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Two drying methods of bovine faeces for estimating *n*-alkane concentration, intake and digestibility: A comparison

F. Sánchez Chopa[a,b,*], L.B. Nadin[a,b], H.L. Gonda[a]

[a] *Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Paraje Arroyo Seco S/N, 7000 Tandil, Buenos Aires, Argentina*

[b] *Consejo Nacional de Investigaciones Científicas y Tecnológicas, Av. Rivadavia 1917, C1033AAJ CABA, Argentina*

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ABSTRACT

An experiment was completed to evaluate effects of two drying methods applied to faecal and herbage samples on estimation of *n*-alkane concentration and the calculated dry matter (DM) intake, faecal output (FO) and apparent *in vivo* DM digestibility (DMD). Four Holstein–Friesian steers (182 ± 12.1 kg initial body weight) housed in individual pens were fed at 8:30 and 16:30 h with ryegrass (*Lolium perenne* L.) hay for 18 d. Refusals were removed and weighed every day at 8:30 h before morning feeding. From days 9 to 18, the steers were dosed twice a day at feeding time, with cellucotton stoppers containing C_{32} (88.49 mg/pellet) and C_{36} (87.12 mg/pellet). During the last 5 d of dosing, total collection of faeces was completed using harnesses. For the *n*-alkane analysis, faecal ($n = 64$) and offered hay ($n = 4$) samples were subdivided into two subsamples and prepared for two alternative drying methods: oven-drying at 60 °C (OD) or freeze-drying (FD). The *n*-alkane profiles of the offered and refused hay oven-dried at 60 °C did not differ. In offered hay subsamples, concentrations of *n*-alkanes were not affected by drying method. In faecal subsamples, drying method affected ($P<0.05$) concentrations of all *n*-alkanes, except for C_{23}, C_{25} and C_{35}, as well as the ratios C_{32}:C_{31} and C_{32}:C_{33}. There were no differences between estimated and measured values when DM intake and apparent *in vivo* DMD were estimated based on the ratio C_{32}:C_{33}. In contrast, when the ratio C_{32}:C_{31} was used, estimated values either differed ($P<0.02$) or tended to differ ($P<00.09$) from measured values for OD and FD subsamples, respectively. Estimates of FO from subsamples OD and FD did not differ from measured values. Faecal recovery increased with increasing carbon-chain length in both drying methods. Results show that oven-drying at 60 °C could replace freeze-drying for sample dehydration to estimate DM intake and apparent *in vivo* DMD when ratio C_{32}:C_{33} was used. However, due to the low number of replicates, further studies should be conducted before use of C_{32}:C_{33} ratio in oven-dried samples can be recommended.

1. Introduction

Knowledge of dry matter (DM) intake and forage digestibility is useful in assessing the nutrient status of grazing animals. The double *n*-alkane marker technique developed by Mayes et al. (1986) allows estimation of DM intake, faecal output (FO), apparent *in vivo* DM digestibility (DMD) and botanical composition of forage based diets (Dove and Mayes, 1996, 2006).

Abbreviations: BW, body weight; DM, dry matter; DMD, DM digestibility; FD, freeze-dried; FO, faecal output; GC, gas chromatography; OD, oven-dried.

* Corresponding author at: Fac. Cs. Veterinarias, UNCPBA, Paraje Arroyo Seco S/N, 7000 Tandil, Argentina. Tel.: +54 249 4439850; fax: +54 249 4439850.
E-mail addresses: fsanchez@vet.unicen.edu.ar (F. Sánchez Chopa), lnadin@vet.unicen.edu.ar (L.B. Nadin), hgonda@vet.unicen.edu.ar (H.L. Gonda).

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To complete *n*-alkane analysis, the faecal and herbage samples must be dried. Mayes et al. (1986) and Dove and Mayes (2006) indicate that the material to be analysed should be freeze-dried, arguing that use of higher temperatures might result in losses of *n*-alkanes leading to an inaccurate estimation of DM intake, FO and the subsequent estimate of apparent *in vivo* DMD. However, routine analysis could be simplified if samples were oven-dried because freeze-drying takes longer and requires equipment which is more expensive to purchase and maintain.

In samples of fresh grasses, Dillon (1993) showed that oven-drying samples did not affect the *n*-alkane content. In contrast, Dove and Mayes (1991) reported that oven-drying samples of lucerne at 70 and 100 °C reduced *n*-alkane concentrations in a temperature dependent manner. Also, in samples of lucerne hay, Oliván and Osoro (1995) stated that, while oven-dried samples up to 40 °C yielded a good estimation of the concentration of *n*-alkanes of long chain length (C_{31}–C_{36}), levels of *n*-alkanes of shorter chain length were reduced.

Oliván and Osoro (1995) found no differences in *n*-alkane concentrations between C_{29} and C_{36} in faecal samples oven-dried at 40 °C but, as observed in herbage samples, concentrations of *n*-alkanes of shorter chain length were reduced. Sandberg et al. (2000) reported that, when drying faecal samples at 60 °C, a reduction in C_{31} occurred in faeces of steers fed lucerne hay, while C_{31} in faeces from steers fed meadow hay was not affected. Moreno et al. (2008) compared the *n*-alkane concentrations in faeces from steers fed winter oats dried at 60 °C or freeze-dried, and showed that the contents of C_{23}, C_{27} and C_{35} were affected by drying temperature. In contrast, Elwert et al. (2006) found no differences in faecal *n*-alkane concentrations when oven-drying at 65 °C or freeze-drying ovine faeces.

Owing to these discrepancies and lack of information, there is a need to evaluate effects of drying temperature on *n*-alkane concentrations in herbage and faecal samples. The objective was to determine if oven-drying at 60 °C could replace freeze-drying in determining the *n*-alkane concentrations in faeces and hay samples and estimating DM intake, FO and forage apparent *in vivo* DMD by comparing estimates against measured values in steers fed chopped ryegrass (*Lolium perenne* L.) hay.

2. Materials and methods

The study was conducted during late winter to early spring of 2007 at the facilities of Facultad de Ciencias Veterinarias de la Universidad Nacional del Centro de la Provincia de Buenos Aires (FCV – UNCPBA; 37°19′S, 59°07′W) in Tandil, Argentina.

For the purposes of this study, samples of faeces collected from 4 of 10 steers from a larger experiment completed to validate the *n*-alkane technique were used. Procedures were conducted according to Council Directive 2010/63/EU guidelines on the protection of animals used for experimental and other scientific purposes.

2.1. Animals, diet and experimental procedures

The experiment lasted 38 d, divided into a pre-experimental period of 20 d, and an experimental period of 18 d. Four Holstein–Friesian steers of 182 ± 12.1 kg of initial body weight (BW) were used. During the pre-experimental period, the steers were in a common paddock and fed ryegrass (*L. perenne* L.) hay *ad libitum*. Afterwards, on day 1 of the experimental period, the steers were individually housed in 18 m^2 pens with free access to water. During the experimental period the steers had an 8 d adaptation period (days 1–8) to the chopped ryegrass hay diet and daily management, followed by a 10 d period (days 9–18) of *n*-alkane dose and sample collection.

To avoid feed selection, ryegrass hay was chopped using a hammer mill chopper (Trapp400; Trapp Metalúrgica LTDA, Santa Fe, Argentina) to pass a 12 mm screen. The amount of ryegrass hay fed to each steer was adjusted during the 8 d adaptation period to achieve 0.10 refusals. Throughout the experimental period, the diet was weighed and offered in equal parts in individual troughs twice a day at 8:30 and 16:30 h.

2.2. Sample collection procedure

2.2.1. Feed samples

Two samples of offered hay were collected daily from days 9 to 18 at feeding to determine its DM content. Also, four samples of hay were collected during the dosing period (*i.e.*, days 14–17) and stored at room temperature (*i.e.*, 20 °C) for further *n*-alkane analysis. Individual steer feed refusals were removed daily at 8:30 h before morning feeding, weighed and individual samples were used to estimate DM content to calculate DM intake. From the refusals, 4 samples were also collected (days 14–17) and stored at room temperature to estimate *n*-alkane concentrations.

2.2.2. Faecal samples

During the last 5 d of *n*-alkane dosing (*i.e.*, days 14–18), total faeces were collected using harnesses. From days 14 to 17, harnesses were changed at 8:00, 16:00 and 22:30 h, and the last day of *n*-alkane dosing, faeces were collected 4 times, changing harnesses every 6 h. At each time, the faeces were weighed, homogenised and two samples of about 400 g each were taken. One sample was used to determine DM content and the other sample was kept at −20 °C until analysis.

2.3. n-Alkane dose

From days 9 to 18, steers were dosed twice a day before feeding with cellucotton stoppers (34.5 mm × 22 mm; Carl-Roth GmbH and Co KG, Karlsruhe, Germany) containing dotriacontane (C_{32} – 88.49 mg/pellet) and hexatriacontane (C_{36} – 87.12 mg/pellet; Sigma–Aldrich, Aldrich Chemical Co., Gillingham, UK). Each dose was prepared by pipetting a controlled amount of 2 solutions of C_{32} and C_{36} in *n*-heptane, respectively, into each cellucotton stopper. The cellucotton stoppers were administered orally using an automatic dosing gun. There was no evidence of regurgitation of the stoppers.

2.4. Analysis

The DM content was determined in offered and individually refused hay, and in faecal samples, by drying at 100 °C to constant weight in order to calculate values of DM intake and FO.

n-Alkane analysis was completed on offered ($n = 4$) and refused ($n = 4$) hay samples, as well as on faecal (16 samples/animal) samples. In order to compare effects of drying methods on *n*-alkane concentrations, faecal and offered hay samples were subdivided into two subsamples. Each subsample was then prepared for *n*-alkane analysis by freeze-drying (FD) or oven-drying at 60 °C (OD).

Once dried, subsamples were finely grounded with an electrical coffee grinder (Connoisserve CG 700, Kin Hip M & P Factory Ltd., Kowloon, Hong Kong). Afterwards, 0.25 g of each faecal and 0.75 g of each hay subsample were placed in borosilicate tubes (o.d. 150 mm × 20 mm) with ∼0.11 g of a solution of ∼0.4 mg/g of *n*-docosane (C_{22}) and ∼0.8 mg/g of *n*-tetriacontane (C_{34}) in *n*-undecane (C_{11}), as internal standards to correct process variation (Oliván and Osoro, 1999). The caps of the tubes were fitted with a Teflon insert. From this analytical step on, and in order to avoid differences due to extraction and quantification by gas chromatography (GC), special care was taken to treat subsamples (OD and FD) from the same sample in pairs. Correction for DM content was by drying 1 g of each subsample in an oven at 95 °C to constant weight.

The *n*-alkane extraction procedure was as described by Mayes et al. (1986) with modifications. The saponification involved a treatment of faeces and ryegrass hay for 16 h in 7 and 10 ml ethanolic 1 M KOH at 90 °C. Upon removal from the dry block heater, tubes were cooled to 65 °C. Afterwards, 5 ml and 8 ml of *n*-heptane (J.T. Baker, Mallinckrodt Chemicals, Phillipsburg, NJ, USA) and 2 ml of distilled water were added to each faecal and forage subsample, respectively. Tubes were shaken vigorously and the *n*-heptane upper phase containing the *n*-alkanes was transferred to new tubes. This procedure was repeated twice on each sample. The *n*-heptane extracts were evaporated in a dry block heater at 90 °C, residues again dissolved in 1.5 ml of *n*-heptane (5 times on each subsample) and then transferred to a ∼5 ml silicagel 60 (Merck, Merck KGaA, Darmstadt, Germany) column, for solid phase separation. The extract eluted from the column was evaporated and kept at room temperature until analysis. The analysis used HP Agilent-6890 GC equipment, with automatic injector and flame detector. The column used was a DB-1 (J&W, Agilent Technologies, Palo Alto, CA, USA; i.d.: 15 m × 0.53 mm; film: 1 μm) using helium as carrier gas with a 10 ml/min flux at constant rate. The extract eluted from silicagel column was redissolved with 400 μl of *n*-heptane (Merck, Merck KGaA), and 0.5 μl were injected without flux division. The chromatography conditions were: injector and detector temperature: 340 °C; oven temperature: 150 °C for 2.5 min; ramp 1: 8 °C/min until 190 °C; ramp 2: 4 °C/min until 270 °C; ramp 3: 3 °C/min until 300 °C for 5 min.

Quantification of odd-numbered *n*-alkanes (C_{23}–C_{35}) used 3 levels of a calibration solution. The calibration solution had *n*-tricosane (C_{23}), *n*-pentacosane (C_{25}), *n*-heptacosane (C_{27}), *n*-nonacosane (C_{29}), *n*-hentriacontane (C_{31}), *n*-dotriacontane (C_{32}), *n*-tritriacontane (C_{33}) and *n*-hexatriacontane (C_{36}) (Sigma–Aldrich, Aldrich Chemical Co.) in *n*-heptane with C_{22} and C_{34} as internal standards; *n*-pentatriacontane (C_{35}) was quantified using the response factor of C_{36}. *n*-Alkane peaks were identified by reference to known standards. The peak areas were converted to *n*-alkane concentrations by using the peak area, the weight of the sample and its DM content, and the weight of internal standards (C_{22} and C_{34}).

2.5. Calculations

Observed DM intake by steer was calculated individually on a daily basis by difference between offered and refused hay. Estimates of DM intake by the *n*-alkane technique were calculated using ratios between *n*-alkane C_{32} (dosed and ingested) and its two naturally occurring adjacent *n*-alkanes C_{31} and C_{33}, from concentrations obtained in ryegrass hay and faecal samples oven and freeze-dried according to the equation of Dove and Mayes (2006). Faecal output was estimated from intake of C_{36} and its concentration in faeces from the OD and FD samples.

Faecal *n*-alkane recovery was calculated as the proportion of ingested alkane which was excreted, using the *n*-alkane concentrations estimated in faeces and offered herbage by both drying methods and observed values of DM intake and FO.

2.6. Statistical analysis

Prior to statistical analysis, data of *n*-alkane concentration in faecal samples were pooled by animal. The effect of drying treatment (OD or FD) on the *n*-alkane concentration and on C_{32}:C_{31} and C_{32}:C_{33} ratios of hay ($n = 4$) and faeces ($n = 4$) was assessed by a Paired Student's *T*-test. To compare the ratios of C_{32}:C_{31} and C_{32}:C_{33} obtained from the OD and FD subsamples, the data were transformed to a normal distribution by exponential transformation.

Table 1
n-Alkane concentration in *Lolium perenne* hay (mg/kg DM) either oven-dried at 60 °C (OD) or freeze-dried (FD).

n-Alkane	Herbage (*n* = 4)		SED[a]	P
	OD	FD		
23	8.9	9.2	0.61	0.66
25	50.7	52.6	4.08	0.68
27	90.3	92.9	7.08	0.74
29	193.8	185.3	8.77	0.40
31	298	304	13.6	0.69
32	6.1	6.6	0.44	0.35
33	58.8	59.1	2.94	0.93
35	5.4	5.7	0.31	0.42
36	2.7	1.8	0.88	0.38

[a] Standard error of the difference.

Paired Student's *T*-tests were performed to compare estimates of DM intake, FO and apparent *in vivo* DMD against measured values, as well as to compare *n*-alkane faecal recovery from OD and FD subsamples.

Statistical analyses used SAS (2008). Differences among means with P<00.05 were accepted as statistically significant. Trends were considered significant when P<00.10.

3. Results

The *n*-alkane profiles of the offered and refused ryegrass hay samples oven-dried at 60 °C did not differ. Therefore, only data from offered hay are presented. In hay subsamples, concentrations of *n*-alkanes were not affected by drying method (Table 1).

In faecal subsamples (Table 2), the concentrations of all *n*-alkanes, except for C_{23}, C_{25}, C_{35} and C_{36}, were significantly lower in OD than FD. While tendencies were observed for C_{25} (P=0.096) and C_{35} (P=0.061) to be lower in OD, the concentration of C_{36} was significantly higher (P<00.01) in OD than in L. The ratios C_{32}:C_{31} and C_{32}:C_{33} were also different between drying methods (Table 2).

The DM intake was 2.99 ± 0.19 kg/100 kg of the BW of the steers. Irrespectively of drying method, when DM intake and apparent *in vivo* DMD were estimated based on the ratio C_{32}:C_{33}, there were no differences between estimated and measured values (Table 3). In contrast, when the ratio C_{32}:C_{31} was used, there were differences between estimated values from OD subsamples and measured values. In FD subsamples, estimates for DM intake (P=0.072) and for apparent *in vivo* DMD (P=0.086) based on ratio C_{32}:C_{31} tended to differ from measured values (Table 3).

Estimates of FO, based on daily intake and faecal concentration of C_{36} in OD and FD subsamples, did not differ from measured values (Table 3).

Faecal recovery increased with increasing carbon-chain length (Table 4). Faecal recovery was numerically lower when samples were OD compared to FD (except for C_{36}), and only a trend to differ between treatments was found for the *n*-alkane C_{31} (Table 4).

4. Discussion

No signs of discomfort were observed and the steers adapted well to the experimental protocol. The lack of differences in the *n*-alkane profile of offered and refused hay (data not shown), showed that there was no selection among different

Table 2
Mean *n*-alkane concentration in faeces (mg/kg DM) oven-dried at 60 °C (OD) or freeze-dried (FD).

n-Alkane	Faeces (*n* = 4)		SED[a]	P
	OD	FD		
23	11.4	12.3	0.64	0.24
25	67.7	76.5	3.68	0.10
27	129.2	154.5	7.72	0.05
29	303	391	23.27	0.03
31	497	607	24.94	0.02
32	79.1	85.9	1.94	0.04
33	106.9	128.7	4.71	0.02
35	11.9	13.3	0.46	0.06
36	74.4	70.9	0.43	0.01
32:31	0.4	0.4	0.01	0.03
32:33	0.9	0.8	0.01	0.02

[a] Standard error of the difference.

Table 3
Dry matter intake (kg DM/d), fecal output (kg DM/d) and apparent *in vivo* DM digestibility estimated by the *n*-alkane technique from oven-dried or freeze-dried samples *versus* measured values ($n = 4$).

	Measured	Oven-dried			Freeze-dried		
		Mean	SED[a]	P	Mean	SED[a]	P
Intake							
C_{32}:C_{31}	5.45	4.42	0.121	<0.004	4.86	0.217	0.072
C_{32}:C_{33}	5.45	5.11	0.299	0.141	5.34	0.258	0.675
Faecal output							
C_{36}	2.37	2.42	0.035	0.257	2.48	0.086	0.292
Digestibility							
C_{36}/C_{32}:C_{31}	0.56	0.45	0.025	0.018	0.49	0.030	0.086
C_{36}/C_{32}:C_{33}	0.56	0.50	0.033	0.136	0.53	0.029	0.398

[a] Standard error of the difference.

morphological components of the diet, such as lamina, pseudostem and inflorescence. Therefore, the *n*-alkane pattern of the hay consumed was assumed to be that of the offered hay.

In contrast to findings of Oliván and Osoro (1995), who found that oven-drying lucerne hay samples at 40 °C diminished the content of C_{25}–C_{30} *n*-alkanes, we did not observe differences due to drying methods in the *n*-alkane content of the offered ryegrass hay. The *n*-alkane content also appears to differ when different drying methods are applied to fresh forage samples. Thus, in fresh lucerne samples, Dove and Mayes (1991) mentioned an *n*-alkane concentration decrease when oven-drying, while Dillon (1993) concluded that oven-drying at 60 °C could replace freeze-drying method in samples of fresh predominantly ryegrass pastures.

Consistent with Oliván and Osoro (1995), the *n*-alkane concentration in faeces was, on average and except for C_{36}, 12% lower in OD than in FD subsamples. However, while Oliván and Osoro (1995) reported that oven-drying at 40 °C reduced the concentration of only C_{24}–C_{28} *n*-alkanes, under the conditions of our experiment, this effect occurred, or tended to occur (P>00.10) for all but C_{23} and C_{36} *n*-alkanes. Moreno et al. (2008) compared effects of drying at 60 °C *versus* freeze-drying faecal samples from steers grazing winter oats, reporting that only C_{23}, C_{27} and C_{35} *n*-alkanes were affected by drying method. In faecal samples from sheep fed mixed diets of lucerne and wheat, Elwert et al. (2006) reported a higher decrease on *n*-alkane concentration when oven-dried at 105 °C, and no differences in *n*-alkanes ranging from C_{25} to C_{33} – except for C_{31} – when drying faeces at 65 °C or freeze-drying. Whether the differences between the temperatures used to oven dry the faecal samples may explain these effects is not clear. In a preliminary study, Sánchez Chopa et al. (2008) examined effects of drying method (*i.e.*, 95 °C, 65 °C and FD) on *n*-alkane losses on 12 of the 64 faecal samples used in our experiment, and no effects due to the drying temperature occurred. In addition, Sandberg et al. (2000) observed that faeces from steers fed lucerne hay oven-dried at 60 °C had a loss of 20% of C_{31} compared to freeze-dried, while faeces from steers fed meadow hay oven-dried at 60 °C or freeze-dried had no differences in C_{31} concentration. Therefore, whether interactions among drying temperatures and herbage species on the *n*-alkane losses exist, remains to be established.

In spite of uncertainties about effects that drying temperature may have on *n*-alkane losses in herbage and faecal samples, it is of major interest to examine effects that the sample drying method may have on estimates of DM intake, FO and apparent *in vivo* DMD. When estimates were calculated from C_{32}:C_{31} ratio, DM intake and DMD differed or tended to differ (for OD and FD subsamples, respectively) from measured values. In contrast, and independently of the drying method, estimates based on C_{32}:C_{33} ratio did not significantly differ from measured values. While these findings suggest that using C_{33} rather than C_{31} would provide reliable estimates in samples oven-dried at 60 °C, the low number of replicates ($n = 4$) used in our study imposes a limitation to the conclusions that could be drawn from our results.

An important assumption when using the double *n*-alkane marker technique is that adjacent *n*-alkanes used in the estimates of intake (dosed even-chain alkane and natural odd-chain alkane) have similar faecal recovery. In previous studies (Mayes et al., 1986; Dove et al., 2000, 2002), *n*-alkane pairs C_{32}:C_{31} and C_{32}:C_{33} had the lowest discrepancy in faecal recovery, and therefore were proposed to estimate DM intake. In our study, however, using the ratio C_{32}:C_{33} for DM intake estimates

Table 4
n-Alkane mean recovery from oven-dried (OD) and freeze-dried (FD) samples expressed as the excreted proportion of ingested *n*-alkane ($n = 4$).

n-Alkane	OD	FD	SED[a]	P
23	0.53	0.55	0.028	0.52
25	0.58	0.62	0.054	0.49
27	0.62	0.71	0.077	0.34
29	0.69	0.91	0.103	0.12
31	0.72	0.87	0.058	0.08
32	0.90	0.96	0.036	0.17
33	0.82	0.95	0.068	0.16
35	0.93	0.98	0.066	0.48
36	0.95	0.93	0.025	0.46

[a] Standard error of the difference.

was more reliable than the ratio C_{32}:C_{31}. It should also be considered that the difference in apparent *in vivo* DMD between OD (ratio C_{32}:C_{31}) and measured values was due to the estimates of DM intake and not to the estimates of FO, since FO was well estimated from OD and FD faecal subsamples. Therefore, differences among estimates based on ratios C_{32}:C_{31} and C_{32}:C_{33} may have been due to differences in faecal recovery among these *n*-alkanes as well as to the effect of the drying temperature on their faecal recovery.

5. Conclusions

Under our experimental conditions, use of alternative drying methods (oven-dry at 60 °C or freeze-dry) did not affect the *n*-alkane content of air-dried (hay) herbage samples, but affected the *n*-alkane concentration of faeces. Use of C_{36} from subsamples oven-dried or freeze-dried gave consistent estimates of FO. Independent of drying methods, use of C_{32}:C_{33} ratio, but not C_{32}:C_{31} ratio, resulted in reliable estimates of DM intake and apparent *in vivo* DMD. However, the low number of replicates ($n = 4$) used in our study may have precluded statistical differences between estimates and measured values. Further studies with a larger number of animals are required to explore effects of different drying temperatures in herbage and faeces samples before the use of the C_{33} *n*-alkane in oven-dried samples could be recommended.

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