

The Application of Biological Markers – A Macaulay Institute success story

■ Summary

Indigestible substances in the diet or those orally administered appear in the faeces and can be used to obtain information about the diet and its utilisation in farm livestock and wild herbivores. Techniques for measuring intake, diet composition, digestibility and rate of passage of material along the digestive tract of free-ranging herbivores have been pioneered at the Macaulay Institute using plant wax compounds and similar substances as faecal markers. In current development work, the range of applications of these compounds is being extended to include the measurement of plant species composition of roots and characterisation of past vegetation cover by examination of such markers in different soil layers. The potential of using the compounds present in the waxy cuticles of insects to determine the diet composition of insectivorous mammals and birds is also being investigated.

■ Context

In order to gain an understanding of the relationships between large herbivores (in particular, ruminant livestock and deer species) and their environment there is a need to obtain quantitative information on the dietary habits of these animals - what they eat, how much they eat and what is the nutritional quality of their diet. Not only does this information tell us about the animals but also the effect that they have upon their habitat through changes in abundance of different vegetation species as a result of defoliation effects and secondary influences such as trampling and return of plant nutrients via faeces and urine. Such knowledge is of considerable importance in the development of research models and decision support systems designed to predict the interactions occurring between large herbivores and their habitats. Quantitative techniques have been successfully developed at the Macaulay Institute to provide dietary information relating to free-ranging herbivores. These techniques are now used around the world.

Dietary Markers

Accurate measurement of the composition, intake and quality of the diets of free-ranging herbivores is not easy to make, especially if the degree of disturbance to the animals is to be kept to a minimum. However, their faeces, which can be collected relatively easily, can provide useful clues. Herbivore faeces mainly consists of residues of the plant material ingested by the animal and a range of such substances of plant origin can be exploited as markers allowing dietary digestibility, intake and composition to be estimated. The hydrocarbons from the waxy cuticles of dietary plants are particularly useful since they are present in most plants, relatively inert and indigestible, and can be easily and accurately analysed by gas chromatography.

The surface wax of most higher plants contains mixtures of saturated straight-chain hydrocarbons (n-alkanes) having 21-35 carbon atoms. Alkanes with odd-numbered carbon chains predominate (>90%). Different plant species have different patterns of individual alkanes, with most herbage species tending to have mainly C29- to C33-alkanes, whereas in many tree and browse species the shorter-chain alkanes predominate.

Measuring diet digestibility

As ingested feed passes along the gut of an animal, material will be removed through digestion and absorption, with the result that the concentration of an indigestible plant marker will be higher in faeces than in the original plant. Thus, representative samples of the diet and faeces can be used to estimate digestibility:

$$\text{Digestibility} = \left(1 - \frac{\text{Marker concentration in diet}}{\text{Marker concentration in faeces}} \right)$$

This concept for digestibility measurement has been recognised for well over 100 years. However, until relatively recently, the method was considered to be unreliable, because relative concentrations of the chosen markers (mainly plant fibre components of variable

chemical composition) could not be determined with adequate consistency. Herbage n-alkanes as digestibility markers, first investigated within the Institute, have the advantages of being discrete compounds which can be analysed with high accuracy and precision. However, they are not perfect markers as they are not completely recovered in faeces. Recovery increases with carbon chain length from less than 50% for C21-alkane to over 95% for C35-alkane. Using a recovery correction, the longer-chain alkanes can be used to give reliable estimates of digestibility.

Measuring intake

In free-ranging herbivores intake can be determined if the digestibility of the diet and the output of faeces are known:

$$Intake = \frac{Faecal\ output}{(1 - Diet\ digestibility)}$$

Digestibility can be determined using the method described above. Although faecal output can be measured by attaching bags to the animals and making total collections, it is more convenient and less disturbing to the animals if markers are used to estimate faecal output and small samples of faeces are collected. If a known amount of an indigestible marker substance, which is normally absent from the diet, is orally-administered to an animal each day (or more frequently), the marker will appear in the faeces, with its concentration stabilising after about 6 days. Then:

$$Faecal\ output = \frac{Dose\ rate\ of\ marker}{Concentration\ of\ dosed\ marker\ in\ faeces}$$

Before n-alkanes were considered as dietary markers, this approach suffered the disadvantages that the method did not allow for variation in diet digestibility among individual animals and could not be used for animals receiving cereal feed supplements or for non-ruminants. At the Institute a major advance was made by realising that intake could be determined using a dosed even-chain alkane marker (to measure faecal output) together with a dietary odd-chain alkane (to measure digestibility). Thus:

$$Intake = \frac{Dose\ rate\ of\ alkane}{\frac{Faecal\ alkane_j\ content}{Faecal\ alkane_j\ content} \times Herbage\ alkane_i\ content - Herbage\ alkane_j\ content}$$

where alkane_i is the chosen dietary odd-chain and alkane_j the dosed even-chain alkane marker. There is also the advantage that the concentrations of both markers are determined in the same analysis. Furthermore, as long as they are the same for both alkanes, the faecal recoveries of the markers need not be quantitative. Work at the Institute has shown this to be the case for C32-(dosed) and C33-(dietary) alkanes in sheep, cattle, goats and deer. In collaboration with other institutions, the method has been further evaluated in other captive plant-eating mammals, including moose, giraffe, pigs, horses, rabbits and wombats.

Measuring intake in wild herbivores

Except for obtaining crude estimates by watching animals graze or browse, the estimation of intake in wild, free-living herbivores has been virtually impossible until recently. The main problem has been the need to capture animals repeatedly in order to carry out intake measurements. For example, the n-alkane technique requires the handling of animals to administer marker doses. However, ways of obtaining intake estimates have been developed which require animals to be handled only once for administration of a marker which

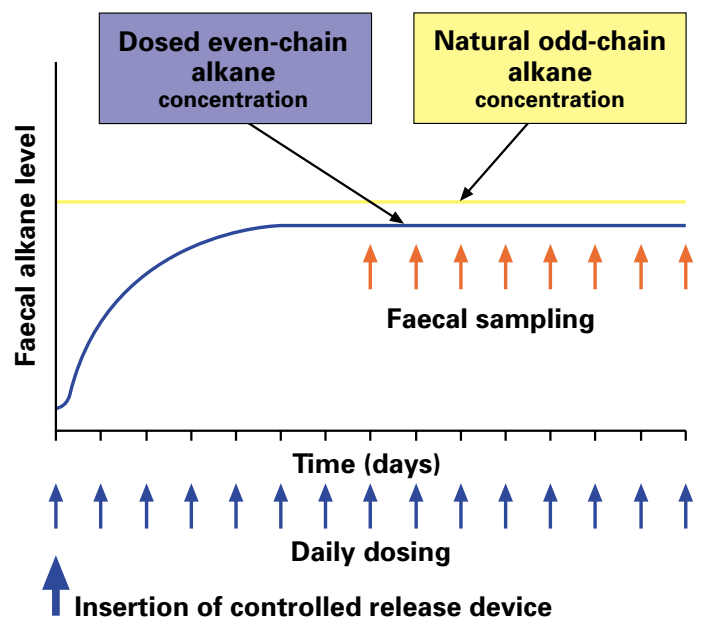


Figure 1. Intake estimation using an even-chain alkane supplied by daily dosing or a controlled release device: this method requires the equilibrium concentrations of dosed and natural dietary alkanes in the feed and faeces

is released at a constant rate daily (Figure 1)

Intraruminal controlled release devices

Controlled-release devices, which remain in the rumen of ruminants after oral administration, were originally designed to deliver anthelmintic drugs over an extended period. The Institute has collaborated with the Division of Plant Industry, CSIRO in Australia in the development and testing of Controlled-released devices impregnated with n-alkanes containing even-chain alkanes for intake measurement. (Dove et al. in press). These devices are now commercially available (Captec Alkane™). Using such devices, together with GPS-tracking equipment to locate faeces, it has been possible for the first time to measure intake in wild moose living in the Swedish boreal forest (Mayes et al., 2001).

Intake measurement using a single marker dose

Controlled release devices can be used for measuring intake in ruminants but not in monogastric herbivores. However, by analysing a series of faeces samples following a single dose of marker, it is possible to estimate the output of faeces. A similar approach can also be used to estimate intake using n-alkane markers; intake can be calculated by replacing the faecal concentration ratio of the

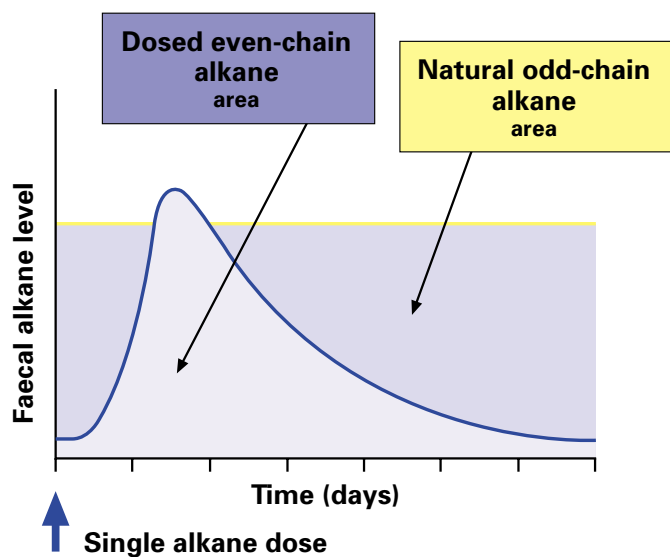


Figure 2. Intake estimation using a single dose of even-chain alkane: this method requires herbage alkane levels and the relative areas under the faecal concentration v time curves of the dosed and natural alkanes

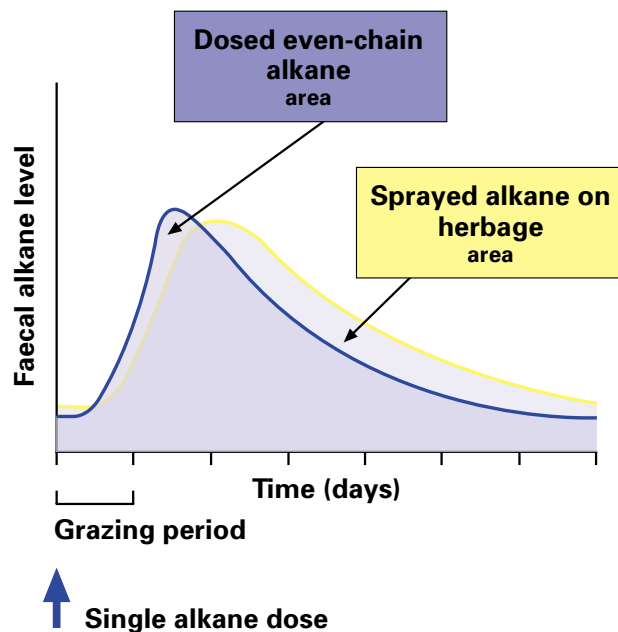


Figure 3. Estimating short-term intake: this method requires the relative areas under the faecal concentration v time curves of an even-chain alkane given as a single dose and a second even-chain alkane sprayed on to the experimental pasture together with the levels of these alkanes in the sprayed herbage

dosed and natural alkanes by the ratio of their areas under faecal concentration v time curves. This has been validated in captive rabbits (Letso, 1996). As long as faeces samples can be collected and identified as coming from known individual animals, intake can be determined following a single dose of alkane marker (Figure 2). Daniels et al. (in preparation) have made such measurements in wild wombats in S.E. Australia, by giving single doses of C32- alkane and glitter of different colours in order to identify which animal produced each faeces sample.

Short-term intake estimation

The n-alkane method is now widely used throughout the world to obtain dietary intakes, representing estimates averaged over a number of days. However in some circumstances, intakes measured over a period of a single day, or less, are required. A suitable method has been developed and tested (Duncan et al., 1999), in which the vegetation, to be grazed over a short experimental period (as little as a few hours), is sprayed with an artificial alkane (e.g. C36-alkane), while the experimental animals are given one or more doses of a

second alkane (e.g. C32-alkane); a series of faeces samples are collected, continuing after the animals have been removed from the sprayed pasture (Figure 3).

The alkane methods for measuring intake require knowledge of the n-alkane concentrations in the animal's diet. Under conditions in which animals have the freedom to select a complex diet of a number of plant components, it is necessary to estimate diet composition in order to determine the n-alkane content of the total diet from the n-alkane levels in the individual components.

Measurement of diet composition

Markers found in faeces can also be used to determine the composition of a herbivore's diet. Diet composition can be estimated

by exploiting differences in the patterns of individual n-alkanes among different plant species and plant parts (Figure 4). From the alkane pattern in an animal's faeces and those of the dietary components, diet composition can be estimated using a least-squares optimisation algorithm. This method has been validated using simple two-component diets, for example rush (*Juncus effusus*)/perennial ryegrass (*Lolium perenne*) mixtures (Figure 5). However, although in theory the number of dietary components that can be discriminated is the same as the number of markers available for use (about 10 for n-alkanes), in practice the reliability of the method declines as the number of components increases. In current research at the Institute, attempts are being made to refine this

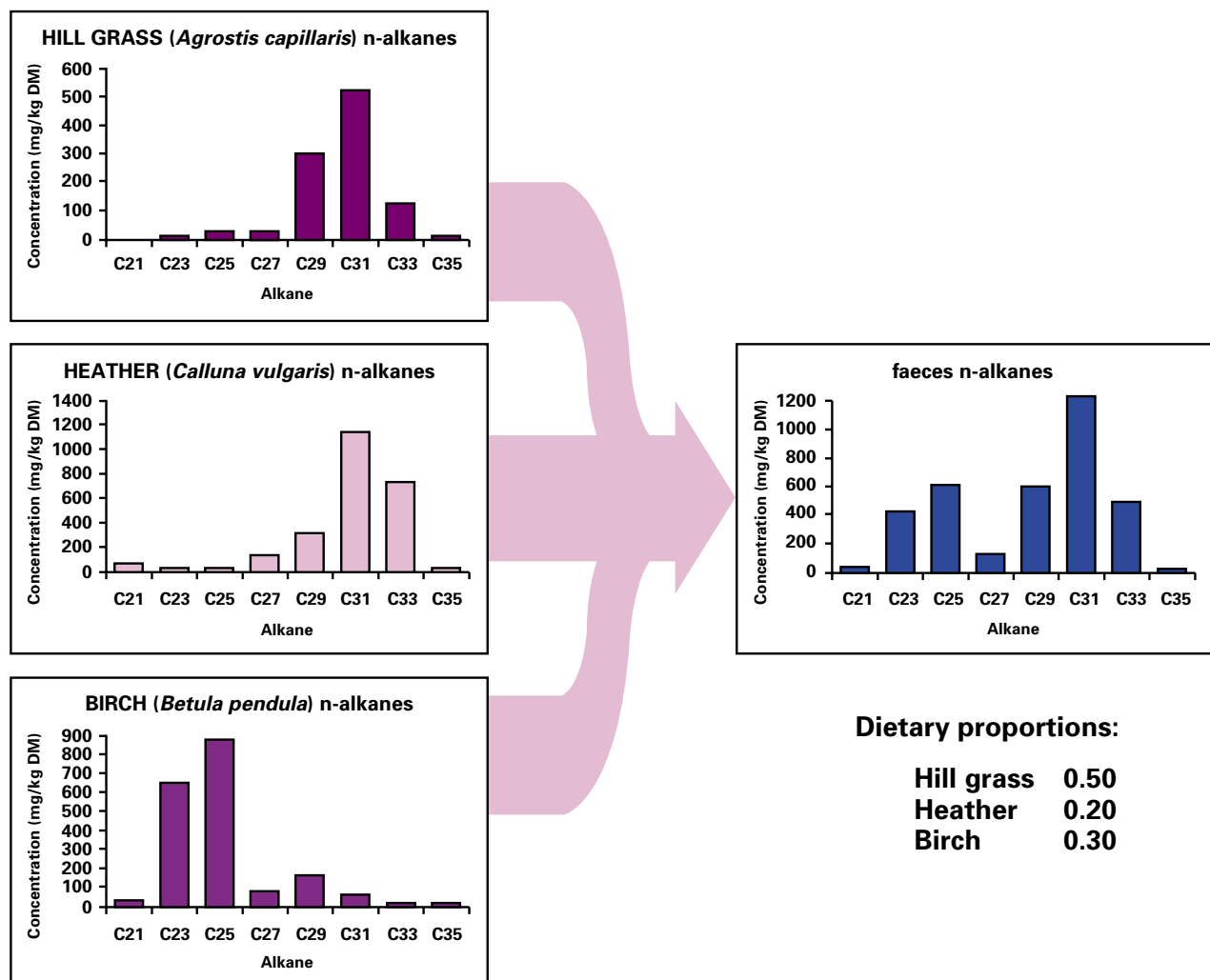


Figure 4. Differences in alkane patterns between plant species can be used to estimate diet composition. For example, the dietary proportions of hill grass, heather and birch can be determined from the alkanes in faeces from red deer

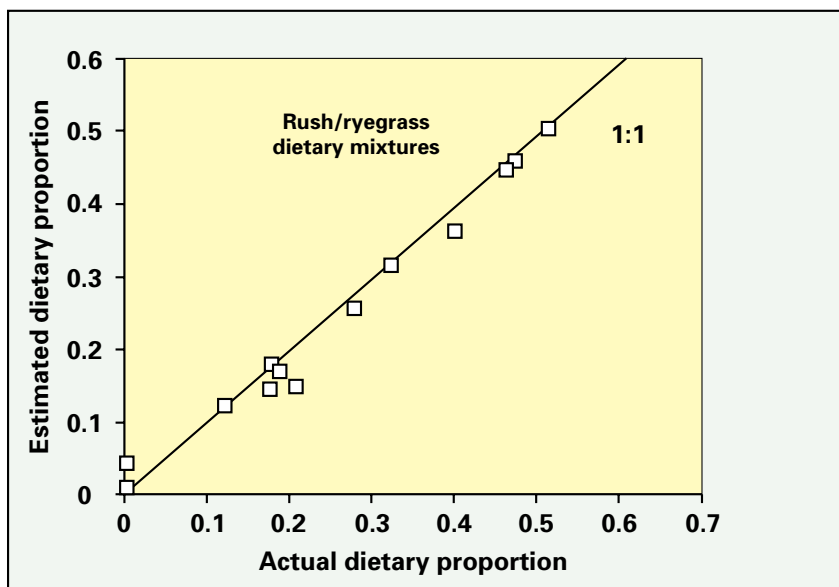


Figure 5. Relationship between actual proportions of rush in the diet of goats and proportions estimated using faecal alkane patterns

approach such that reliable diet composition estimates can be made under conditions in which animals may select highly complex dietary mixtures.

Such work includes the development of improved calculation and statistical methods for diet composition estimation, and the concept of grouping species in order to reduce the number of estimated components has been explored. However, the main emphasis of this research has been to search for different plant markers. Plant wax usually contains many classes of compounds which may have potential as diet composition markers (Figure 6). To be a useful diet composition marker the compounds must be accurately analysable, be consistently recoverable in faeces and show differences in composition between dietary plant species. Of the compound types that have been investigated, the long-chain fatty alcohols have shown the most promise to date. They have been particularly useful for diets containing plant species with low levels of n-alkanes, including white clover, Scots pine and certain grasses such as timothy, cocksfoot and *Phalaris* spp.

Alkanes as passage-rate markers

The rate at which dietary material passes along the digestive tract of a herbivore is an important factor influencing the digestion and absorption of dietary nutrients, and the animal's intake. Passage rate, usually measured as mean retention time, is normally estimated

from a faecal concentration v time excretion curve of an indigestible marker given as a single dose. Ideally a suitable marker should remain associated with dietary material throughout its passage along the gut. Unfortunately, most exogenous markers do not meet this criterion. However, it has been shown that the natural n-alkanes of plant diets remain attached to particulate material in the gut and thus would be suitable passage rate markers, if it were not for the fact that such n-alkanes would normally be present in the diet and faeces. If the animals were given a single feed of dietary material which was labelled with an isotope of carbon or hydrogen, passage-rate could be determined from the excretion curve of the isotope

levels in the faecal alkanes. It has been shown that the method can be used by feeding plant material labelled either with ^{14}C or ^3H -isotopes. Furthermore, this approach offers the potential for estimating simultaneously passage rates of different dietary components labelled with separate isotopes (Mayes et al. 1997).

A much simpler approach is to use artificial even-chain alkanes as passage-rate markers. Such estimates have been obtained using a single feed of plant material sprayed with artificial alkanes. It is apparent that the estimates of mean retention time obtained are slightly lower than estimates made using isotopically-labelled plant material, suggesting that the artificial alkanes were not behaving as ideal markers. Work is currently underway to examine whether correction factors can be used to remove any bias in estimates of mean retention time.

Beyond herbivores

The relative long-term stability of plant wax compounds, species specificity and ease of analysis offers the opportunity for them to be exploited as markers for a range of applications within many disciplines in addition to that of herbivore ecology. The principle of using the alkane patterns in different plant species to estimate diet composition has been used to determine the species composition of samples of mixed vegetation (Dove, 1992). Despite much lower concentrations in plant roots, alkanes have been used as markers to

estimate the species composition of mixed root mats (Dawson et al., 2000). Alkanes have also been found in the organic matter of soil and the patterns appear to reflect the patterns in the vegetation growing on that soil. The possibility of n-alkane patterns in different soil layers being used to describe the vegetation cover throughout the last few thousand years is currently being explored at the Institute. Plants are not the only living organisms that have waxy cuticles. Insects, their larvae and many other arthropods have complex mixtures of long-chain hydrocarbons (mainly branched-chain alkanes) in the cuticular wax and, like plants, differ greatly between species. Since the digestive tracts of mammalian and avian insectivores are not profoundly different from those of herbivores, it may be expected that insect hydrocarbons will appear in the faeces of their predator, thus offering the opportunity of applying n-alkane marker techniques for measuring diet composition, digestibility and perhaps intake in insect-eating mammals and birds. Preliminary studies in collaboration with Aberdeen University have shown that faeces from wild bats contain complex hydrocarbon patterns. Furthermore, the hydrocarbon pattern in the faeces of captive bats was the same as that of their mealworm diet. Currently the application of the approach to birds is being investigated.

Class	Chain length	Occurrence	Concentration
HYDROCARBONS			
n-Alkanes	C_{21} to C_{37} Odd	Most plants	Low - high
n-Alkanes	7- C_{23} to C_{31} Odd	Flower parts	Low - high
	9- C_{23} to C_{31} Odd	Flower & Tree leaves	Low - high
	1- C_{22} to C_{30} Even	Tree leaves	Low - Medium
Iso-Alkanes	C_{29} to C_{33} Odd	Rare	Low
ALCOHOLS			
Primary	HO C_{20} to C_{34} Even	Most plants	Medium - high
Secondary	10 - C_{29} - ol	Gymnosperms	Medium - high
FATTY ACIDS			
Saturated	HOOC C_{20} to C_{34} Even	Most plants	Low - Medium

Figure 6. Classes of plant wax compounds that have been considered as faecal markers for determining diet composition

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