RAPA Revista Argentina de Producción Animal

TEMPORAL VARIATION IN FAECAL *N*-ALKANE RATIOS AND ESTIMATION OF PASTURE INTAKE IN CALVES

Variación temporal de la relación entre los n-alcanos fecales y Estimación del consumo de pastura en terneros

Bakker, M.L.[1], Alvarado, P.I.[1], Gonda, H.L.[2], Hidalgo, L.G.[2], Inza, M.C.[1], Wade, M.H.[2], Dalla Valle, D.E.[2], Otero, M.J.[1], Yuño, M.M.[1]

Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina

SUMMARY

The temporal variation (between and within sampling days) in the faecal *n*-alkane ratios (C_{32}/C_{31} and C_{32}/C_{33}), and the effect of AM or PM faecal sampling on the estimation of pasture intake, was studied in Holstein-Friesian calves in two independent trials. In Study 1, calves strip-grazed at different pasture allowances: Low or High (2.5 or 7.5 kg DM/100 kg LW/day). In Study 2, calves strip-grazed at different grazing time schedules but same total daily pasture allowance (4-h: one fresh grazing area/day for 4 h (11:00 AM to 3:00 PM) at a pasture allowance of 4.2 kg DM/100 kg LW, or 2x2-h: two fresh grazing areas/day, each one for 2 h (11:00 AM to 1:00 PM and 3:00 PM to 5:00 PM) at a pasture allowance of 2.1 kg DM/100 kg LW). In both trials, calves (LW range: 117-173 kg) were dosed orally with C_{32} embedded in paper stoppers twice a day. The variation between days in the faecal *n*-alkane ratios, estimated from the coefficient of variation (CV, %) over days 7 to 12 (Study 1) or 6 to 10 of dosing (Study 2), was not significantly different between pasture allowances (Low: 17%, High: 14%) or grazing time schedules (4-h: 16%, 2x2-h: 12%). In both studies, AM had significantly lower faecal *n*-alkane ratios than PM ($p<0.01$), therefore, significantly higher pasture intakes were estimated from AM compared with PM (Study 1: $p<0.01$, Study 2: $p<0.001$). The magnitude of the variation within sampling days in the faecal *n*-alkane ratios indicates that, in calves, more than one faecal sample per day should be taken to obtain a more reliable estimation of pasture intake under similar grazing conditions. In Study 1, the time required for the faecal concentration of C_{32} to reach stable levels was very short (day 2 of dosing), and suggests that the faecal sampling in calves might begin earlier than in present recommendations.

Key words. *n*-alkanes, grazing, intake, calves.

RESUMEN

Se estudió la variación temporal (entre y dentro de los días de muestreo) de la relación entre los *n*-alcanos fecales (C_{32}/C_{31} y C_{32}/C_{33}) y el efecto del horario de muestreo de materia fecal (AM o PM) en la estimación del consumo de pasto en terneros Holstein-Friesian en dos ensayos independientes. En el Estudio 1, los terneros pastorearon en franjas con diferentes asignaciones: Baja o Alta (2,5 o 7,5 kg MS/100 kg PV/día). En el Estudio 2, los terneros pastorearon en franjas con diferentes horarios de pastoreo pero con una misma asignación de pastura total diaria (4-h: una franja de pastoreo/día durante 4 h (11:00 AM a 3:00 PM) con una asignación de 4,2 kg MS de forraje/100 kg LW, o 2x2-h: dos franjas de pastoreo/día, cada una durante 2 h (11:00 AM a 1:00 PM y 3:00 PM a 5:00 PM) con una asignación de 2,1 kg MS de forraje/100 kg PV). En ambos ensayos, los terneros (rango PV: 117-173 kg) se dosificaron oralmente dos veces al día con C_{32} embebido en tapones de papel. La variación entre días de la relación entre los *n*-alcanos fecales, estimada a partir del coeficiente de variación (CV, %) durante los días 7 a 12 (Estudio 1) o 6 a 10 de dosificación (Estudio 2), no fue significativamente diferente entre las asignaciones de forraje (Bajo: 17%, Alto: 14%) o los horarios de pastoreo (4-h: 16%, 2x2-h: 12%). En ambos estudios, las relaciones entre los *n*-alcanos fecales en AM fueron significativamente menores que en PM ($p<0,01$), por lo tanto, los consumos de pasto estimados con AM fueron significativamente mayores que con PM (Estudio 1: $p<0,01$, Estudio 2: $p<0,001$). La magnitud de la variación dentro de los días de muestreo de las relaciones entre los *n*-alcanos fecales indica que

---

Recibido:
Aceptado:

1. Médicos Veterinarios, Docentes de la Facultad de Ciencias Veterinarias, UNCPBA, E-mail: bakker@vet.unicen.edu.ar
2. Ingenieros Agrónomos, Docentes de la Facultad de Ciencias Veterinarias, UNCPBA.

Abbreviations: ADF, acid detergent fiber expressed exclusive of residual ash; C_{22}, *n*-docosane; C_{31}, *n*-hentriacontane; C_{32}, *n*-dotriacontane; C_{33}, *n*-tritriacontane; C_{34}, *n*-tetratriacontane; CP, crude protein; CV, coefficient of variation; DM, dry matter; IVDDM *in vitro* digestible DM; LW, live weight; FDN, neutral detergent fiber assayed without a heat stable amylase and expressed inclusive of residual ash; PI: pasture intake; SI: supplement intake.

en terneros se debe recolectar más de una muestra fecal por día para obtener una estimación de consumo de pastura más confiable en condiciones de pastoreo similares. En el Estudio 1, el tiempo requerido para que la concentración fecal de C_{32} llegue a niveles estables fue muy corto (día 2 de dosificación), y sugiere que el muestreo fecal en terneros podría comenzar antes de lo que se recomienda actualmente.
Palabras clave. *n*-alcanos, pastoreo, consumo, terneros.

Introduction

The need to measure intake by ruminants under grazing conditions has led to the development of a wide variety of herbage and animal based techniques. Mayes et al (1986) proposed the *n*-alkanes for the estimation of herbage intake. This technique, in contrast to the widely used chromic oxide technique, does not require an independent assessment of digestibility, nor does it rely on the quantitative recovery of the dosed *n*-alkane used as the marker. Two general recommendations are given for the estimation of daily forage intake in grazing cattle dosed orally with the *n*-alkane *n*-dotriacontane (C_{32}) embedded in paper stoppers: 1) to dose twice a day during 10-12 consecutive days, with each dose given preferably before a main grazing period, 2) to sample faeces twice a day, during 5-7 consecutive days at, or very close to, the time of dosing, starting from day 5-7 of dosing, or once the faecal concentration of C_{32} has reached stable levels (Mayes et al, 1986; Mayes and Dove, 2000; Oliván et al, 2007). The estimation of daily forage intake is based on the daily dose of C_{32}, the mean contents of *n*-hentriacontane (C_{31}) or *n*-tritriacontane (C_{33}) and C_{32} in forage, and the ratio between the mean contents of dosed C_{32} and forage C_{31} or C_{33} in faeces (i.e. C_{32}/C_{31} or C_{32}/C_{33}) (Mayes et al, 1986; Dove and Mayes, 1991). Taking several samples of faeces, with or without pooling before the analysis, becomes necessary because there is a degree of temporal variation (between and within sampling days) in the faecal *n*-alkane ratios (Mayes et al, 1986; Dove and Mayes, 1991, 1996; Vulich and Hanrahan, 1992, 1995). The temporal variation has been associated mainly with the incomplete mixing of the dosed alkane with the gastrointestinal content and is affected by various animal, feeding and dosing related factors, such as the size of gastrointestinal compartments, diet type, intake level, ingestive behaviour, dynamics of digesta, type of carrier matrix of the dose and dosing frequency (Dillon, 1993; Mayes and Duncan, 1999; Mayes and Dove, 2000; Sibbald et al, 2000; Giráldez et al, 2004). Under grazing conditions, temporal changes in ingestive behaviour and pasture intake rate might be of major relevance to the degree of variation in the *n*-alkane ratios. However, little information is available on this subject to assist in the design of dosing and sampling protocols for a particular breed and animal age under particular grazing conditions.

The aim of this work was to analyse the temporal variation (between and within sampling days) in the faecal *n*-alkane ratios, and the effect of the time of faecal sampling on the estimation of pasture intake, in Holstein-Friesian calves dosed orally with C_{32} embedded in paper stoppers twice a day, and strip-grazed at different pasture allowances (Study 1) or grazing time schedules (Study 2), to evaluate a suitable sampling protocol for using the *n*-alkanes technique under similar conditions.

Materials and Methods

Pasture, animals, and general management

The two studies were conducted at the Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina (37°19'S; 59°08'W), on a paddock (6 ha) sown in 1999 with a pasture composed mainly of perennial ryegrass (*Lolium perenne* L. cv. Yatsyn), tall fescue (*Festuca arundinacea* Schreb. cv. El Palenque), white clover (*Trifolium repens* L. cv. El Lucero) and birdsfoot trefoil (*Lotus corniculatus* L. cv. San Gabriel). More details on the characteristics of the pasture used in each study are given in corresponding sections.

Before each study, a group of Holstein-Friesian castrated male calves (100-200 kg LW) were selected from a local private herd and adapted to the pasture and grazing management. During the adaptation period, the calves were continuously grazed on a fenced area of the paddock which was not used during the trial, and regularly walked to the facilities and passed through a crush where dosing and sampling was done. The calves were vaccinated and dewormed according to the current veterinary health plan. Individual LW was recorded before and after each study with an electronic balance after an overnight enclosure of 12 h without food and water.

Study 1

Experimental design

The study was conducted on June and lasted twenty days. The first five days were considered for adaptation to daily strip management and pasture allowance, and the last fifteen days for dosing and sampling.

The pasture was divided with electric fencing into four adjacent strips (two of 10 x 200 m and two of 30 x 200 m), to arrange two different pasture allowances strips, replicated twice: Low and High (2.5 and 7.5 kg DM/100 kg LW/day, respectively). A 3 m wide alley was built alongside each strip with electric fencing, where all the grass was removed to ground level and an automatic water trough was installed at the approximate mid-point of its total length. The length of each fresh grazing area was periodically adjusted to maintain the required pasture allowance. For that purpose, the mean DM availability (kg DM/ha) of two or three consecutive fresh grazing areas was estimated before grazing, by cutting five quadrats (20 x 20 cm) at random to ground level on each one with hand shears, and drying the material in a forced-air oven at 100 °C until constant weight.

Thirty one calves (118 ± 17 kg LW) were used, from which the heaviest 16 (131 ± 14 kg LW) were selected for dosing. The calves of both groups were further divided into two groups according to LW and randomly assigned to the four strips, resulting three strips with eight and one strip with seven calves. The calves were moved daily to a fresh grazing area at 8:00 AM, by moving front and back electric fences along the strips, and remained on it until the next day, except during the dosing and sampling (7:00-8:00 AM and 5:00-6:00 PM).

Dosing, sampling, measurements and sample analysis

From day 6 to 17, the 16 calves received 360 mg/d of C_{32} (SIGMA-ALDRICH, St. Louis, MO, USA), divided into two equal doses of 180 ± 3 mg, each at 7:00-8:00 AM and 5:00-6:00 PM. Each dose was prepared by pipetting a controlled amount of a solution of C_{32} in *n*-heptane into a paper stopper (cellucotton stopper, 34.5 x 22.0 mm, Carl-Roth, Karlsruhe, Germany). Each paper stopper was administered orally using an automatic dosing gun. There was no evidence of regurgitation during the study.

Samples of faeces were collected by rectal grab sampling from all 16 calves on days 6 to 12 of dosing at the same time as dosing (7:00-8:00 AM and 5:00-6:00 PM). In addition, samples of faeces were similarly collected from eight calves (two from each pasture strip) on days 2 to 5 of dosing (to estimate the time required for the faecal concentration of C_{32} to reach stable levels) and during the two days after the end of dosing (AM only, to estimate the time to clear the C_{32} dose). The samples were frozen at -20 °C, freeze-dried and analysed for C_{31}, C_{32} and C_{33} contents.

Samples of pasture were collected before grazing from the fresh grazing areas corresponding to days 6, 8 and 10 of dosing, by cutting five quadrats (20 x 20 cm) at random to the mean postgrazing height of the area grazed on the previous day with hand shears. Samples from each strip and day were pooled, mixed, and a subsample was frozen at –20 °C, freeze-dried and analysed for C_{31}, C_{32} and C_{33} contents. Another subsample was dried in a forced-air oven at 60 °C for 48 h, ground to pass through a 1 mm screen, and analysed for CP (AOAC, 1995; method 984.13), NDF (Van Soest et al, 1991) without a heat stable amylase and inclusive of residual ash, ADF (AOAC, 1995; method 973.18), and IVDDM (Tilley and Terry, 1963). The remaining fresh material was pooled across days by strip, and manually separated into different plant species and live or dead material, dried in a forced-air oven at 100 °C until constant weight, and the proportion of each fraction in the grazed horizon was estimated. Mean DM availability and DM content of the pasture during the study was estimated from all the samples taken to adjust daily pasture allowance as explained in 2.2.1.

In order to estimate the pregrazing and postgrazing heights, twenty five measurements were taken at random (pregrazing) or on grazed patches (postgrazing) on days 5, 7 and 9 of dosing with a sward stick (Barthram, 1986).

Analyses of C_{31}, C_{32} and C_{33} contents (mg/kg DM) in samples of faeces and pasture were done on the freeze-dried material and corrected to DM after drying ~1 g subsamples in an oven at 100 °C until constant weight. The general procedure for the extraction and analysis of *n*-alkanes followed that of Mayes et al (1986) with modifications as explained by Hatt et al (2002), except that tubes (150 x 20 mm) and all glassware, caps and liners were rinsed with *n*-heptane prior to use. Briefly, ~0.5 g (faeces) or ~1.0 g (pasture) of sample were weighed into a screw-capped borosilicate tube, 0.2 g of a solution containing 0.8 mg/g C_{22} (*n*-docosane) and 0.8 mg/g C_{34} (*n*-tetratriacontane) in *n*-undecane was added as internal standard and the extraction was performed with 7 mL (faeces) or 10 mL (pasture) of 1 M ethanolic potassium hydroxide solution and 2 x 7 mL (faeces) or 2 x 10 mL (pasture) of *n*-heptane. Purified extracts were dissolved again with 400 µL of *n*-heptane and analysed by gas chromatography in a Hewlett Packard series 6890 with a flame-ionization detector and automatic injection, using a DB-1 column (J&W) (15 m x 0.53 mm internal diameter, film thickness 1 µm) and helium as the carrier gas (10 mL/min). Column temperature was 2.5 min at 150 °C, 8 °C/min to 190 °C, 4 °C/min to 270 °C, 3 °C/min to 300 °C and 5 min at 300 °C, the injector and detector were maintained at 340 °C. The detector response was calibrated with three levels of a standard mixture of *n*-alkanes run every after sixteen samples. Paper stoppers, one out of every batch of a hundred, were sampled at random. These stoppers were individually analysed for C_{32} in screw-capped borosilicate tubes after the addition of a known amount of C_{34} as internal standard and 30 mL of *n*-heptane, using a similar procedure to that of Duncan et al (1999), except that the tubes were placed in an ultrasonic waterbath at 65 °C for 2 h and were manually shaked every 15 minutes. An aliquot of the extract diluted with *n*-heptane was analysed as described in the preceding paragraph.

Calculations and statistical analysis

The time required for the faecal concentration of C_{32} to reach stable levels was estimated by analysing the differences in the faecal *n*-alkane ratios (C_{32}/C_{31} and C_{32}/C_{33}) between each of the days from 2 to 6 and the mean of days 7 to 12 of dosing for AM, PM and the mean of AM and PM (AM-PM) samples. The analysis was performed using *proc mixed* (SAS, v. 9.1.3), considering day as repeated measurement, pasture allowance and its interaction with day as fixed, and animals within pasture allowance as random, with a covariance structure autoregressive order 1 chosen for final inference.

The rate of decrease of the faecal *n*-alkane ratios after the last dose of C_{32} (*k,* %/d) was estimated for each calf using the last four days (AM) of faecal sampling, by fitting the individual data to a monoexponential decay curve $y = y_0 + A*e^{-kt}$, where y_0 is the mean *n*-alkane ratio measured in the pasture. The time to clear the C_{32} dose was estimated by solving t for $y = y_0 + y_0*0.15$, where 0.15 represents the mean coefficient of variation in the faecal *n*-alkane ratios over days 7 to 12 of dosing. The differences in *k* between the faecal *n*-alkane ratios were analysed by a paired t test (α = 0.05, n = 8), and those between the pasture allowances by a t test (α = 0.05, n = 8).

The variation between days in the faecal *n*-alkane ratios for AM and PM samples, was estimated by calculating the coefficient of variation (CV, %) for each calf over days 7 to 12 of dosing. The effect of the pasture allowance (Low vs High) and the sampling time (AM vs PM) on the CV was analysed using *proc mixed* (SAS v. 9.1.3), considering sampling time as repeated measurement, pasture allowance and its interaction with sampling time as fixed, and animals within pasture allowance as random.

The individual pasture intake (PI) was estimated either from C_{31} or C_{33}, using either AM or PM samples, calculated as described in the preceding paragraph, with the formulae:

PI (kg DM/day) = $D_j/((F_j/F_i)*H_i - H_j)$ (Mayes and Dove, 2000),

where D_j is the mean daily dose of C_{32}, F_i is the mean content of C_{31} or C_{33} in faeces, F_j is the mean content of C_{32} in faeces, H_i is the mean content of C_{31} or C_{33} in pasture, and H_j is the mean content of C_{32} in pasture. The mean content of each *n*-alkane in pasture for each of the four strips was calculated by averaging the values from the grazing areas sampled.

The effect of the pasture allowance (Low vs High) and the sampling time (AM vs PM) on the faecal *n*-alkane ratios and the estimated pasture intake (based on values averaged over days 7 to 12 of dosing) was analysed using *proc mixed* (SAS v. 9.1.3), considering sampling time as repeated measurement, pasture allowance and its interaction with sampling time as fixed, and animals within pasture allowance as random.

Study 2

Experimental design

The study was conducted on July and lasted seventeen days. The first seven days were considered for adaptation to grazing time schedule and supplementation, and the last ten days for dosing and sampling.

The pasture was divided by electric fencing into two adjacent strips (15 x 200 m), to arrange two different grazing time schedules within each strip, replicated twice: 4-h: one fresh grazing area per day for 4 h (11:00 AM to 3:00 PM) at a pasture allowance of 4.2 kg DM/100 kg LW and 2x2-h: two fresh grazing areas per day, each one for 2 h (11:00 AM to 1:00 PM and 3:00 PM to 5:00 PM) at a pasture allowance of 2.1 kg DM/100 kg LW. The length of each fresh grazing area was periodically adjusted to maintain the required pasture allowance as described in Study 1, except that four quadrats were taken to estimate the mean DM availability. The alleys and automatic water troughs were arranged as described in Study 1.

Sixteen calves (158 ± 15 kg LW) were used, from which twelve were selected at random for dosing. The calves of both groups were further divided at random into two groups and randomly assigned to each strip and grazing time schedule, resulting four groups of four calves each. The calves were moved to each fresh grazing area by moving front and back electric fences along the strips. During the non-grazing hours, all the calves were kept in pens with free access to water, and were supplemented in individual feeders with 0.85 kg DM/100 kg LW/day of corn grain, divided into two equal portions (4-h: 10:00 AM and 3:00 PM, 2x2-h: 10:00 AM and 5:00 PM).

Dosing, sampling, measurements and sample analysis

From day 8 to day 17 of the study, 12 calves (three from each group) received 170 mg/d of C_{32}, divided into two equal doses of 85 ± 3 mg, each at 9:00-10:00 AM and 6:00-7:00 PM. The doses were prepared and administered as described in Study 1.

Samples of faeces were collected by rectal grab sampling from all 12 calves on days 6 to 10 of dosing at the same time as dosing (9:00-10:00 AM and 6:00-7:00 PM). The samples were frozen, freeze-dried and analysed for alkane contents as described in Study 1.

Samples of pasture were collected before grazing from the fresh grazing areas corresponding to days 5, 7 and 9 of dosing, by cutting four quadrats (20 x 20 cm) to the mean postgrazing height of the area grazed on the previous day (estimated from fifty measurements within grazed patches) with hand shears as described in Study 1. Samples from each strip, grazing time schedule and day were pooled, mixed and a subsample was frozen, freeze-dried and analysed for alkanes as described in Study 1.

Samples of corn grain daily offered were collected, pooled, mixed and analysed for alkanes as described in Study 1, except that these were not freeze-dried. Individual refusals were collected daily and weighed.

Calculations and statistical analysis

The variation between days in the faecal *n*-alkane ratios for AM and PM samples was estimated by calculating the CV (%) for each calf over days 6 to 10 of dosing, The effect of the grazing time schedule (4-h vs 2x2-h) and the sampling time (AM vs PM) on the CV was analysed using *proc mixed* (SAS, v. 9.1.3), considering sampling time as repeated measurement, grazing time schedule and its interaction with sampling time as fixed, and animals within grazing time schedule as random.

The PI was estimated as described in Study 1, with the formulae that allow for a known supplement intake (SI, kg DM/d):

PI (kg DM/d) = $[(D_j + SI*S_j)*F_i/F_j - SI*S_i]/(H_i - F_i/F_j*H_j)$ (Mayes et al, 1986),

where S_j is the mean content of C_{32}, and S_i is the mean content of C_{31} or C_{33} in the corn grain.

The effect of the grazing time schedule (4-h vs 2x2-h) and the sampling time (AM vs PM) on the *n*-alkane ratios and the estimated pasture intake (based on values averaged over days 6 to 10 of dosing) were analysed using *proc mixed* (SAS, version 9.1.3), considering sampling time as repeated measurement, grazing time schedule and its interaction with sampling time as fixed, and animals within grazing time schedule as random.

Results

Study 1

Pasture characteristics

The DM availability and DM content of the pasture were 4089 ± 397 kg DM/ha and 250 ± 70 g DM/kg of fresh pasture, respectively. The pregrazing and postgrazing heights, botanical composition, total live material, and the contents of CP, NDF, ADF, IVDDM, C_{31}, C_{32} and C_{33} in the grazed horizon are shown in Table 1.

Temporal variation in faecal n-alkane ratios

The temporal variation in the faecal *n*-alkane ratios is shown in Figure 1. From the beginning of dosing, the faecal *n*-alkane ratios increased very rapidly on both pasture allowances, and no significant differences ($p>0.05$) were detected between each of the days from 2 to 6 and the mean of days 7 to 12 of dosing for AM, PM and AM-PM.

There were no significant differences ($p>0.05$) in k estimated from C_{32}/C_{31} or C_{32}/C_{33} nor in those estimated from the different pasture allowances. After the last C_{32} dose, the faecal *n*-alkane ratios decreased at an overall k = 81 ± 11%/d, excluding one calf which had a much higher value (k = 166%/d). The time to clear the C_{32} dose was, on average, 111 ± 26 h (range: 58-141 h), the lowest value corresponded to the calf with the highest k. The estimates based on C_{32}/C_{31} were, on average, 5 hours significantly higher ($p<0.01$) than those on C_{32}/C_{33}, but no significant differences were detected between the pasture allowances. The data from individual calves fitted reasonably well to the decay curve (R^2 = 0.9088 to 0.9995), and no significant differences were found between individual curves when grouped by *n*-alkane ratio and pasture allowance. The parameters for the shared model (all calves) were: C_{32}/C_{31}-Low: A = 0.509 ± 0.029, k = 80.7 ± 10.3%, R^2 = 0.9215; C_{32}/C_{31}-High: A = 0.305 ± 0.019, k = 93.1 ± 12.5%, R^2 = 0.9142; C_{32}/C_{33}-Low: A = 0.630 ± 0.036, k = 81.1 ± 10.3%, R^2= 0.9226; C_{32}/C_{33}-High: A = 0.376 ± 0.027, k = 93.1 ± 14.8%, R^2= 0.8847.

The mean variation between days in the faecal *n*-alkane ratios was not significantly different between Low (C_{32}/C_{31} = 17 ± 6%, C_{32}/C_{33} = 18 ± 6%) and High (C_{32}/C_{31} = 13 ± 6%, C_{32}/C_{33} = 14 ± 6%) pasture allowance (although lower individual CV values were more frequent at the High pasture allowance), nor between AM (C_{32}/C_{31} = 16 ± 7%, C_{32}/C_{33} = 17

Table 1. Pasture characteristics (Study 1).
Tabla 1. *Características de la pastura (Estudio 1).*

Pasture allowance (kg DM/100 kg LW/day)	Low: 2.5	High: 7.5
Pregrazing height (mm)	194 ± 33	204 ± 41
Postgrazing height (mm)	27 ± 11	90 ± 21
Botanical composition of the grazed horizon	Fraction of standing DM	
Lolium perenne	0.432 ± 0.134	0.649 ± 0.035
Festuca arundinacea	0.194 ± 0.183	0.028 ± 0.017
Trifolium repens	0.077 ± 0.068	0.141 ± 0.013
Lotus corniculatus	0.154 ± 0.107	0.087 ± 0.012
other (mainly *Trifolium pratense*)	0.143 ± 0.010	0.095 ± 0.028
total live material	0.628 ± 0.076	0.696 ± 0.011
Chemical composition and IVDDM of the grazed horizon	g/kg DM	
CP	122 ± 11	134 ± 11
NDF	498 ± 24	490 ± 43
ADF	284 ± 12	266 ± 21
IVDDM	679 ± 35	718 ± 29
Alkane contents in the grazed horizon	mg/kg DM	
C_{31}	223 ± 24	233 ± 23
C_{32}	12 ± 1.1	12 ± 1.3
C_{33}	168 ± 13	183 ± 43

ADF: acid detergent fiber expressed exclusive of residual ash, CP: crude protein, C_{31}: *n*-hentriacontane, C_{32}: *n*-dotriacontane, C_{33}: *n*-tritriacontane, DM: dry matter, IVDDM: *in vitro* digestible dry matter, LW: live weight, NDF: neutral detergent fiber assayed without a heat stable amylase and expressed inclusive of residual ash. Values are the mean ± standard deviation (for more details see section 2.2. Study 1).

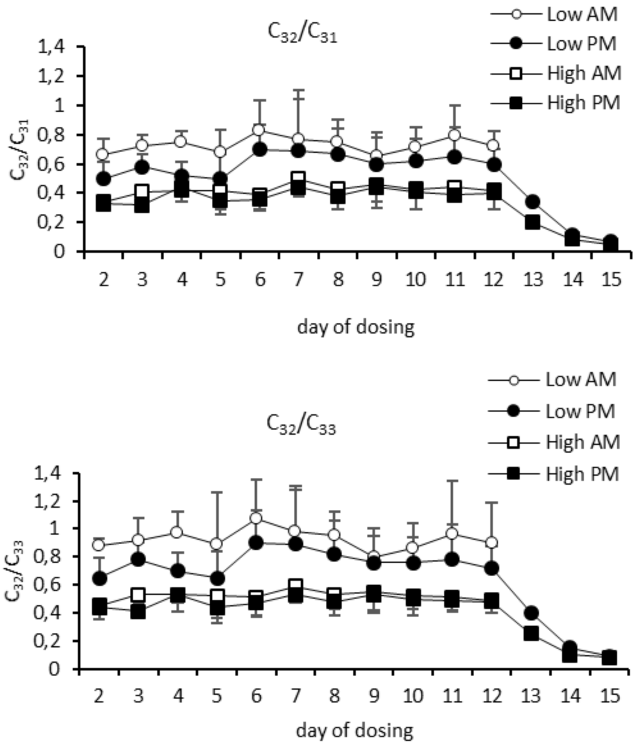

Figure. 1. Temporal variation (between and within sampling days) in faecal *n*-alkane ratios (C_{32}/C_{31} and C_{32}/C_{33}) in Holstein-Friesian calves (131 ± 14 kg LW) dosed with C_{32} twice a day, and strip-grazed at a Low (2.5 kg DM/100 kg LW/day) or High (7.5 kg DM/100 kg LW/day) pasture allowance (Study 1), AM or PM faecal sampling. Each value is the mean ± standard deviation of 8 calves.

Figura. 1. *Variación temporal (entre y dentro de los días de muestreo) de las relaciones entre los n-alcanos fecales (C_{32}/C_{31} y C_{32}/C_{33}) en terneros Holstein-Frisian (131 ± 14 kg PV) dosificados con C_{32} dos veces al día, y en pastoreo de franjas con asignación Baja (2,5 kg MS/100 kg PV/día) o Alta (7,5 kg MS/100 kg PV/día) (Estudio 1), muestreo de materia fecal AM o PM. Cada valor es la media ± desvio estándar de 8 terneros.*

± 6%) and PM (C_{32}/C_{31} = 14 ± 6%, C_{32}/C_{33} = 15 ± 7%) samples, and there was no indication of interaction. Considering all calves and both sampling times, the range for the individual CV values was wide (5-28%).

There were significant differences in the faecal *n*-alkanes ratios between AM and PM samples (p<0.01), and between Low and High pasture allowance (p<0.01), but there was no indication of interaction. The faecal *n*-alkane ratios in AM samples were lower than those in PM ones, the mean relative difference was 11% and 6% for Low and High pasture allowance, respectively, except for two calves, one from each pasture allowance, in which the faecal *n*-alkane ratios in AM samples were, on average, 4% higher than those in PM ones.

Pasture intake estimated from AM or PM faecal sampling

The pasture intake estimated from AM or PM samples is shown in Table 2. There were significant differences in the estimated pasture intake between AM and PM samples (p<0.01), and between Low and High pasture allowance (p<0.001), but there was no indication of interaction. The pasture intake estimated from AM samples were higher than those from PM ones, the mean relative difference was 11% and 8% for Low and High pasture allowance, respectively, except for two calves, one from each pasture allowance, in which the pasture intakes estimated from AM samples were, on average, 6% lower than those from PM ones.

Study 2

Pasture and supplement characteristics

The DM availability and DM content of the pasture during the study were 1774 ± 749 kg DM/ha and 39 ± 7 kg DM/100 kg of fresh pasture, respectively. The mean postgrazing height was 60 mm and no significant differences between grazing time schedules were detected (data not shown). The contents of C_{31}, C_{32} and C_{33} in the grazed horizon and the corn grain were 227 ± 79, 7.5 ± 3.2, 144 ± 43 and 0.72 ± 0.01, 0.48 ± 0.25, 0.35 ± 0.13 mg/kg DM, respectively. There were no refusals of corn grain, and a maximum amount of 50 g fresh was recovered, occasionally, from individual feeders after each supplementation.

Variation between and within days in the faecal n-alkane ratios

The variation between and within days in the faecal *n*-alkane ratios is shown in Figure 2. The mean variation between days in the faecal *n*-alkane ratios was not significantly different between 4-h (C_{32}/C_{31} = 15 ± 8%, C_{32}/C_{33} = 17 ± 9%) and 2x2-h (**C_{32}/C_{31}** = 12 ± 6%, C_{32}/C_{33} = 12 ± 6%) grazing time schedule (although lower individual CV values were more frequent at the 2x2-h grazing time schedule), nor between AM (C_{32}/C_{31} = 13 ± 6%, C_{32}/C_{33} = 15 ± 8%) and PM (C_{32}/C_{31} = 14 ± 8%, C_{32}/C_{33} = 14 ± 7%) samples, and there was no indication of interaction. Considering all calves and both sampling times, the range for individual CV values was wide (3-34%).

There were significant differences in the faecal *n*-alkanes ratios between AM and PM samples (p<0.001), but no significant differences were detected between 4-h and 2x2-h grazing time schedule (p=0.4), and there was no indication of interaction. The faecal *n*-alkane ratios in AM samples were lower than those in PM ones, the mean relative difference was 19% and 14% for 4-h and 2x2-h grazing time schedule, respectively.

Table 2. Pasture intake (kg DM/calf/day) estimated by faecal *n*-alkanes ratios (C_{32}/C_{31}; C_{32}/C_{33}) at different sampling times (AM or PM) in Holstein-Friesian calves dosed with C_{32} twice a day, and strip-grazed at different pasture allowances (Low or High) (Study 1) or at different grazing time schedules (4-h or 2x2-h) (Study 2).

Tabla 2. *Consumo de pastura (kg MS/ternero/día) estimada con la relación de los n-alcanos fecales (C_{32}/C_{31}; C_{32}/C_{33}) en diferentes horarios de muestreo (AM o PM) en terneros Holstein-Friesian dosificados con C_{32} dos veces al día, y en pastoreo de franjas con diferentes asignaciones de pastura (Baja o Alta) (Estudio 1) o con diferentes horarios de pastoreo (4-h o 2x2-h) (Estudio 2).*

Study 1: Sampling time						
n-alkane ratio	C_{32}/C_{31}			C_{32}/C_{33}		
Faecal sampling	AM	PM	Mean	AM	PM	Mean
Low: 2.5	2.79	2.49	2.64	2.81	2.52	2.66
High: 7.5	4.71	4.32	4.51	5.17	4.79	4.98
Mean	3.75	3.40		3.99	3.65	
AM vs PM	S.E.M.: 0.175	p = 0.001		S.E.M: 0.196	p = 0.007	
Low vs High	S.E.M: 0.240	p = 0.001		S.E.M: 0.267	p < 0.001	
Study 2: Grazing time schedule						
n-alkane ratio	C_{32}/C_{31}			C_{32}/C_{33}		
Faecal sampling	AM	PM	Mean	AM	PM	Mean
4-h	2.52	2.05	2.28	2.25	1.78	2.01
2x2-h	2.95	2.37	2.66	2.55	2.06	2.31
Mean	2.73	2.21		2.40	1.92	
AM vs PM	S.E.M: 0.170	p < 0.001		S.E.M: 0.136	p < 0.001	
4-h vs 2x2-h	S.E.M: 0.230	p = 0.280		S.E.M: 0.184	p = 0.281	

C_{31}: *n*-hentriacontane, C_{32}: *n*-dotriacontane, C_{33}: *n*-tritriacontane, DM: dry matter, LW: live weight, Differences were analysed using *proc mixed* with sampling time as repeated measurement (for more details see section 2.2. Study 1 and section 2.3. Study 2).

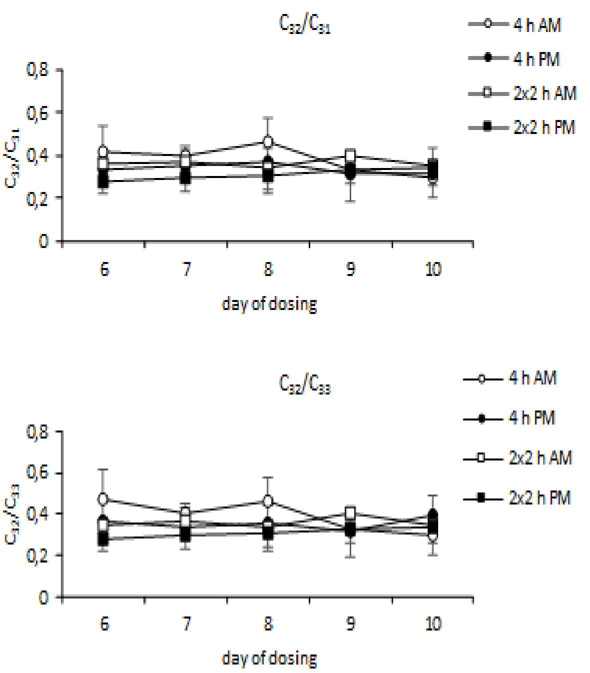

Figure. 2. Temporal variation (between and within sampling days) in faecal *n*-alkane ratios (C_{32}/C_{31} and C_{32}/C_{33}) in Holstein-Friesian calves (158 ± 15 kg LW) dosed with C_{32} twice a day, and strip-grazed at different grazing time schedules: 4-h (one fresh grazing area per day for 4 h (11:00 AM to 3:00 PM) at 4.2 kg DM/100 kg LW) or 2x2-h (two fresh grazing areas per day, each one for 2 h (11:00 AM to 1:00 PM and 3:00 PM to 5:00 PM) at 2.1 kg DM/100 kg LW) (Study 2), AM or PM fecal sampling. Each value is the mean ± standard deviation of 6 calves.

Figura. 2. Variación temporal (entre y dentro de los días de muestreo) de las relaciones entre los *n*-alcanos fecales (C_{32}/C_{31} y C_{32}/C_{33}) en terneros Holstein-Frisian (158 ± 15 kg PV) dosificados con C_{32} dos veces al día, y en pastoreo de franjas a diferentes horarios de pastoreo: 4-h (una franja de pastoreo por día durante 4 h (11:00 AM a 3:00 PM) a razón de 4,2 kg MS/100 kg PV) o 2x2-h (dos franjas de pastoreo por día, cada una durante 2 h (11:00 AM a 1:00 PM y 3:00 PM a 5:00 PM) a razón de 2,1 kg MS/100 kg PV) (Estudio 2), muestreo de materia fecal AM o PM. Cada valor es la media ± desvío estándar de 6 terneros.

Pasture intake estimated from AM or PM faecal sampling

The pasture intake estimated from AM or PM samples is shown in Table 2. There were significant differences in the estimated pasture intake between AM and PM samples (p<0.001), but no significant differences were detected between 4-h and 2x2-h grazing time schedule (p=0.3), and there was no indication of interaction. The pasture intake estimated from AM samples were higher than those from PM ones, the mean relative difference was 21% and 18% for 4-h and 2x2-h grazing time schedule, respectively.

Discussion

The rapid increase in the faecal *n*-alkane ratios to a maximum by day 2 of dosing (Study 1) would indicate that the time required for the faecal concentration of C_{32} to reach stable levels may be very short in Holstein-Friesian calves within 100-200 kg of LW grazing on good quality pastures, and this would not be affected by the pasture allowance within the range studied. This finding differs from the general recommendation (Mayes et al, 1986; Mayes and Dove, 2000; Oliván et al, 2007), however, since no other information is available, it seems reasonable that young animals, with high gastrointestinal passage rates (as indicated by the high decreasing rate (k = 81%/d) of the faecal *n*-alkane ratios in Study 1), may need less time for the faecal *n*-alkane ratios to achieve the plateau. The results suggest that, under similar conditions, the faecal sampling might begin several days earlier than in present recommendations, which would be advantageous when the pasture characteristics change rapidly with time, and further data are needed to confirm this observation.

Considering both studies, the variation between days in the faecal *n*-alkane ratios was more related to the individual calves than to sampling time, pasture allowance or grazing time schedule, as shown by the wide range for the individual CV (Study 1: 5-28%, Study 2: 3-34%). When the relationship between the individual CV and the LW was analysed, a weak positive correlation was detected only for PM in Study 1 (r = 0.54, P<0.05), and AM-PM in Study 2 (r = 0.65, p<0.05). Despite the lack of statistical significance, there was an indication that the variation between days in the faecal *n*-alkane ratios might be lower at higher daily pasture allowances and/or less restricted grazing time schedules, as shown by the fact that the lowest mean CV was seen at the High pasture allowance (Study 1: 8%) and also at the 2x2-hours grazing time schedule (Study 2*:* 11%). The variation between days in the faecal *n*-alkane ratios seen in this study can be considered relatively low, as compared to that reported by Mayes et al (1986) for housed sheep under controlled feeding (±5% deviation from a known mean) and, as argued by Dillon (1993), who found a good correlation between the variation between days in the *n*-alkane contents in pasture and that in faeces, it might be entirely explained by this fact.

The variation within days in the faecal *n*-alkane ratios was marked in both studies and, as a result, a higher pasture intake was estimated from AM (Study 1: +9%, Study 2: +20%), except for the two calves in Study 1. When the relationship between the individual relative difference in the faecal *n*-alkane ratios between AM and PM and the LW was analysed, no association was found in any of the studies. The mean relative difference in the estimated pasture intake between AM and PM increased in the order: High (+8%), Low (+11%) pasture allowances (Study 1), 2x2-h (+18%) and 4-h (+21%) grazing time schedules (Study 2). These results suggest that the effect of a strong restriction in the daily time available for grazing (Study 2) on the pasture intake estimated either from AM or PM might be greater than that of the pasture allowance when the daily time available for grazing is unrestricted (Study 1). This observation agrees with that of Dillon (1993, Experiment 2), from a study with dairy cows receiving different feeding patterns of cut herbage, where a more even feeding pattern favoured a lower variation between and within days in the faecal *n*-alkanes ratios, and the higher variation was noticed when the herbage allowance was offered for only 8 h per day.

Several authors have discussed that the feeding pattern, and the subsequent digesta kinetics, might be involved in the variation within days in the faecal *n*-alkane ratios, and postulated that this might relate to a variation of the dosed alkane, due to its tendency to associate with the liquid phase of the digesta (Dove and Mayes, 1991 and 1996; Mayes and Dove, 2000; Sibbald et al, 2000), but few (perhaps, only the work of Dillon, 1993) have attempted to study directly the relationship between the feeding pattern and the faecal excretion of *n*-alkanes). As far as we know, no study has been done yet to consider the effect of the ingestive behaviour on the temporal variation in the faecal *n*-alkane ratios under grazing conditions. Unfortunately, in the current studies we did not register the ingestive behaviour.

Conclusions

The results of this study indicate that more than one faecal sample per day should be taken to obtain a more reliable estimation of pasture intake in strip-grazed Holstein-Friesian calves dosed with C_{32} twice a day, particularly at low pasture allowances and/or when there is a strong restriction in the daily time available for grazing. The time required for the C_{32} to reach stable levels was very short, and suggests that the faecal sampling might begin earlier than in present recommendations, which would be advantageous when the pasture characteristics change rapidly with time.

Acknowledgments

We wish to thank Mr. Hugo Inza who kindly provided the calves from his private dairy farm for these studies. This research work was funded by the *Agencia Nacional de Promoción Científica y Tecnológica* (ANPCyT) of Argentina, under the project N° 08-09788.

References

AOAC. 1995. Official Methods of Analysis, 16th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

BARTHRAM, G.T. 1986. Experimental techniques: the HFRO sward stick. In: Hill Farming Research Organization, Biennal Report 1984-1985, Edimburgh, UK, pp. 29-30.

DILLON, P. 1993. The use of *n*-alkanes as markers to determine herbage intake, botanical composition of available or consumed herbage and in studies of digesta kinetics with dairy cows. Doctoral Thesis, National University of Ireland, Dublin, Ireland.

DOVE, H. and MAYES, R.W. 1991. The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. Australian J. Agr. Res. 42: 913-952.

DOVE, H. and MAYES, R.W. 1996. Plant wax components: a new approach to estimating intake and diet composition in herbivores. J. Nutr. 126: 13-26.

DUNCAN, A.J., MAYES, R.W., LAMB, C.S., YOUNG, S.A. and CASTILLO, I. 1999. The use of naturally occurring and artificially applied *n*-alkanes as markers for estimation of short-term diet composition and intake in sheep. J. Agric. Sci. 132: 233-246.

GIRÁLDEZ, F.J., LAMB, C.S., LÓPEZ, S. and MAYES, R.W. 2004. Effects of carrier matrix and dosing frequency on digestive kinetics of even-chain alkanes and implications on herbage intake and rate of passage studies. J. Sci. Food Agric. 84: 1562-1570.

HATT, J.M., GISLER, R., MAYES, R.W., LECHNER-DOLL, M., CLAUSS, M., LIESEGANG, A. and WANNER, M. 2002. The use of dosed and herbage *n*-alkanes as markers for the determination on intake, digestibility, mean retention time and diet selection in Galapagos tortoises (*Geochelone nigra*). Herpetol. J. 12: 45-54.

MAYES, R.W., LAMB, C.S. and COLGROVE, P.M. 1986. The use of dosed and herbage *n*-alkanes as markers for the determination of herbage intake. J. Agric. Sci. 107: 161-170.

MAYES, R.W. and DUNCAN, A.J. 1999. New developments in the use of plant-wax markers to determine intake. In: H. Dove, S.W. Coleman (Eds.), Emerging Techniques for Studying the Nutritional Status of Free-ranging Herbivores. Satellite Meeting of the Vth International Symposium on the Nutrition of Herbivores, San Antonio, Texas, USA (CD-ROM).

MAYES, R.W. and DOVE, H. 2000. Measurement of dietary nutrient intake in free-ranging mammalian herbivores. Nutr. Res. Rev. 13: 107-138.

OLIVÁN, M., FERREIRA, L.M.M., CELAYA, R. and OSORO, K. 2007. Accuracy of the n-alkane technique for intake estimates in beef cattle using different sampling procedures and feeding levels. Livestock Sci. 106: 28-40.

SAS (Statistical Analysis Systems), version 9.1.3. Institute Inc., Cary, NC, USA.

SIBBALD, A.M., DAVIDSON, G.C. and MAYES, R.W. 2000. Effect of dosing regime on intake estimation using the *n*-alkane technique in sheep feed pelleted grass meal. J. Sci. Food Agric. 80: 1206-1210.

TILLEY, J.M.A. and TERRY, R.A. 1963. A two-stage technique for the in vitro digestion of forage crops. J. British Grassld. Soc. 18: 104–111.

Van SOEST, P.J., ROBERTSON, J.B. and LEWIS, B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597.

VULICH, S.A. and HANRAHAN, J.P. 1992. Estimation of intake by the grazing animal with special reference to the *n*-alkane technique. In: 43rd Annual Meeting of the European Association of Animal Production, Madrid, Spain, pp. 1-12.

VULICH, S.A. and HANRAHAN, J.P. 1995. Faecal sampling for the estimation of herbage intake using *n*-alkanes: evaluation of sample pooling and the use of rectal grab samples. J. Agric. Sci. 124: 79-86.