Review

ALKANES AS MARKERS IN NUTRITIONAL STUDIES WITH WILD RUMINANT AND NON-RUMINANT ANIMALS

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ABSTRACT: Knowledge of information relative to the digestibility, intake and botanical and morphological composition of the diet is important in nutritional studies, since it provides the basis for understanding aspects related to the ingestive behavior and selectivity of animals. N-alkanes have been used successfully as markers in studies with many species of animals, particularly domesticated ruminants, most of the times as replacements for conventional markers as chromium oxide for example. However, for wild ruminants and non-ruminant animals information on this technique is still scarce and, as a consequence, its potential for use unknown. This review reports the use of this technique in studies of feed digestibility, intake and diet composition with wild ruminants and non-ruminant animals, summarizing results and inferring on the feasibility and applicability of the technique.
Key words: digestibility, diet composition, feed intake, forages, hydrocarbons

ALCANOS COMO INDICADORES EM ESTUDOS NUTRICIONAIS COM RUMINANTES SELVAGENS E ANIMAIS NÃO-RUMINANTES

RESUMO: O conhecimento de informações relativas à digestibilidade, consumo, composição botânica e morfológica da dieta é importante em estudos de nutrição, pois fornece a base para a compreensão de aspectos relativos ao comportamento ingestivo e a seletividade dos animais. N-alcanos têm sido usados com sucesso como indicadores em estudos com várias espécies de animais, particularmente ruminantes domésticos, muitas vezes como substitutos a marcadores convencionais como o cromo por exemplo. No entanto, no caso de ruminantes selvagens e animais não-ruminantes as informações sobre essa técnica são ainda escassas e, consequentemente, seu potencial de uso desconhecido. Esta revisão borda o uso dessa metodologia em estudos de digestibilidade, consumo e estimativa da composição da dieta em ruminantes selvagens e animais não-ruminantes, sumarizando resultados e inferindo sobre a viabilidade e aplicabilidade da técnica.
Palavras-chave: consumo, composição da dieta, digestibilidade, forragens, hidrocarbonetos

INTRODUCTION

N-alkanes have been commonly used in nutritional studies with the purpose of estimating intake, digestibility and botanical and/or morphological composition of the forage consumed by domestic ruminants. For tropical and sub-tropical forage species this technique has been adopted quite regularly (Oliveira & Prates, 2000; Oliveira et al., 2007) and methodology for extraction and determination adequately adapted (Oliveira, 2004). However, information is needed regarding the use of these markers for evaluating feeding strategies as well as management of wild ruminant and non-ruminant animals, either in conservationist farms or captivity.

Ruminant and non-ruminant animals, herbivores or browsers, domesticated or wild, depend on the amount and concentration of nutrients in the forages they consume (Mayes & Dove, 2000) or in other feeds such as grains and fruits available in the environment. The life cycle of animals is influenced by their individual nutritional "status" and by the type and amount of feed they consume which, in turn, affect the species distribution within an ecosystem. In this context, a quantitative understanding of the interaction between animal responses (such as feeding behavior, intake and performance) and their diet is essential for developing management practices that ensure the equilibrium and sustainability of any ecosystem. The objective of this review paper was to gather and systematize the available information on the use of *n*-alkanes in experiments with wild ruminant and non-ruminant animals as a means of evaluating its potential for future research and experimentation.

Use of *n*-alkanes in nutritional studies with wild ruminants

Hatt et al. (1998), concerned with the lack of information on feeding strategies of wild animals kept in captivity, used *n*-alkanes to estimate intake, digestibility and diet composition for giraffes (*Giraffa camelopardalis* L.). Animals were kept in separate pens in a "giraffe house" at night with access to an outside paddock with a sand surface during the day. They were fed clover (*Trifolium hybridum* L.) hay *ad libitum*, commercial concentrate, vitamin supplement and a 1:1 mixture of kneaded oats (*Avena sativa* L.) and wheat (*Triticum aestivum* L.) bran. They also received cabbage (*Brassica oleracea* L.) and fresh browse (*Quercus ilex* L.) on a daily basis, as well as potatoes (*Solanum tuberosum* L.), carrots (*Daucus carota* L.) and bananas (*Musa* L.) three times per week comprising less than 2% of their diet.

The *n*-alkanes C_{28}, C_{32} and C_{36} were sprinkled on the commercial concentrate used (pellets) and hand-fed to animals once a day (100 mg of each alkane per 100 kg BW). Samples of each type of feed were analyzed to determine natural alkane profiles. Eight days after the beginning of dosing animals with alkanes, fecal samples were collected daily from each animal during a 2-week period and total dietary intake and composition estimated. Estimates of diet composition were made using the method described by Salt et al. (1994) and corrections for fecal recovery of alkanes made using data for sheep (Mayes et al., 1986). Estimates of intake were made using the C_{33}:C_{32} pair of n-alkanes. Fecal output was calculated using C_{36} and diet digestibility calculated accordingly. Only foodstuffs with a 10 mg kg^{-1} DM minimum concentration of at least one of the alkanes used were included in calculations of diet composition and no attempt was made to estimate intakes of potatoes, carrot and banana because of the extremely low alkane concentrations. Intake values calculated with the C_{33}:C_{32} pair were lower than the observed and so was the corresponding digestibility of the diet. Direct and indirect estimates of diet composition were similar only for cabbage, browse and pellets, with those for clover hay and oat/wheat mix being extremely different. The authors attributed those results to diurnal variations in the ratio between C_{33} and C_{32} fecal concentrations (C_{33}:C_{32}) and to the relatively low concentration of alkanes in the oat/wheat mix.

Clauss et al. (2001) also worked with giraffes in an experiment designed to investigate the importance of the animal's nutritional state on the occurrence of a disease called "peraguda" mortality syndrome, characterized by depletion of body fat reserves. Four different substances (Co-EDTA, acid detergent lignin, acid insoluble ash and C_{36} *n*-alkane) were used as markers in a digestibility study with four giraffes kept in captivity. The base diet was alfalfa (*Medicago sativa* L.) hay plus a commercial concentrate, a vitamin E supplement and controlled amounts of *Fagus sylvatica* L., *Acer pseudoplatanus* L. and *Corylus avellana* L.. Co-EDTA was supplied in a banana piece and C_{36} was impregnated in pellets of the commercial concentrate used. Animals were housed separately during the night and two of them were housed individually all the time during a 20-day period. The pellets with C_{36} were supplied first, twice a day, in order to guarantee consumption. During the 7-day measurement period feeds and orts were measured daily and fecal samples collected directly from the ground several times a day. Total fecal collection was not made in order not to disturb the animals. Feedstuffs used were analyzed for crude protein, fat, crude ash, neutral and acid detergent fiber and lignin. Fecal concentration of C_{36} was corrected for incomplete recovery using the value of 0.95 determined by Mayes et al. (1986) with sheep. Fecal output and digestibility were calculated. The authors concluded that diet digestibility values obtained using C_{36} were more precise than those obtained with other markers when compared to available figures in the literature for similar diets. As a consequence, the resulting estimates of fecal output and digestibility had "biases" that were not independent from each other.

Gedir & Hudson (2000a) studied pregnant female deer (*Cervus elaphus* canadensis L.) kept in small paddocks without vegetation and receiving individual and electronically monitored amounts of alfalfa in cubes through an automatic feed monitoring system to estimate forage intake, digestibility and fecal output. Controlled-release capsules of *n*-alkanes containing C_{32} and C_{36} were used during a two-week trial. Samples of freshly voided feces were collected daily from each animal commencing on day five after animals were dosed with capsules. These were freeze-dried and then submitted to *n*-alkanes extraction. The *n*-alkane C_{36} was used to determine herbage digestibility and correction for its fecal recovery was made using the value 0.96. Estimates of fecal output were made using the resulting digestibility values and the electronic measurements of intake. The accuracy of intake estimates using *n*-alkanes was assessed by plotting a regression between estimated and observed values using the C_{31}:C_{32} and C_{33}:C_{32} pairs. The results revealed an overestimation of 2.2 and 6.5% for C_{33}:C_{32} and C_{31}:C_{32} pair, respectively, suggesting that the technique could be used as a means of getting reliable estimates of intake for the species of deer studied, particularly when animals were fed uniform diets. In that work, there

was no total collection of feces so accuracy of the digestibility values obtained was unknown since they were also estimated and not measured.

Gedir & Hudson (2000b) worked with females of the same animal species under grazing conditions. Estimates of intake during four different physiological phases of the reproductive cycle (beginning and end of gestation, peak and end of lactation) were performed using either controlled-release capsules containing C_{32} or measurements of ingestive behaviour as biting rate, bite mass and grazing time. Intake estimates from ingestive behaviour measurements were larger than those using *n*-alkanes, leading authors to conclude that the results of the experiment were encouraging for the use of alkanes. However, more research is needed to generate reliable estimates of intake for wild herbivores. In this work, Gedir & Hudson (2000b) simulated grazing in order to estimate bite mass and used a correction factor to adjust for the difference between the width of the animals' dental pad and the observer's hand, suggesting that this simulation might have influenced estimates of intake obtained.

In Gedir & Hudson (2000a,b), the *n*-alkanes extraction procedure that was used omitted one step in the standard procedure. Specifically, the saponification with alcoholic KOH solution was not carried out. Alkanes were extracted from freeze-dried fecal and feed samples adding 10 mL hexane (HPLC grade) and 5 mL distilled water, transferring separated hexane layer to a 20 mL scintillation vial and then repeating the extraction with another 8 mL hexane. This turned the method faster than the original procedure described by Mayes et al. (1986).

Use of *n*-alkanes in nutritional studies with non-ruminant animals

Gudmundsson & Halldorsdottir (1995) used Arctic salmons (*Salvelinus alpinus* L.) to evaluate the use of the *n*-alkanes C_{28} and C_{32} as external markers to estimate digestibility and did not find differences among digestibility values for either fat or protein obtained with the *n*-alkanes method and two other markers (chromium oxide and celite), suggesting that *n*-alkanes could be used as markers for the determination of digestibility of feedstuffs for fish. The authors did note however that there was low *n*-alkanes concentration in most of the feeds used. This constraint would practically eliminate the possibility of using *n*-alkanes as internal indicators, but it would create opportunity to use almost any long-chain alkane as an external marker in studies with fish. Laredo et al. (1991) stated that concentrations of *n*-alkanes lower than 50 mg kg^{-1} DM could lead to analytical errors and result in poor estimates of intake and digestibility.

With the objective of studying the use of *n*-alkanes to estimate intake, digestibility and diet selection of pigeons (*Columba livia*, Gmelin), Hatt et al. (2001) kept individual birds in stainless steel cages fitted with two wooden perches. *N*-alkanes C_{28}, C_{32} and C_{36} were incorporated into a commercial mixture of seeds before pelleting. Two experiments were carried out. In the first, digestibility and intake were determined. The experimental period lasted 10 days, with 6 days for adaptation and 4 days for total excreta collection. Feeds offered and refused were weighed and sampled. Estimates of intake were made using the C_{31}:C_{32} pair, with and without adjustment for fecal recovery. The supplied *n*-alkanes did not interfere with the digestive process and consumption of the birds. Estimate of mean daily intake, adjusted for fecal recovery, was very close to the observed value (24.7 vs. 24.6 g day^{-1}, respectively), but it was underestimated in 16% when no adjustment of fecal recovery was used. In relation to the determination of diet composition, the procedure of the least-squares non negative described by Dove & Moore (1995) was used. The mean difference between estimated and observed values was 2.5%. In that experiment, fecal recovery of C_{31} present in the food was very different from that of the supplied C_{32} (68 vs. 81%), resulting in an inaccurate estimate of intake and the need for a corresponding correction. However, the results might have been influenced by the difference in ingestion of the two studied *n*-alkanes, since concentration of C_{31} in the feed was much smaller than the amount supplied to the animals. That research pioneers estimates of intake and diet composition of birds, and it was also important because it resulted in a modification of the methodology for extraction of alkanes. Specifically, it allowed for a ten-fold decrease in sample size and amount of reagent used.

McLean et al. (1996) used *n*-alkanes as markers for measuring intake and passage rates in horses (*Equus caballus* L.) consuming either hay or silage offered *ad libitum*. Forage intake was overestimated using the C_{33}:C_{36} pair, particularly for hay, leading to the conclusion that further research, based on a larger number of animals and different feedstuffs, was required in order to validate alkanes as markers for estimating intake in horses. The use of natural and synthetic adjacent *n*-alkanes to estimate feed intake is based on the assumption of similar fecal recoveries of both alkanes (Mayes et al., 1986). In this study with horses, McLean et al. (1996) used a pair of *n*-alkanes with a three carbon difference in chain length. Since fecal recoveries were not measured and increased with increasing length of carbon chain, estimates of intake may have been compromised.

Gudmundsson & Thorhallsdottir (1998) used synthetic C_{32} and C_{36} and natural C_{25} to C_{35} *n*-alkanes (plant constituents) as markers to determine intake and digestibility of three different types and varying quantities of hay fed to Icelandic toelter horses. Fecal recoveries ranged from 114.7% for C_{25} to 83.1% for C_{30}, decreasing as the length of carbon chain increased. This is quite the opposite of most reports available (e.g. lower recovery for shorter chained alkanes), mainly for ruminants, and could be a consequence of partial digestion of short chain alkanes in the rumen (Mayes et al., 1988), which did not happen in non-ruminant animals. For the C_{31} and C_{33} used as internal markers, fecal recoveries were 90.1 and 86.8%, respectively, and for C_{32} and C_{36}, commonly used as external markers, values were 91.3 and 87.0%, respectively. The C_{31}:C_{32} pair resulted in the smallest variation in the data set, but *n*-alkanes overestimated the intake of hay.

Aware of the difficulty of measuring dry matter intake, digestibility and fecal output of animals in field conditions (Ordakowski et al., 2001), carried out an experiment with horses consisting of two digestion trials where five *n*-alkanes of odd carbon chains (C_{25}, C_{27}, C_{29}, C_{31} and C_{33}), natural from the forage, were tested as internal markers for digestibility estimates of four diet types (hay or hay plus concentrates). Fecal recoveries were calculated from fecal and diet concentrations of *n*-alkanes and the respective amounts of feces produced and feed consumed. Alternatively, fecal recovery was also estimated as the slope of a linear regression between the amount of *n*-alkanes in the feces and that in the diet. Fecal recoveries varied from 71 to 92% and did not have any relationship with length of carbon chain. A correction factor was used for the measured fecal recoveries of *n*-alkanes, and resulting digestibility estimates were not different from those obtained from total fecal collection. OrdaKowski et al. (2001) concluded that satisfactory estimates of dry matter digestibility could be obtained using *n*-alkanes. However, they emphasized that incomplete fecal recovery suggested that horses could be capable of metabolizing *n*-alkanes in some extent and, therefore, adjustments would have to be made accordingly. Because fecal recovery did not increase with increasing length of carbon chain, as it did for ruminants, a single value of fecal recovery obtained by linear regression could be used. This is a favorable finding for grazing experiments but should not be adopted across a wider range of studies.

Also working with horses, O'Keefe & McMeniman (1998) used seven diets and C_{32} and C_{36} *n*-alkanes as external markers to estimate diet composition according to the procedure described by Dove & Moore (1995). There was no variation in fecal recovery in relation to the length of the alkane chain, and diet composition was accurately estimated under these circumstances. Fecal recovery of *n*-alkanes natural to plants was greater than that of dosed *n*-alkanes by ruminants. Further, fecal recovery of the supplied *n*-alkanes and of homologous natural ones in the diet was quite different when compared with values generated in experiments with cattle (*Bos* sp.). In both experiments with horses (O'Keefe & McMeniman, 1998 and Ordakowski et al., 2001) and studying the same *n*-alkanes, mean fecal recovery was 81.0 ± 6.8% for the first and 89.2 ± 7.1% for the second, suggesting the need for caution about the use of a single value of fecal recovery for the *n*-alkanes studied, even considering the existence of reports in the literature indicating that there is no increase in fecal recovery with the increase in length of the carbon chain of alkanes.

Alkanes were also used in studies with small non-ruminant herbivores known as being selective animals. Hulbert et al. (2001) studied the causes of seasonal variation in the diet of hares (*Lepus timidus* L.) through evaluation of individual patterns of habitat selection. These were made by radio telemetry and resulted in a range of diet composition similar to that obtained using *n*-alkanes to estimate botanical composition of the ingested forage. Vegetation in the experimental area was separated into three classes according to its relative position to sea level. These varied from forage-grass dominated areas (*Lolium perenne* L.) to forests. Animals (118) were grouped according to sex and females further divided in reproductive and non-reproductive. From these, sub-groups of 18 animals were allocated to each of the three vegetation areas according to previous observations and monitored by radio telemetry technique. Forage mass in each vegetation area was monitored every three months and separated into component species. From 41 animals, including those with radio equipment, fecal samples were collected during spring/summer and autumn/winter periods with the objective of comparing animals' diets with the available forage in each vegetation area. Ingested species were separated from the fecal samples and the annual profile of the diets determined, including monthly variation in diet composition. The method seemed to be appropriate to estimate variation in diet composition because animals had a relatively simple diet and the profiles of *n*-alkanes were sufficiently different among the ingested species. No adjustments for fecal recovery of *n*-alkanes were made because necessary measurements could not be performed under the grazing conditions of the experiment, so values were used from available data in the literature for other non-ruminant herbivores.

Martins et al. (2002a) evaluated *n*-alkanes as markers and mathematical techniques to describe the complex diets selected by wild rabbits (*Oryctolagus cuniculus* L.) in southeast Portugal. Vegetation consisted mainly of cork oak (*Quercus suber* L.), holm oak (*Quercus rotundifolia* L.), some crops of triticale (x Triticosecale Wittmack) and oat (*Avena sativa* L.), patches of gum cistus (*Cistus ladanifer* L.) or natural pasture (most being *Agrostis pouretii* Willd., *Bromus hordaceus* L., *Echium plantagineum* L., *Leontodon taraxacoides* Vill., *Ornithopus compressus* L., *Rhagadiolus stellatus* L., *Trifolium angustifolium* L. and *Vulpia geniculata* L.). As a result, a new and more precise least-squares optimization procedure was developed to calculate each dietary component. Martins et al. (2002b) assessed changes in diet composition across seasons in relation to the reproductive cycle in wild rabbits (*Oryctolagus cuniculus* L.) and related those changes to herbage mass availability and habitat structure using the *n*-alkanes technique. The population of wild rabbits was able to adapt itself to seasonal variations in quality and quantity of feeding resources in order to ensure high quality diets and the *n*-alkane technique was effective in determining that.

Woolnough (1998), cited by Dove & Mayes (1999), compared some methods to estimate botanical composition of the diet in kangaroos (*Macropus giganteus*, Shaw) and vombates (*Lasiorhinus krefftii*, Owen). One of these techniques was *n*-alkanes. There was no difference between estimates using *n*-alkanes and those obtained from microhistological analysis of fecal samples, although for this last method there was a large proportion of material that could not be identified. Additionally, the results using *n*-alkanes indicated that the pattern of selection for the two animal species studied was similar in the experimental area used.

Wilson et al. (1999) used pregnant sows (*Sus scroffa* L.) to measure fecal recovery of *n*-alkanes from ingested grass and from dosed n-alkane (C_{32}) under controlled indoor conditions, with or without the addition of soybean oil in the diet. There was no effect of soybean (*Glycine max* L.) oil addition on fecal recovery of alkanes nor was there a consistent pattern of diurnal variation in fecal *n*-alkane concentration. Fecal recoveries were incomplete and ranged from 43.3% for C_{24} to 98.6% for C_{32}. No relationship was detected between length of carbon chain and fecal recovery of the alkanes studied, but fecal recovery of dosed alkanes was greater than that of natural alkanes in the diet. As a consequence, if intake estimates were made, they would not be adequate because of the discrepancies in fecal recovery of *n*-alkanes (e.g. C_{31} = 74.1%, C_{32} = 98.6% and C_{33} = 76.9%).

Rivera Ferre et al. (1999) used the C_{31}:C_{32} pair of *n*-alkanes to estimate grass intake by sows grazing paddocks of ryegrass (*Lolium perenne* L.) and fed two levels of concentrate twice a day, in two periods. No difference in forage intake was detected in any of the periods, and authors used fecal recovery values provided by Wilson et al. (1999) to calculate the estimates of grass intake.

Herbage intake of individual growing pigs was measured by Mowat et al. (2001) in an outdoor organic production system based on grass x white clover (*Trifolium repens* L.) pastures. Animals were supplemented individually with a concentrate and herbage samples separated into grass and clover components. Diet composition was estimated using a least squares optimization algorithm and *n*-alkanes concentrations corrected for incomplete recoveries using the values obtained by Wilson et al. (1999). Pasture represented only 4% of the daily organic matter intake when concentrate was available *ad libitum* and there was no evidence of a consistent pattern of selection for any of the plant species comprising the pastures.

The first reported use of *n*-alkanes as markers in digestive studies with reptiles was that of Hatt et al. (2002). N-alkanes C_{28}, C_{32} and C_{36} were used as external markers to estimate intake, digestibility and diet composition in Galapagos tortoises (*Geochelone nigra*, Quoy & Gainard) and the results revealed that the C_{31}:C_{32} pair overestimated intake under those conditions. The digestibility calculated from total fecal collection data and using C_{36} were not different and diet composition estimates were considered satisfactory, with n-alkanes proving to be a valuable option for performing estimates of this nature.

FINAL REMARKERS

N-alkanes have been used with varying degrees of success to estimate digestibility, feed intake and diet composition and the results vary according to the animal species used. Extraction and analysis of *n*-alkanes demand as much effort as for other markers, with a difference: the marker is quantified at once following a single method, gas chromatography. The use of chromium oxide, for example, requires the determination of chromium as well as *in vitro* dry matter digestibility of the consumed herbage, two techniques with inherent bias that may combine and influence the final outcome of the analysis. The use of markers in domestic animals (e.g. cattle, pigs, horses) is relatively less complicated because it is easier to determine fecal recovery. In wild animals (e.g. deer, giraffe, pigeon) this degree of control is not as possible, suggesting that *n*-alkanes could be a feasible alternative under

those circumstances. Even when long-chained alkanes like C_{35}, for example, occur in low concentrations, the technique can still be used if the analytical procedure is carried out precisely. Evidences are not conclusive about alkanes being a more accurate method than other markers, however, depending on the restrictions imposed by the experimental protocol used, they can be an interesting and effective alternative.

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