J. Range Manage.
53:159-163 March 2000

N-alkane as an internal marker for predicting digestibility of forages

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Abstract

Independent digestion trials with 5 forages were conducted to compare n-alkane with indigestible acid-detergent fiber (IADF) as internal markers to predict in vivo dry matter digestibility (digestibility). Forages were mixed grasses from subirrigated meadow (meadow), meadow regrowth (regrowth), native range (range), mature mixed grass hay from meadow, and alfalfa (*Medicago sativa* L.) hay. Meadow, regrowth, and range diets were immature grasses harvested 0.5 hours before feeding. Feces from the meadow hay and alfalfa hay trials were divided to compare freeze drying and oven drying (60°C). All diets were subjected to in vitro fermentation for 0, 48, or 96 hours. N-alkane was separated from samples by 4.5-hour saponification with alcoholic KOH followed by extraction with n-hexane. Indigestible ADF was measured by 96-hour in vitro fermentation followed by ADF extraction. Digestibility estimated by markers was compared with in vivo digestibilities. N-alkane based digestibilities were lower ($P < 0.01$) than in vivo digestibility for all diets. N-alkanes provided higher estimates of digestibilities than IADF for meadow ($P < 0.01$), regrowth ($P = 0.06$), and alfalfa hay ($P = 0.06$), and lower digestibility for meadow hay ($P = 0.02$). Digestibilities calculated using n-alkanes for range tended to be higher ($P = 0.14$) than IADF values. Freeze drying increased ($P<0.01$) the amount of n-alkane extracted from alfalfa hay, but did not affect ($P = 0.1$) the amount extracted from meadow hay. N-alkane disappeared ($P < 0.001$) from residue collected after 48 hours of in vitro fermentation, but no additional disappearance ($P = 0.78$) was evident at 96 hours. Neither marker was completely recoverable, although recovery of n-alkane was higher than indigestible ADF for 4 of the 5 forages tested.

Key Words: indigestible, ADF, hydrocarbon, beef cattle

Resumen

Se condujeron ensayos individuales de digestibilidad con 5 forrajes para comparar el n-alcano con la fibra indigestible ácido-detergente (IADF) como marcadores internos para predecir la digestibilidad in vivo de la materia seca (digestibilidad). Los forrajes evaluados fueron: zacates mezclados de una pradera subirrigada (pradera), rebrote de la pradera (rebrote), pastizal nativo (pastizal), heno de zacates mezclados maduros de la pradera y heno de alfalfa (*Medicago sativa* L.). Las dietas de pradera, rebrote y pastizal consistieron de zacates inmaduros cosechados 0.5 horas antes de ofrecerlos como alimento. Las heces fecales de los ensayos de los henos de pradera y alfalfa se dividieron para comparar el secado por congelamiento y el secado en horno (600 C). Todas las dietas se sometieron a fermentación in vitro por 0, 48 y 96 horas. El n-alcano fue separado de las muestras mediante una saponificación de 4.5 horas con KOH alcohólico seguido por una extracción con n-hexano. La ADF indigestible se midió mediante una fermentación in vitro de 96 horas seguida por la extracción de ADF. La digestibilidad estimada por los marcadores se comparó con las digestibilidades in vivo. En todas las dietas, la estimación de la digestibilidad basada en n-alcano fue menor ($P < 0.01$) que la digestibilidad in vivo. Las estimaciones de digestibilidad obtenidas con n-alcanos fueron mayores que las obtenidas con IDAF, esto para pradera ($P < 0.01$), rebrote ($P = 0.06$) y heno de alfalfa ($P = 0.06$), y menor para heno de pradera ($P = 0.02$). Las digestibilidades del pastizal calculadas usando n-alcano tendieron a ser mayores ($P = 0.14$) que los valores obtenidos con IADF. El secado por congelamiento aumento ($P < 0.01$) la cantidad de n-alcano extraída del heno de alfalfa, pero no afecto ($P = 0.01$) la cantidad extraída del heno de pradera. El n-alcano desapareció ($P < 0.001$) del residuo colectado después de 48 horas de fermentación in vitro, pero no se evidencio una mayor desaparición de n-alcano en la fermentación de 96-horas. Ningún marcador fue completamente recuperable, aunque la recuperación de n-alcano fue mayor que la IADF en 4 de los 5 forrajes evaluados.

In vitro dry matter disappearance and internal markers such as indigestible acid-detergent fiber (IADF) and lignin are common methods for estimating forage digestibility in cattle. However, these methods often fail to accurately predict in vivo digestibility (Galyean et al. 1986). Mayes et al. (1986) proposed that long chained hydrocarbons (n-alkanes) may accurately predict in vivo digestibility.

Naturally occurring n-alkanes found in most pasture species contain odd-numbered carbon chains with 25 to 35 carbon atoms. Because fecal recovery of n-alkanes improves with increasing chain length, tritriacontane ($C_{33}H_{68}$) is commonly used to estimate digestibility (Mayes et al. 1986a). Tritriacontane and penttriacontane ($C_{35}H_{72}$) are not present in some tropical forages (Laredo et al. 1991). However, there is evidence that long chain n-alkanes disappear during gastrointestinal passage (Mayes et al. 1986). Mayes et al. (1988) determined in sheep that a site of disappearance of n-alkanes is from the small intestine. In contrast, with dairy cattle Ohajuruka and Palmquist (1991) estimated that 15% of a ruminally infused synthetic n-alkane marker disappeared in the rumen. To correct for incomplete recovery of n-alkane markers in the feces, Mayes et al (1986) proposed dosing

Published with the approval of the director of the University Nebraska-Lincoln, Institute of Agr. and Natur. Resources, Agric. Res. Div. as Journal Ser. No. 12440.
Manuscript accepted 30 May 1999.

animals twice daily with synthetic even-chained n-alkanes. However, for many studies on range or pasture, dosing of synthetic n-alkanes may not be practical. Little is known on how method of drying (i.e., oven vs. freeze drying) affects n-alkane extraction from samples. Objectives of this study were to: 1) identify which n-alkanes were present in sufficient quantities to be used as internal markers for range, meadow and alfalfa, 2) compare the effectiveness of n-alkane with IADF in estimating in vitro and in vivo digestibility when externally dosing an even-chain n-alkane is not practical because of pasture size and animal distribution, 3) determine if n-alkane disappearance occurs in the rumen, and 4) evaluate effects of different drying methods on n-alkane extraction.

Material and Methods

Animals and Feeding

Five yearling steers (body weight = 425 kg ± 13) were housed individually in 3 x 3 m pens for 5 independent digestion trials using immature mixed grasses from subirrigated meadow (meadow), meadow regrowth (regrowth), and native sandhills range (range), mature mixed grass hay from meadow, and alfalfa (*Medicago sativa* L.) hay. Meadow, range, and meadow regrowth trials were conducted in 1995 using vegetative grasses, beginning 1 June, 1 July, and 15 July, respectively, at the Gudmundsen Sandhills Laboratory located 11 km northeast of Whitman, Neb. The meadow hay and alfalfa hay trials were conducted in September 1996 at the West Central Research and Extension Center, North Platte, Neb.

Dominant vegetation for the meadow, meadow regrowth, and meadow hay was Kentucky bluegrass (*Poa pratensis* L.), slender wheatgrass [*Elymus trachycaulum* (Link) Gould ex Shinn.], quackgrass [*Elytergia repens* (L.) Nevski], redtop (*Agrostis stolonifera* L.), timothy (*Phleum pratense* L.), several species of sedges (*Carex* spp.), smooth bromegrass (*Bromus inermis* Leyss.), and reed canarygrass (*Phalaris arundinacea* L.). Other common species were prairie cordgrass (*Spartina pectinata* Link), rushes (*Juncus* spp. and *Eleocharis* spp.), big bluestem (*Andropogon gerardii* Vitman), indiangrass *[Sorgastrum nutans* (L.) Nash], switchgrass (*Panicum virgatum* L.), and several species of clover (*Trifolium* spp).

The forage collected from the range site was dominated by warm-season grasses, including little bluestem [*Schizachyrium scoparium* (Michx.) Nash], prairie sandreed [*Calamovilfa longifolia* (Hook.) Scribn.], sand bluestem (*Andropogon hallii* Hack.), and switchgrass (*Panicum virgatum* L.). Other common species were blue grama [*Bouteloua gracilis*(H.B.K.) Lag. ex Griffiths], hairy grama (*Bouteloua hirsuta* Lag.), sand dropseed [*Sporobolus cryptandrus* (Torr.) Gray], prairie junegrass [*Koeleria pyramidata* (Lam.) Beauv.], needleandthread (*Stipa comata* Trin. & Rupr.), western ragweed (*Ambrosia psilostachya* DC.), Schweinitz flatsedge (*Cyperus schweinitzii* Torr.), and sun sedge (*Carex heliophila* Mack.). More detail of meadow and range vegetation and soils is given by Adams et al. (1998).

Each trial consisted of a 10-day diet adaptation period followed by 5 days of total fecal collection. Each forage was limit-fed twice daily at 1.0% of body weight per feeding with the forage from meadow, meadow regrowth, and range harvested 0.5 hours before feeding. Feed samples were collected and frozen before each feeding. Refusals were collected and frozen before the morning feeding, and feces were collected and frozen twice daily.

Laboratory analysis

Diets, refusals, and feces from each trial were freeze dried and ground in a Wiley Mill to pass through a 1-mm screen. Samples were composited by dry weight across days on an individual animal basis. Feces from the meadow hay and alfalfa hay trials were subsampled and either freeze dried or dried in a forced air oven (60°C) to compare the effect of drying method on n-alkane extraction. Laboratory analyses included dry matter, organic matter (AOAC 1990), and IADF (Berger et al. 1979, Cochran et al. 1986) for all samples and NDF (Van Soest et al. 1991), ADF (Van Soest 1963), crude protein (AOAC 1990), and in vitro dry matter disappearance (Tilley and Terry 1963) using modified procedures as described by Hollingsworth-Jenkins et al. (1996) for the diet samples.

Hentriacontane disappearance in the rumen was examined by using a modified Tilley and Terry (1963) method for in vitro dry matter disappearance. Samples weighing 1.8 g were measured into three , 30-ml polypropylene in vitro tubes in 0.6 g increments, inoculated with a mixture of rumen fluid:McDougall's buffer (McDougall 1948), and incubated in a 39°C water bath for either 48 or 96 hours. The contents from the 3 tubes were filtered through filter paper (Whatman[1] 541), and the residues were combined to form a single sample and saved for later n-alkane analysis.

Alkane Analysis

Subsamples weighing either 1 g for feces or 2 g for forage were placed in a 75-ml tube (fitted with a screwcap and teflon liner) with 0.6 ml of a 1,000 mg/liter^{-1} solution of dotriacontane ($C_{32}H_{66}$) n-hexane as an internal standard. Each tube was then placed in a 90°C water bath to saponify samples for 4.5 hours with 10 ml alcoholic KOH. After saponification, liquid-liquid extraction was performed by adding 7 ml of n-hexane and 2 ml of H_2O, shaking vigorously, centrifuging (1,000xg for 10 min), and transferring the n-hexane layer to a prepared column for solid phase separation. The column was prepared by first placing 2 g of silicic acid per column in a 110°C oven to activate the silicic acid. Then the silicic acid was suspended in solution using 10 ml of n-hexane and placed in an extraction column. The extract eluted from the column was evaporated to dryness, reconstituted with 2 ml of n-hexane, and placed into a glass vial for later analysis using gas chromatography. Ten samples from the meadow, meadow regrowth, and range trials selected randomly were reconstituted with 2 ml of a triacontane ($C_{30}H_{26}$) standard (0.3 mg per ml of n-hexane) to determine the recovery rate of dotriacontane during the extraction procedure.

Calculations and Statistical Analysis

In vivo DMD and estimated values of DMD using IADF and n-alkanes as internal markers were calculated following procedures outlined by Schneider and Flatt (1975). N-alkane concentrations were determined using the following formula with 0.6 mg representing 0.6 ml of a standard solution containing 1.0 mg of dotriacontane per ml of n-hexane:

$$\text{mg of n-alkane/kg sample} = \frac{\text{peak area of alkane}}{\text{peak area of internal standard}} \times \frac{0.6 \text{ mg} \times 100}{\text{sample weight} \times \text{DM}} \quad (1)$$

Dry matter digestibility coefficients and marker recovery for each trial and drying

[1]Whatman Labsales, P.O. Box 1359, Hillsboro, Ore. 97123.

methods for the meadow hay and alfalfa hay trials were analyzed as a randomized complete block, with steers as blocks using the General Linear Model Procedure of SAS (1990).

Results

Because the n-alkane, tritriacontane, did not produce any detectable peaks using gas chromatography for the freshly harvested forages, the next smaller, odd-chained n-alkane, hentriacontane ($C_{31}H_{64}$), was used. The recovery of the dotriacontane internal standard averaged 82%. The chemical composition of the 5 diets are given in Table 1. For all 5 diets, digestibility estimates calculated using n-alkane ratio were lower ($P < 0.01$) than in vivo DMD (Table 2). Comparison of digestibilities estimated using the n-alkane ratios and IADF ratios showed that the n-alkane ratio predicted higher DMD for meadow ($P < 0.01$), meadow regrowth ($P = 0.06$), and alfalfa hay ($P = 0.06$), and lower DMD for meadow hay ($P < 0.02$). Forage digestibilities for native range using n-alkane ratio tended to be higher ($P = 0.14$) than IADF ratio values. Indirect comparisons between in vivo digestibility and digestibilities estimated using an n-alkane and IADF showed that for meadow, range, meadow regrowth, and alfalfa hay, n-alkane provided a better estimate of in vivo digestibility. Intake and fecal output estimates are shown in Table 3.

Although statistical comparisons between in vitro dry matter disappearance and the other methods of estimating digestibility were not possible because in vitro methods produce a single estimate that does not account for the variation between animals, in vitro dry matter disappearance appeared to produce estimates of digestibility comparable to the n-alkane ratio method for the immature, freshly harvested forages and higher estimates for the alfalfa and meadow hay.

Fecal recoveries of hentriacontane and IADF are shown in Table 4. While marker recoveries were not consistent across forages, n-alkane recoveries for the forages that were freshly harvested were higher ($P < 0.02$) than IADF recoveries. Both markers had similar recoveries for alfalfa hay ($P = 0.14$), and IADF recovery rate was higher for meadow hay ($P = 0.07$). The amounts of hentriacontane extracted from the meadow hay fecal samples were similar ($P > 0.10$) for freeze drying (0.235 g kg^{-1}) and oven drying (0.240 g kg^{-1}). However, freeze drying increased ($P < 0.01$) the amount of n-alkane extracted from alfalfa hay (0.316 g kg^{-1}) compared with oven drying (0.249 g kg^{-1}).

Hentriacontane amounts found in residues collected by filtration after a 48-hour in vitro fermentation period decreased ($P < 0.001$) over 0.1 g kg^{-1} (Table 5). However, samples incubated for 96 hours produced residues similar ($P = 0.78$) to those produced after 48 hours of incubation. In terms of hentriacontane recovery, an average of 18.8% was recovered for the residues obtained after 48 and 96 hours of incubation.

Table 1. Chemical composition of fresh harvested forage from subirrigated meadow, subirrigated meadow regrowth, native sandhills range, mature hay from subirrigated meadow and alfalfa hay diets.

Diet	Dry matter	Organic matter	NDF	ADF	Crude protein	Hentriacontane
	(DM, %)					(g kg^{-1} dm)
Fresh meadow forage harvested in June	96.1	91.4	62.8	33.0	10.3	0.076
Fresh native range forage harvested in July	96.7	93.5	70.0	32.7	11.3	0.081
Fresh meadow regrowth harvested in July	96.4	87.9	63.8	34.0	11.3	0.093
Alfalfa hay	96.2	87.8	54.3	36.3	19.1	0.162
Meadow hay	96.8	90.5	67.1	38.9	7.7	0.175
Standard error of means	0.1	0.1	0.3	0.3	0.3	0.008

Table 2. In vivo, hentricontane, indigestible ADF (IADF), and in vitro dry matter disappearance (IVOMD) calculations of apparent dry matter digestibiliy estimates of fresh harvested forage from subirrigated meadow, subirrigated meadow regrowth and native sandhills range, mature hay from subirrigated meadow, and alfalfa hay.

Diet	In vivo	Hentria-contane	p-value[a]	IADF	p-value[b]	In vitro DMD
		(DM, %)				
Fresh meadow forage harvested in June	67.5	62.9	0.004	57.2	0.001	61.5
Fresh native range forage harvested in July	70.5	61.8	0.004	58.3	0.14	58.3
Fresh meadow regrowth harvested in July	70.7	57.5	0.002	51.0	0.06	57.8
Alfalfa hay	60.2	50.0	0.01	43.8	0.06	58.2
Meadow hay	55.1	36.2	0.0001	42.6	0.01	47.1
Standard error of means	2.9	4.5		2.7		0.3

[a]Comparison between in vivo digestibility and digestibility predicted using hentriacontane.
[b]Comparison between digestibility predicted using hentriacontane and IADF.

Table 3. Fecal output of dry matter, actual dry matter intake, hentriacontane intake and dry matter intake predicted by hentriacontane used to calculate digestibilities[a].

	Intake		Fecal Output	
Diet	Hentriacontane predicted dry matter	Actual dry matter	Hentriacontane	Dry matter
	(kg day^{-1})	(kg day^{-1})	(g day^{-1})	(kg day^{-1})
Fresh meadow forage harvested in June	4.8	5.5	0.36	1.8
Fresh native range forage harvested in July	5.3	7.0	0.42	2.0
Fresh meadow regrowth harvested in July	5.8	8.5	0.50	2.4
Alfalfa hay	6.1	7.5	1.05	3.0
Meadow hay	5.6	8.0	1.09	3.6

[a]Data in table is for reader information, no statistics performed.

Table 4. Recoveries of indigestable acid detergent fiber (IADF) and hentriacontane from the feces of steers fed fresh harvested forage from subirrigated meadow, subirrigated meadow regrowth, native sandhills range, mature hay from subirrigated meadow, and alfalfa hay.

Diet	Alkane	IADF	p-value
Fresh meadow forage harvested in June	87.2	75.6	0.01
Fresh native range forage harvested in July	76.0	69.9	0.002
Fresh meadow regrowth harvested in July	67.6	57.9	0.02
Alfalfa hay	80.4	71.1	0.14
Meadow hay	70.5	78.4	0.07
Standard error of means	5.2	5.2	

Discussion and Conclusions

Although n-alkane recovery increases with increasing chain length (Mayes et al. 1986), hentriacontane was selected as the internal marker to estimate DMD because tritriacontane was not detected in the freshly harvested forages. The inability of tritriacontane to be detected in this study was attributed to lack of column sensitivity where n-alkane amounts less than 20 mg kg^{-1} are not detectable. This agrees with Casson et al. (1990) who suggested that odd chain n-alkane concentrations should be at least 50 mg kg^{-1} DM for accurate prediction of DMD. Additionally, Laredo et al. (1991) concluded that for some tropical forages tritriacontane was not present in sufficient quantity for intake to be estimated using dotriacontane: tritriacontane ratios.

Although hentriacontane consistently underestimated the in vivo digestibilities for all forages examined, it offered an improvement over digestibilities estimated with IADF for freshly harvested forages. The differences between digestibilities estimated with hentriacontane and IADF can be explained by examining fecal recoveries. An exception is that hentriacontane and IADF had similar fecal recoveries but different digestibility estimates for alfalfa hay. Fecal recoveries of hentriacontane for freshly harvested range and meadow forages were lower than those reported for hays. In contrast to freshly harvested forages, fecal recovery of hentriacontane for alfalfa and meadow hays were within ranges reported for other hays (Dove et al. 1989, Ohajuruka and Palmquist 1991). Differences between digestibilities predicted using hentriacontane and in vitro dry matter disappearance were small for meadow, meadow regrowth, and range.

Cochran et al. (1986) reported that fecal IADF recoveries from animals grazing immature forages are lower than for animals fed mature forages. However, hentriacontane recoveries appeared to be greater for the studies using freshly harvested forage. Additionally, Owens and Hanson (1992) stress that markers be chemically discrete with a specific method of analysis. When comparing the 2 markers, one benefit n-alkanes have over IADF is that n-alkanes meet this requirement, because their composition remains the same over a variety of forages. Another concern is IADF can have additional sources of error due to variations found among donor animals and handling of innoculum (Horton et al. 1980, Fahey and Berger 1988).

Replacing freeze-drying of fecal samples with oven drying would decrease the amount of drying time and increase the number of samples handled. While hentriacontane amounts in feces from steers fed meadow hay were not affected by drying method, oven drying reduced the amount of hentriacontane recovered from the feces of steers on an alfalfa hay diet by 20%. In a review of n-alkanes as markers, Dove and Mayes (1991) indicated drying method affects herbage n-alkane concentrations and that further research was needed to determine the effect of drying method on n-alkane concentrations in feces. During oven drying, the high temperatures may subject hentriacontane to either marker degradation or chemical reactions that make complete extraction difficult. Because hentriacontane concentrations vary with drying methods in both forage and feces, it is recommended that samples should be freeze dried for n-alkane analysis.

In vitro fermentation was used to determine if hentriacontane was degraded in the rumen. Filter paper rated to retain particles greater than 25µm was used to isolate the residue since Mayes et al. (1988) indicated that n-alkanes are associated with the particulate phase of digesta. Initial examination of the results indicated that hentriacontane is highly degraded in the rumen. However, the in vivo studies showed an average total tract recovery of 76.3%. Hentriacontane recovery in the residue left after in vitro fermentation was approximately 60 percentage units lower. While in vitro fermentation could degrade hentriacontane to a greater extent than gastrointestinal passage, large differences are unlikely. Because the hentriacontane amounts found in the residues remained unchanged between the 48-hour and 96-hour incubation times, we propose that the low recovery was due to association of the marker with the liquid phase, which was lost during filtration rather than degradation as suggested by Mayes et al. (1988). Further examination is recommended to determine which digesta phase hentriacontane associates with during gastrointestinal passage.

Locating the site of n-alkane disappearance is important when evaluating their use as potential internal markers. If disappearance is isolated to the lower tract, the marker may be used to estimate forage dry matter digestibility in the rumen. Faichney (1975) indicated that a marker needs to be intimately associated with the material it is marking. If n-alkanes are to be reliable as internal markers, it is important that the digesta phase association of n-alkanes be determined.

We concluded that digestibility was underestimated by n-alkanes and hentriacontane recovery was not consistent across forages. Freeze-drying should be used to dry fecal samples for n-alkane analysis. In grazing situations where internal markers are desired, naturally occurring n-alkanes may be a more reliable alternative than IADF for estimating DMD of immature forages.

Table 5. Hentriacontane in residue after 0, 48, and 96 hours of in vitro fermentation.

Time	Hentriacontane g/kg	SE[c]	p-value
Control, 0 hours	.184	61.8	
48 hours	.031	14.9	.0002[a]
96 hours	.038	10.9	.78[b]

[a]Comparison of control with 48-hour fermentation.
[b]Comparison of 48- and 96-hour fermentation.
[c]Standard error of means.

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