD	Recovery Rates (%)			CV (%) within experiment		
	(%)	C_{29}	C_{31}	C_{33}	C_{29}	C_{31}	C_{33}
Mixed pasture - 48 h incubation							
Experiment 1	63.6	64.3 ± 8.05	70.3 ± 6.47	71.6 ± 5.61	17.7	13.0	11.1
Experiment 2	60.5	48.4 ± 6.95	60.7 ± 3.72	66.4 ± 2.85	20.3	8.7	6.1
Experiment 3	62.5	68.3 ± 1.36	78.2 ± 1.52	71.8 ± 1.13	2.8	2.7	2.2
Mixed pasture - 72 h incubation							
Experiment 1	64.7	80.2 ± 2.63	85.6 ± 3.30	84.6 ± 4.53	4.6	5.4	7.6
Experiment 2	64.1	60.4 ± 18.5	63.1 ± 2.98	70.5 ± 2.12	43.4	6.7	4.3
Mixed pasture - undigested/filtered							
Experiment 2		38.7 ± 3.29	42.0 ± 2.29	49.0 ± 1.70	12.0	7.7	4.9
Experiment 3		46.4 ± 4.98	52.7 ± 5.42	52.8 ± 4.65	15.2	14.6	12.4
Alfalfa - 48 h incubation	69.8	74.4 ± 1.19	81.5 ± 1.34	81.8 ± 0.97	2.3	2.3	1.7
Alfalfa - undigested/filtered		49.6 ± 2.57	57.2 ± 1.94	61.0 ± 1.50	7.3	4.8	3.5
Orchardgrass	71.4	52.9 ± 2.22	6.4 ± 0.45	10.8 ± 0.81	5.7	3.9	4.0
Smooth Bromegrass	66.6	19.2 ± 0.77	9.4 ± 0.26	36.3 ± 1.02	3.2	5.8	9.7
Timothy	60.7	107.3 ± 3.42	55.4 ± 3.21	23.8 ± 2.31	5.9	9.9	10.6
Tall Fescue	64.2	77.8 ± 4.03	27.5 ± 1.20	105.6 ± 3.59	8.6	7.3	7.2
Birdsfoot Trefoil	65.1	135.3 ± 8.26	82.7 ± 4.27	76.7 ± 3.92	7.3	6.2	4.8
Across experiments					CV (%) between experiments		
Mixed pasture - 48 h incubation	62.2	60.3 ± 6.08	69.7 ± 5.08	69.9 ± 1.77	14.3	10.3	3.6
Mixed pasture - 72 h incubation	64.4	70.3 ± 5.72	74.3 ± 6.50	77.6 ± 4.07	14.1	15.2	9.1
Mixed pasture - undigested/filtered		42.6 ± 2.23	47.3 ± 3.08	50.9 ± 1.11	9.1	11.3	3.8

In experiment 3, mixed pasture forage samples incubated for 0 h had n-alkane recovery rates of 46.4, 52.7 and 52.8% for C_{29}, C_{31} and C_{33}, respectively. Mixed pasture forage samples incubated for 48 h had n-alkane recovery rates of 68.3, 78.2 and 71.8% for C_{29}, C_{31} and C_{33}, respectively. Alfalfa samples incubated for 0 h had n-alkane recovery rates of 49.6, 57.2 and 61.0% for C_{29}, C_{31} and C_{33}, respectively. Alfalfa samples incubated for 48 h had n-alkane recovery rates of 74.4, 81.5 and 81.8% for C_{29}, C_{31} and C_{33}, respectively. Individual plant species samples incubated for 48 h had n-alkane recovery rates ranging from 6.4 to 135.3% (Table 11). Orchardgrass had the lowest n-alkane recovery rates (< 53%), which may be due in part to the n-alkane concentration being less than 14.6 mg kg^{-1} DM. This concentration is lower than the suggested threshold level required for accurate n-alkane estimations of 50 mg kg^{-1} DM (Sandberg et al. 2000).

The CV for mixed pasture samples incubated for 48 h within an experiment ranged from 2.2 to 20.3% (Table 11). The CV for mixed pasture samples incubated for 72 h within an experiment ranged from 4.3 to 43.4%. Mixed pasture samples had higher CV values than the individual plant species which ranged from 1.7 to 10.6%. There may have been higher variation with the mixed pasture samples because the IVDMD was lower than most of the individual plant species. The mixed pasture samples may not have been as homogeneous as the individual plant species samples.

The CV for mixed pasture samples between experiments ranged from 3.6 to 14.3% for 48 h incubation and ranged from 9.1 to 15.2% for 72 h incubation. Variation between experiments may have been due to differences in rumen fluid, however there was just as much variation within experiments.

The n-alkane recovery rates of mixed pasture forage incubated at 48 and 72 h in the in

vitro experiments were 20.6 to 37.5 percentage units lower than the in vivo n-alkane recovery rates of 97.8, 97.4 and 90.5% for C_{29}, C_{31} and C_{33}, respectively. A T-test was conducted with an assumption of non-equal variance for in vivo n-alkane recovery rates from the n-alkane recovery experiment and the in vitro n-alkane recovery rates. The n-alkane recovery rates for in vivo compared to the n-alkane recovery rates for in vitro for C_{29}, C_{31} and C_{33} were not equal ($P > 0.05$), with a few exceptions (Table 12). There were exceptions because there was so much variability in the in vitro experiments with standard errors ranging from 5.61 to 18.53. Sandberg et al. (2000) also found that in vitro C_{31} recovery rates were 60 percentage units lower than in vivo values.

Undigested and filtered mixed pasture and alfalfa samples were tested to try to identify why the in vivo and in vitro n-alkane recovery rates were not the same. There was a 36% DM loss in mixed pasture and alfalfa samples during the filtration process. It is not known what n-alkane was lost with this particulate matter, which could have contributed to the lower n-alkane recovery rate. The ratio of C_{27}, C_{29} and C_{31} was the same before and after filtering, so there was not preferential loss of a particular n-alkane (Table 13). The n-alkane recovery rates for the undigested/filtered mixed pasture and alfalfa samples were between 17.7 and 24.8 percentage units lower than the samples incubated for 48 h (Table 11). This indicates that major loss of the natural n-alkanes occurred in the filtration step. Mayes et al. (1988) observed that the natural n-alkanes were associated with the particulate phase of digesta, since 95% were precipitated by centrifugation. Therefore, if the natural n-alkanes are associated with the particulate matter, they should have had a higher recovery. The glass fibre filters used had a pore size of 2.5 μm, which is the pore size typically used in the IVDMD

Table 12. Results of T-test comparison of mean n-alkane recovery rates for in vivo and in vitro experiments. Probability values < 0.05 indicate that means were significantly different, $n = 3$

	Pr > t		
	C_{29}	C_{31}	C_{33}
48 h incubation			
Experiment 1	0.05	0.04	0.07
Experiment 2	0.02	0.00	0.01
Experiment 3	<.0001	0.00	0.00
72 h incubation			
Experiment 1	0.01	0.05	0.33
Experiment 2	0.18	0.00	0.00

Table 13. The ratios of C_{27}, C_{29} and C_{31} in mixed pasture and alfalfa samples before and after filtering

	C_{27}	C_{29}	C_{31}
Mixed pasture	0.35	0.94	1.00
Undigested/filtered	0.32	0.86	1.00
48 h	0.11	0.75	1.00
72 h	0.11	0.90	1.00
Alfalfa	0.07	0.61	1.00
Undigested/filtered	0.05	0.52	1.00
48 h	0.07	0.58	1.00

procedure. This size was used because the material that passes through that pore size is considered to be soluble material. There may have been small particles, with natural n-alkanes associated with them, passing through the filter, causing a decrease in the n-alkane recovery. There has not been much research on which digesta phase the natural n-alkanes associate with. Therefore, another explanation could be that the natural n-alkanes might not exclusively associate with the particulate phase, as Mayes et al. (1988) had suggested.

After filtering, two samples with accompanying glass fibre filters were composited into a tube for n-alkane extraction and analysis. The undigested/filtered mixed pasture samples had on average 1.4 g of sample going into the tube for n-alkane extraction, whereas the digested samples had on average 0.9 g for 48 h incubation and 0.8 g for 72 h incubation. The undigested/filtered alfalfa samples had on average 1.7 g of sample going into the tube for n-alkane extraction, whereas the digested samples had on average 0.7 g for 48 h incubation. The undigested/filtered samples had more sample in the tube, which may have been too much for the tube. The samples may not have been able to mix properly and therefore, all of the n-alkanes in the samples may not have been extracted.

The undigested/filtered mixed pasture samples had CV of up to 15.2% and alfalfa samples had CV of up to 7.3% within experiments (Table 11). This again demonstrates that alfalfa samples had lower variation than the mixed pasture samples. The CV for undigested/filtered mixed pasture samples between experiments ranged from 3.8 to 11.3%, suggesting that it was difficult to get good repeatability with mixed pasture samples.

CONCLUSIONS

The in vitro n-alkane recovery rates were low. It was determined that large losses of n-alkanes occurred during the filtration step. The modified IVDMD procedure did not produce n-alkane recovery rates similar to those in vivo values from the three steers.

GENERAL DISCUSSION

The total n-alkane content of the pasture forage in this experiment was 92 mg kg^{-1} DM, which was lower than the total n-alkane content of 681 mg kg^{-1} DM found in typical grass pastures of Australia (Dove and Mayes 1996). The low n-alkane content of mixed grass pasture forage in this study appears to be typical of Canadian pasture forages (Boadi et al. 2002). The n-alkane content was low because the predominant grass, orchardgrass, had a low individual n-alkane concentration (< 50 mg kg^{-1} DM). Boadi et al. (2002) found that typical Canadian pasture forages such as reed canarygrass (*Phalaris arundinaecea* L.), orchardgrass, timothy, Russian wildrye (*Psathyrostachys* (Nevski) *juncea* Fisch.), little bluestem (*Andropogon gerardii* Vitman) and Indiangrass (*Sorghastrum nutans* (L.) Nash) had very low individual n-alkane concentrations (< 50 mg kg^{-1} DM). These levels are below the suggested minimum n-alkane concentration of 50 mg kg^{-1} DM for accurate estimations using n-alkanes (Sandberg et al. 2000). It is not known how this threshold value was established. Sandberg et al. referenced this value from Casson et al., but this is an Australian reference that was difficult to access. More research needs to be done to determine what n-alkane concentration can be used accurately in estimations. Canadian pasture forages having n-alkane concentrations (< 76.3 mg kg^{-1} DM) above this threshold level include big bluestem (*Andropogon gerardii* Vitman), sideoats grama (*Bouteloua curtipendula* (Michx.) Torr.), smooth bromegrass, intermediate wheatgrass (*Thinipyrum intermedium* Host) and birdsfoot trefoil. The n-alkane concentrations reported by Boadi et al. (2002) were higher (Table 1) than those reported in Table 10. The ratio of C_{31} to C_{33} also differed between the studies, suggesting that the n-alkane profiles for a plant species can differ. For example, the ratio of C_{31} to C_{33} for orchardgrass was 1.0 in Table 10, but was 2.4 in Boadi et al.'s study (2002). This difference could reflect problems with

analysis when levels are low or a plant response to excess water in our study.

Estimations for dry matter intake, digestibility and forage to concentrate ratio made using the C_{31}:C_{32} ratio were lower than those from the C_{33}:C_{32} ratio. The n-alkane concentration of C_{33} was 13.0 and 10.7 mg kg^{-1} DM in trial 1 and 2, respectively. This concentration was much lower than the suggested threshold of 50 mg kg^{-1} DM (Sandberg et al. 2000). It was for this reason that the C_{31}:C_{32} ratio was used in this study, which is contrary to other experiments where the C_{33}:C_{32} ratio was used (Berry et al. 2000). However, Laredo et al. (1991) also found low levels of C_{33} in tropical forages and suggest using the C_{31}:C_{32} ratio for intake estimates.

Pasture forage DMI estimations were lower when using the C_{31}:C_{32} ratio compared to the C_{33}:C_{32} ratio. Pasture forage DMI decreased as concentrate DMI increased using both n-alkane ratios. However, the Cr/in vitro marker technique produced pasture forage DMI estimates that were similar across dietary treatments. The Cr/in vitro technique forage DMI estimates were 7% lower than C_{31}:C_{32} n-alkane estimates at low levels of concentrate and 27% higher at the high level of concentrate. Dove et al. (2000) also found that estimates of forage DMI based on the Cr/in vitro and n-alkane procedures were not the same, and that the relationship between these estimates was not consistent with grazing ewes. The relationship between estimates appeared to be associated with both the level of intake and the physiological state of the grazing animals. At high intakes, the intake estimates using the n-alkane marker technique were lower, and at low intakes, they were higher than the intake estimates using the Cr/in vitro marker technique. Other studies have found the Cr/in vitro technique forage DMI estimates to be consistently higher than estimates from the n-alkane

procedure (Malossini et al. 1996; Moshtaghi Nia and Wittenberg 2002), with cattle consuming forage with and without supplemental feeding.

The targeted forage to concentrate ratios were 4:1, 1.9:1 and 1:1 for the L, M and H treatments, respectively. Forage quality was good in trial 1, with a crude protein content of 14.2% and IVDMD of 72.3%. Forage quality had declined in trial 2, with a crude protein content of 10.0% and IVDMD of 58.2%. Trial 1 had higher quality pasture forage. The forage to concentrate ratio was significantly different across dietary treatments, using both C_{31}:C_{32} and C_{33}:C_{32} forage DMI estimates. Forage to concentrate ratio estimates using the C_{31}:C_{32} ratio were 3.3:1, 2.9:1 and 1.4:1 in trial 1, for L, M and H, respectively. In trial 2, forage to concentrate ratio estimates were 4.1:1, 1.8:1 and 1.0:1, for L, M and H, respectively, which were closer than the estimates from trial 1 to the targeted values. As concentrate intake for treatments was similar for both trials, this indicates that grazing conditions of trial 1 appear to have resulted in more variation relative to forage DMI.

Total diet DMD estimates using the Cr/in vitro technique were significantly different across dietary treatments, with the H group having the highest total diet DMD estimates and the L group having the lowest. Total diet DMD estimates were similar across dietary treatments when n-alkane marker technique was used. It has been suggested that there is a decrease in forage DMD with increasing levels of concentrate (Reeves et al. 1996). The decreased digestibility has been attributed it to a reduction in cellulolytic activity of rumen microorganisms, leading to a decrease in cellulose digestibility (Mayne and Gordon 1984; Agnew et al. 1996). However, Jones-Endsley et al. (1997) suggested that the decrease in forage DMD is caused by an increased rate of passage of digesta due to the addition of concentrate to the diet. The total diet DMD estimates were influenced by marker technique,

as the Cr/in vitro marker technique did not produce the same ranking of treatments as the n-alkane estimations. This is due to using IVDMD values for the forage and the concentrate with the Cr/in vitro technique. The IVDMD values do not take into account individual animal differences, as the rumen fluid used in this method is from one animal. The total diet DMD is then calculated by multiplying the forage IVDMD by the proportion of forage in the total diet and adding it to the product of the concentrate IVDMD multiplied by the proportion of concentrate in the diet. The correct way to account for adding concentrate to the diet and its effect on total diet DMD has not been determined. Le Du and Penning (1982) assumed a constant digestibility of forage and concentrate DM independent of concentrate intake. However, Langlands (1975) and Milne et al. (1981) have shown that allowance must be made for an effect of the supplement on the digestibility of the forage. With the n-alkane marker technique total DMD is calculated without having to estimate how much the concentrate changes the digestibility of the forage. The forage, concentrate and faeces samples are analysed for n-alkane concentration and these results are used to calculate total diet DMD. The faecal n-alkane concentrations are corrected for incomplete recovery of n-alkanes. The n-alkane marker technique produces results for individual animals, unlike the Cr/in vitro method.

The n-alkane marker technology has the potential to estimate diet composition because of the characteristic differences between plant species in the patterns of n-alkane concentrations. Hameleers and Mayes (1998) found that the n-alkane technique accurately predicted the ratio of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) consumed by Holstein Friesian cows. Diet selection could not be determined in our experiments because we did not have n-alkane recovery rates for the individual plant species. The pasture was

predominately orchardgrass, which had very low n-alkane concentration. Dove and Mayes (1991) state that the greater the differences between plant species in n-alkane composition, the greater is the sensitivity of the estimation. The total n-alkane content of the plant species should be similar, but the patterns of the n-alkane profile should be markedly different. Unfortunately, this was not the case with our pasture, as orchardgrass had very low levels of total n-alkane (< 100 mg kg^{-1} DM) compared to the other plant species in the pasture mixture that ranged from 176.6 to 766.6 mg kg^{-1} DM. Given the low level of total n-alkane content of orchardgrass, it is not recommended to use pastures that are dominated by orchardgrass, to conduct studies if the n-alkane technique is to be used for diet selection. The n-alkane marker technique can estimate diet composition with simple pasture mixtures containing two species, however no information is available relative to estimation of diet composition for typical complex pasture mixtures.

Faecal recovery rates of natural n-alkanes increased with increasing carbon-chain length in a curvilinear fashion (Figure 3), except for C_{33}, which is similar to that described previously (Dove and Mayes 1996; Moshtaghi Nia and Wittenberg 2002). The n-alkane recovery rates from three steers were 97.8 ± 1.7, 97.4 ± 2.4 and 90.5% ± 1.4 for C_{29}, C_{31} and C_{33}, respectively. The low recovery rate for C_{33} may have been due to the low concentration of C_{33} in the pasture forage averaging 13.0 mg kg^{-1} DM in trial 1 and 10.7 mg kg^{-1} DM in trial 2. The concentration of C_{31} was 30.5 mg kg^{-1} DM in trial 1 and 45.6 mg kg^{-1} DM in trial 2. The low forage concentration of C_{33} and the fact that C_{33} recovery from the gastrointestinal tract did not follow the curvilinear pattern of other n-alkanes has resulted in the conclusion that the C_{31} n-alkane should be used for estimates because of the higher recovery rate and the higher concentration in this study.

There was little animal-to-animal variation for faecal n-alkane recovery rates, as the coefficient of variation for the three steers was 6.0, 2.3, 1.7, 2.5, and 1.6 for C_{25}, C_{27}, C_{29}, C_{31} and C_{33}, respectively. The curvilinear pattern of n-alkane recovery rates behaved similarly among animals. Dove and Mayes (1996) noted that errors from animal-to-animal variation in faecal recovery of n-alkanes are likely to be small because it is the relative recoveries among n-alkanes which is important.

Ohajuruka and Palmquist (1991) discovered that ruminal loss of n-alkanes does occur. It is for this reason that faecal recovery rates of the n-alkanes must be determined. The most common method to determine n-alkane recovery is the use of a separate group of housed animals fed the same diet ingredients as pastured animals with known n-alkane intake. Total faecal collections are conducted with subsamples and analysed for n-alkane concentration (Marais 2000). Recovery rates for C_{31} have ranged from 59.5 to 90.9% in cattle (Ohajuruka and Palmquist 1991; Moshtaghi and Wittenberg 2002) and are different with different feedstuffs. The determination of n-alkane recovery rates can be quite cumbersome, as pasture forage has to be cut and brought to the animals. Total faecal collection also requires a great deal of work and a facility where this can be done.

A fast and simple method to estimate in vivo n-alkane recovery rates is required for the n-alkane marker technology. For this purpose, a modified Tilley and Terry (1963) in vitro DMD method was tested. This method was chosen because it has long been used for rapid estimation of feed constituent disappearance in the ruminant gastro intestinal tract. Therefore, if forage samples could be subjected to this simulation of digestion and the resulting residue is analysed for n-alkane content, it was proposed that the result would be representative of the n-alkane disappearance that occurs in vivo.

The in vitro technique produced low estimates of n-alkane recovery rates. Pasture forage samples incubated for 48 h had n-alkane recovery rates of 60.3, 69.7 and 69.9% for C_{29}, C_{31} and C_{33}, respectively and samples incubated for 72 h had n-alkane recovery rates of 70.3, 74.3 and 77.6% for C_{29}, C_{31} and C_{33}, respectively. Variation for n-alkane recovery rate within experiments ranged from 2.2 to 20.3% for 48 h incubation and 4.3 to 43.4% for 72 h incubation. The samples were of mixed pasture forage containing five different plant species, so samples may not have been homogeneous, leading to variability between the samples.

The alfalfa sample had n-alkane recovery rates of 74.4, 81.5 and 81.8% for C_{29}, C_{31} and C_{33}, respectively. The coefficent of variation ranged from 1.7 to 2.3% within an experiment. The alfalfa samples had low variability and had better n-alkane recovery rate estimates than the mixed pasture forage samples. The alfalfa sample may have behaved differently because it had higher levels of n-alkanes, which resulted in less analytical error.

The in vivo n-alkane recovery rates for mixed pasture forage were 97.8, 97.4 and 90.5% for C_{29}, C_{31} and C_{33}, respectively. T-tests showed that in vitro estimates were not equal to the in vivo n-alkane recovery rates. Sandberg et al. (2000) found in vitro estimates to be 60 percentage units lower than in vivo n-alkane recovery rates, using a similar in vitro technique. Our results were better, however this suggests that n-alkane loss occurred in the modified in vitro technique.

Undigested and filtered mixed pasture forage and alfalfa samples were tested to examine why the in vivo and in vitro n-alkane recovery rates were not equal. The undigested and filtered mixed pasture forage and alfalfa samples had n-alkane recovery rates that were 20.8 to 27.7% lower than those from digested samples. The filtration step resulted in a 36% DM loss of sample for both mixed pasture forage and alfalfa samples. It is not known what n-alkane

was lost with the particulate matter that was lost during filtration. This loss could have attributed to the lower n-alkane recovery rates. This could also explain why Sandberg et al. (2000) had very low n-alkane recovery rate. It is expected that they had larger losses of particulate matter because they used glass fibre filters with a pore size of 25 μm and our experiment used filters with a pore size of 2.5 μm. The ratio of C_{27}, C_{29} and C_{31} was the same before and after filtering, so there was not a preferential loss of particular n-alkanes. Another possibility for lower n-alkane recovery rates with the undigested and filtered sample versus digested samples is related to the higher sample weight (1.6 g versus 0.8 g, respectively) going into the tubes for n-alkane extraction. The undigested and filtered samples may have had too much sample for the tube and may not have mixed properly. This may have made it more difficult to extract all the n-alkanes from the samples.

In future experiments using the n-alkane marker technique, it would be advisable to use a pasture that contained species with a higher level of n-alkanes. The plant species in the pasture should have similar total n-alkane concentration, but have a different n-alkane profile, in order to calculate diet composition. Further, a better understanding of recovery rates and a quick method to estimate in vivo n-alkane recovery rates is required. The in vitro technique for estimating recovery rates needs to be examined. Studies should be done to find out where the excessive n-alkane loss is occurring. A possibility would be to examine the n-alkane content of the digested sample without filtering. The solution could be partially freeze-dried and analysed for n-alkane content. We found lower variability when alfalfa samples were used in the in vitro technique, rather than the mixed pasture forage samples. Perhaps a single plant species should be used when determining a suitable in vitro technique that will estimate in vivo recovery rates. In vivo n-alkane recovery rates were determined in steers in these

experiments, but then the recovery rates were applied to lactating dairy cows. It is not known if this is an acceptable practice or not. Therefore, research should be done to compare n-alkane recovery rates from steers and lactating dairy cows.

The n-alkane marker technique has the potential to be advantageous for research requiring DMI and DMD estimations. However, further research is required to validate this technique on Canadian pastures. The n-alkane marker technology is important as it should be able to provide insight into individual intake behaviour by examining the individual intake and digestibility. Each animal is unique when it comes to DMI on pasture, response to supplementation, the diet they select and even diet digestibilities. Refinement of the n-alkane marker technology can give researchers the ability to estimate these parameters on an individual animal basis. This may lead to the commercial application of the n-alkane marker technique to aid in designing supplementation programs for individual animals.

CONCLUSIONS

The n-alkane marker technique produced very different estimates of DMI and DMD than the Cr/in vitro technique. Pasture forage DMI estimations were similar across treatments using the Cr/in vitro marker technique, but decreased with increasing level of concentrate using the n-alkane marker technique. Total diet DMD estimations increased with increasing level of concentrate using the Cr/in vitro marker technique, but were similar across treatments using the n-alkane marker technique. Recovery rates of n-alkanes for a typical Canadian grass pasture were similar among animals and increased with increasing chain length, except for C_{33}. The modified IVDMD procedure did not produce n-alkane recovery rates similar to those in vivo values from the n-alkane recovery experiment. It was determined that large losses of n-alkanes occurred during the filtration step of the in vitro procedure. If a quick in vitro procedure is found to estimate in vivo n-alkane recovery rates, the n-alkane marker technique will be advantageous as a tool in examining individual animal response for pastured cattle.

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Calculation of n-alkane concentration

For C_{25} to C_{35} (except C_{31} & C_{33}):

$$\frac{AreaSample}{AreaES} * \frac{WeightES(mg)}{VolumeES(ml)} * \frac{VolumeSample(ml)}{WeightSample(g)} * 1000g\ kg^{-1} * \frac{C34\,AreaES}{C34\,AreaSample} * \frac{WeightIS(g)}{VolumeIS(ml)} * \frac{VolumeES(ml)}{C34WeightES(mg)} * Factor(mg\ g^{-1})$$

For C_{31} & C_{33}:

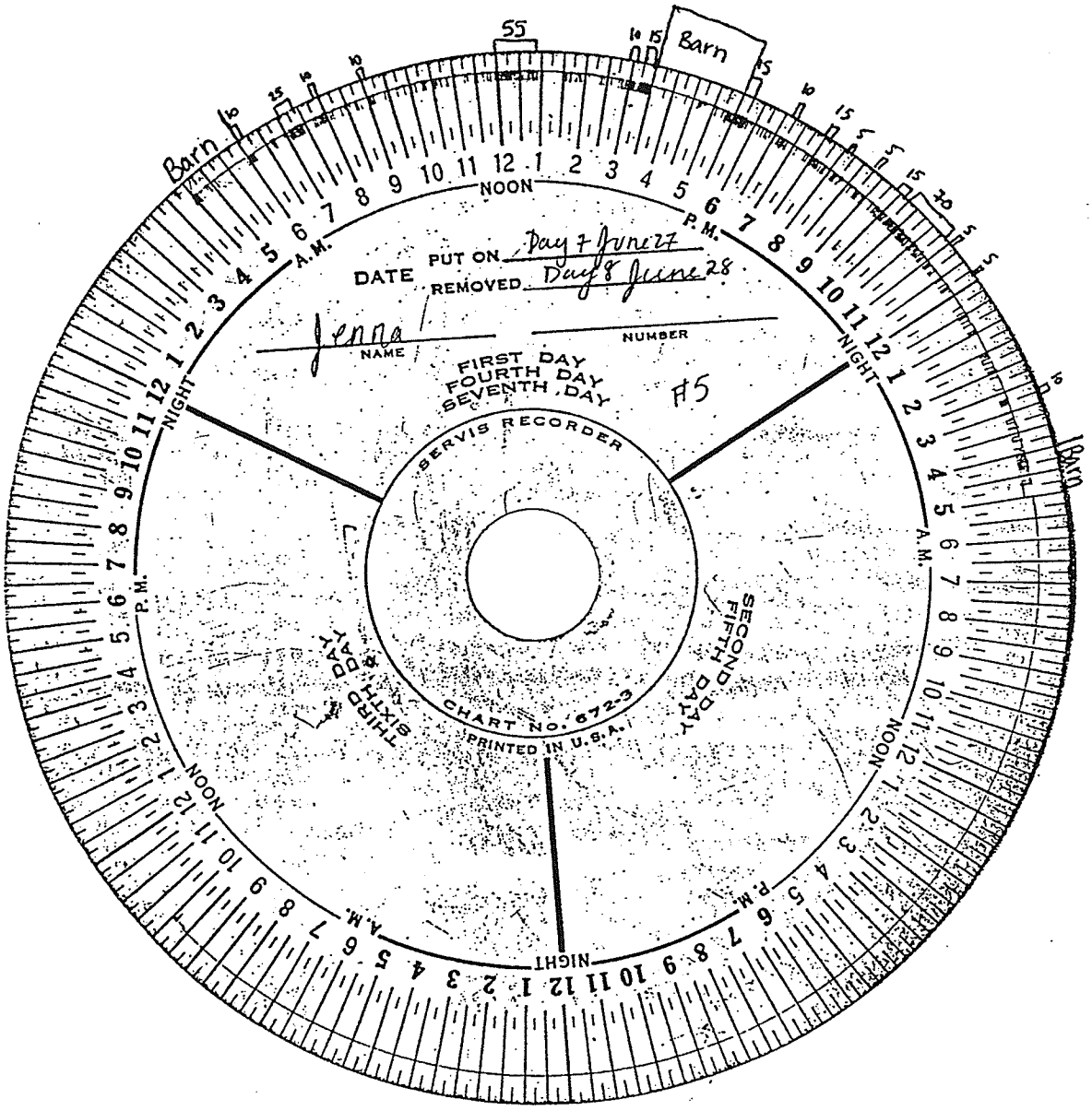
$$\frac{AreaSample}{C34\,AreaSample} * \frac{VolumeSample(ml)}{WeightSample(g)} * 1000g\ kg^{-1} * \frac{WeightIS(g)}{VolumeIS(ml)} * Factor(mg\ g^{-1}) = mg\ kg^{-1}(dm\ basis)$$

where ES is external standard (contains C_{24}, C_{25}, C_{26}, C_{27}, C_{28}, C_{29}, C_{30}, C_{32}, C_{34}, C_{35}, C_{36}) and IS is internal standard (contains C_{34}).

Table 14. The n-alkane concentrations for plant species

	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{36}
	mg kg^{-1} DM										
Trial 1											
Orchardgrass	1.2	5.9	0.0	48.0	0.0	7.5	1.2	12.6	3.0	13.0	6.0
Smooth bromegrass	4.9	144.0	8.1	138.3	9.2	179.0	14.4	224.9	6.9	30.7	6.2
Timothy	2.7	47.0	4.5	96.0	6.7	69.7	5.1	49.0	3.7	8.2	6.0
Tall fescue	1.9	6.6	2.0	11.7	3.1	73.4	7.7	194.6	5.8	43.5	6.2
Birdsfoot trefoil	2.1	11.6	2.2	36.8	3.0	31.4	2.2	27.9	4.0	28.6	6.1
Trial 2											
Orchardgrass	4.2	9.5	4.6	11.3	3.9	15.0	3.7	19.3	3.9	7.6	6.1
Smooth bromegrass	2.1	19.8	3.5	50.3	8.3	121.3	9.6	218.2	4.4	12.4	5.5
Timothy	1.1	7.8	6.2	29.6	2.5	37.8	2.9	22.6	2.8	4.4	5.7
Tall fescue	2.8	3.1	3.2	8.6	4.1	45.5	9.0	183.1	5.9	51.4	6.3
Birdsfoot trefoil	1.3	7.7	2.2	26.3	9.8	89.0	3.7	16.6	3.3	10.5	6.2

An example of a waxed vibracorder chart used to measure grazing time



Day grazing time = 135 min

Night grazing time = 155 min

Analysis of variance (ANOVA) for actual concentrate dry matter intake in Manuscript I

Concentrate dry matter intake

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	4.90	4.90	17.06	0.41
Treatment (T)[z]	2	903.54	451.77	1571.70	0.0001*
P*T [z]	2	18.70	9.35	32.53	0.27
Cow(P*T)	23	157.08	6.83	23.76	0.0001*
Day (D)	6	3.25	0.54	1.89	0.09
D*T	12	4.29	0.36	1.24	0.26
Error	156	44.84	0.29		

[z]Test of hypotheses using Type III MS for cow(P*T) as error term.

Analysis of variance (ANOVA) for pasture forage intake estimations in Manuscript I

C_{31}

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	0.16	0.16	0.03	0.96
Treatment (T)[z]	2	341.50	170.75	35.18	0.11
P*T [z]	2	225.86	112.93	23.27	0.22
Cow(P*T)	21	1444.70	68.80	14.17	< 0.0001*
Day (D)	6	302.56	50.43	10.39	< 0.0001*
D*T	12	47.91	3.99	0.82	0.63
Error	144	698.92	4.85		

C_{33}

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	124.80	124.80	22.29	0.18
Treatment (T)[z]	2	471.93	235.96	42.13	0.04*
P*T [z]	2	141.16	70.58	12.60	0.35
Cow(P*T)	21	1334.00	63.52	11.34	< 0.0001*
Day (D)	6	179.32	29.89	5.34	< 0.0001*
D*T	12	47.76	3.98	0.71	0.74
Error	144	806.44	5.60		

Cr

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	662.41	662.41	404.64	0.0001*
Treatment (T)[z]	2	36.10	18.05	11.03	0.56
P*T [z]	2	134.66	67.33	41.13	0.13
Cow(P*T)	22	656.93	29.86	18.24	< 0.0001*
Day (D)	6	58.76	9.79	5.98	< 0.0001*
D*T	12	24.26	2.02	1.23	0.26
Error	150	245.56	1.64		

[z]Test of hypotheses using Type III MS for cow(P*T) as error term.

Analysis of variance (ANOVA) for total intake (as %BW) estimations in Manuscript I

C_{31}

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	0.83	0.83	9.55	0.54
Treatment (T)[z]	2	1.44	0.72	8.30	0.72
P*T [z]	2	5.61	2.80	32.33	0.29
Cow(P*T)	21	44.32	2.11	24.33	< 0.0001*
Day (D)	6	1.39	0.23	2.67	0.02*
D*T	12	1.73	0.14	1.66	0.08
Error	144	12.49	0.09		

C_{33}

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	0.06	0.06	0.30	0.89
Treatment (T)[z]	2	0.90	0.45	2.41	0.84
P*T [z]	2	2.56	1.28	6.89	0.61
Cow(P*T)	21	52.97	2.52	13.56	< 0.0001*
Day (D)	6	6.71	1.12	6.02	< 0.0001*
D*T	12	1.87	0.16	0.84	0.61
Error	144	26.79	0.19		

Cr

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	9.26	9.26	135.34	0.07
Treatment (T)[z]	2	32.03	16.01	234.05	0.01*
P*T [z]	2	3.11	1.55	22.69	0.54
Cow(P*T)	22	54.41	2.47	36.15	< 0.0001*
Day (D)	6	1.76	0.29	4.28	0.0005*
D*T	12	1.18	0.10	1.44	0.15
Error	150	10.26	0.07		

[z]Test of hypotheses using Type III MS for cow(P*T) as error term.

Analysis of variance (ANOVA) for total diet dry matter digestibility estimation in Manuscript I

C_{31}

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	5.34	5.34	0.79	0.71
Treatment (T)[z]	2	56.37	28.19	4.16	0.47
P*T [z]	2	15.00	7.50	1.11	0.82
Cow(P*T)	21	765.89	36.47	5.38	< 0.0001*
Day (D)	6	491.18	81.86	12.08	< 0.0001*
D*T	12	41.79	3.48	0.51	0.90
Error	144	976.17	6.78		

C_{33}

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	181.53	181.53	18.96	0.03*
Treatment (T)[z]	2	38.09	19.05	1.99	0.57
P*T [z]	2	17.77	8.88	0.93	0.77
Cow(P*T)	21	689.34	32.83	3.43	< 0.0001*
Day (D)	6	862.59	143.77	15.02	< 0.0001*
D*T	12	44.82	3.73	0.39	0.97
Error	144	1378.52	9.57		

Cr

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	3697.31	3697.31	8374.23	< 0.0001*
Treatment (T)[z]	2	533.15	266.58	603.78	< 0.0001*
P*T [z]	2	121.25	60.63	137.31	0.0003*
Cow(P*T)	22	111.08	5.05	11.44	< 0.0001*
Day (D)	6	22.67	3.78	8.56	< 0.0001*
D*T	12	5.46	0.46	1.03	0.42
Error	150	66.23	0.44		

[z]Test of hypotheses using Type III MS for cow(P*T) as error term.

Analysis of variance (ANOVA) for comparison of marker technique in Manuscript I

Pasture Forage DMI

Source	DF	SS	MS	F-Value	Pr > F
Method (M)[z]	2	95.33	47.67	0.87	0.43
M*Treatment (T)[z]	4	560.02	140.01	2.55	0.05
M*Trial (P)[z]	2	377.89	188.94	3.43	0.04*
P*T[z]	2	366.97	183.48	3.34	0.04*
M*P*T[z]	4	28.40	7.10	0.13	0.97
cow(M*P*T)	66	3630.41	55.01	11.32	< 0.0001*
Error	504	2448.02	4.86		

Total DMI as %BW

Source	DF	SS	MS	F-Value	Pr > F
Method (M)[z]	2	6.84	3.42	1.47	0.24
M*Treatment (T)[z]	4	16.13	4.03	1.74	0.15
M*Trial (P)[z]	2	9.13	4.56	1.97	0.15
P*T[z]	2	8.21	4.10	1.77	0.18
M*P*T[z]	4	1.84	0.46	0.20	0.94
cow(M*P*T)	66	153.27	2.32	18.12	< 0.0001*
Error	504	64.60	0.13		

Total Diet DMD

Source	DF	SS	MS	F-Value	Pr > F
Method (M)[z]	2	1361.53	680.76	25.70	< 0.0001*
M*Treatment (T)[z]	4	417.13	104.28	3.94	0.01*
M*Trial (P)[z]	2	2061.23	1030.62	38.90	< 0.0001*
P*T[z]	2	71.44	35.72	1.35	0.27
M*P*T[z]	4	74.44	18.61	0.70	0.59
cow(M*P*T)	66	1748.43	26.49	3.29	< 0.0001*
Error	504	4062.63	8.06		

[z]Test of hypotheses using Type III MS for cow(M*P*T) as error term.

Analysis of variance (ANOVA) for forage : concentrate ratio in Manuscript I

C_{31}

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	2.41	2.41	5.18	0.44
Treatment (T)[z]	2	206.82	103.41	221.93	< 0.0001*
P*T [z]	2	26.52	13.26	28.46	0.05
Cow(P*T)	22	84.71	3.85	8.26	< 0.0001*
Day (D)	6	4.50	0.75	1.61	0.15
D*T	12	6.39	0.53	1.14	0.33
Error	150	69.89	0.47		

C_{33}

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	10.25	10.25	16.05	0.13
Treatment (T)[z]	2	244.03	122.02	191.08	< 0.0001*
P*T [z]	2	16.55	8.28	12.96	0.16
Cow(P*T)	22	89.79	4.08	6.39	< 0.0001*
Day (D)	6	7.47	1.25	1.95	0.08
D*T	12	8.94	0.75	1.17	0.31
Error	150	95.78	475.46		

[z]Test of hypotheses using Type III MS for cow(P*T) as error term.

Analysis of variance (ANOVA) for grazing times in Manuscript I

AM

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	349007.38	349007.38	135.58	0.0001*
Treatment (T)[z]	2	2556.98	1278.49	0.50	0.82
P*T [z]	2	693.53	346.77	0.13	0.95
Cow(P*T)	23	148409.82	6452.60	2.51	0.04*
Error	15	38612.5	2574.17		

PM

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	3031.13	3031.13	0.53	0.44
Treatment (T)[z]	2	2433.19	1216.60	0.21	0.78
P*T [z]	2	12227.78	6113.89	1.06	0.30
Cow(P*T)	23	110985.57	4825.46	0.84	0.66
Error	15	86500.00	5766.67		

Total

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	417088.88	417088.88	46.74	0.0001*
Treatment (T)[z]	2	8837.52	4418.76	0.50	0.75
P*T [z]	2	15694.95	7847.48	0.88	0.61
Cow(P*T)	23	355649.85	15463.04	1.73	0.14
Error	15	133862.50	8924.17		

[z]Test of hypotheses using Type III MS for cow(P*T) as error term.

Analysis of variance (ANOVA) for milk quality and composition in Manuscript I

Milk Yield

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	980.13	980.13	395.62	0.02*
Treatment (T)[z]	2	2625.63	1312.82	529.91	0.0016*
P*T [z]	2	57.10	28.55	11.52	0.83
Cow(P*T)	23	3515.36	152.84	61.69	0.0001*
Day (D)	6	75.35	12.56	5.07	0.0001*
D*T	12	34.15	2.85	1.15	0.33
Error	156	386.48	2.48		

Milk Fat

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	0.46	0.46	6.31	0.31
Treatment (T)[z]	2	2.29	1.15	15.58	0.09
P*T [z]	2	2.46	1.23	16.75	0.08
Cow(P*T)	23	10.00	0.43	5.92	< 0.0001*
Day (D)	1	0.00	0.00	0.06	0.81
D*T	2	0.11	0.05	0.73	0.49
Error	26	1.91	0.07		

Milk Protein

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	0.90	0.90	506.82	0.03*
Treatment (T)[z]	2	0.54	0.27	151.08	0.22
P*T [z]	2	0.13	0.07	37.02	0.67
Cow(P*T)	23	3.77	0.16	92.29	< 0.0001*
Day (D)	1	0.08	0.08	44.79	< 0.0001*
D*T	2	0.00	0.00	0.03	0.97
Error	26	0.05	0.00		

Milk SNF

Source	DF	SS	MS	F-Value	Pr > F
Trial (P)[z]	1	2.76	2.76	1331.13	0.0039*
Treatment (T)[z]	2	1.19	0.60	287.12	0.13
P*T [z]	2	0.61	0.30	146.09	0.34
Cow(P*T)	23	6.17	0.27	129.36	< 0.0001*
Day (D)	1	0.07	0.07	33.95	< 0.0001*
D*T	2	0.00	0.00	0.18	0.83
Error	26	0.05	0.00		

[z]Test of hypotheses using Type III MS for cow(P*T) as error term.

Table 15. In vivo n-alkane recovery rates for three steers

	Recovery Rates (%)				
	C_{25}	C_{27}	C_{29}	C_{31}	C_{33}
Steer 1	80.5	85.6	95.4	94.1	92.4
Steer 2	69.7	81.1	99.1	99.5	88.9
Steer 3	77.6	84.8	99.0	98.6	90.1