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DETERMINING DIET COMPOSITION ON COMPLEX SWARDS USING n-ALKANES AND LONG-CHAIN FATTY ALCOHOLS

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Abstract. We conducted an experiment to quantify the accuracy of methods based on n-alkanes and long-chain fatty alcohols for determining the diet composition of animals grazing complex swards. We cut forage from two indigenous vegetation communities, a *Molinia caerulea*-dominated grassland and a *Calluna vulgaris*-dominated dwarf-shrub community, and offered it to mature ewes in different ratios in a zero-grazing experiment. Nine dietary categories were identified within the forage offered: *Molinia caerulea*, *Festuca* spp., *Juncus effusus*, *Carex* spp., *Calluna vulgaris*, *Erica tetralix*, *Vaccinium myrtillus*, and dead grass. Samples of each of these categories together with fecal samples from each individual animal were analyzed for n-alkane and long-chain fatty alcohol concentrations. We analyzed the data using optimization software to minimize the sum of squares differences in the proportional profiles of n-alkanes and fatty alcohols in the diet and feces. Different combinations of n-alkane and fatty alcohols were investigated to assess which gave the most accurate measures of diet composition from the fecal profile. The most accurate estimates were obtained using combinations of the n-alkanes C25, C29, C31, and C33 and the long-chain fatty alcohols 1-C24-ol, 1-C28-ol, and 1-C30-ol, and these gave values for Lin's concordance correlation coefficient between estimated and actual values of >0.98. Our results demonstrate that n-alkanes and long-chain fatty alcohols can be used to estimate several components within the diet of animals grazing complex swards. Diet composition information obtained using this methodology has wide-ranging applications in terms of the assessment of the impact of grazing animals on particular ecosystems or the quantification of nutrient supply to the animal from different selection choices.

Key words: *diet composition; fatty alcohols; foraging strategy; grazing; n-alkanes; ruminants; selection.*

Introduction

Selection of dietary components by herbivores occurs at a number of hierarchical levels. First, in a given feeding bout an animal can choose a vegetation type or community on which to feed; second, within a vegetation type or community it can choose among plant types or species to eat; and third, it may choose which morphological units of those plants to eat (Jarman and Sinclair 1979, Senft et al. 1987). Depending on the choices made by a given animal, the composition of the diet selected may be substantially different with respect to the proportions of various species or plant parts present within a particular community or sward (Grant et al. 1985). The scope for selection depends on the heterogeneity of the vegetation from which the animal is feeding and the spatial distribution of different plant components. The digestibility of different plant species within vegetation communities, their spatial distribution (Spalinger et al. 1988), and the seasonality of plant growth (Arnold et al. 1966) can all be limiting to nutrient intake and result in the uneven distribution of grazing pressure both within and between plant communities (Hunter 1962, Jarman and Sinclair 1979, Grant and Maxwell 1988). Furthermore, in all situations the relationship between the animal and the vegetation it is feeding upon is dynamic (Lundberg and Astrom 1990), and biomass extraction through ingestion to provide nutrient gain for the animal removes material from a number of pools of photosynthetic mass and may alter the subsequent growth form of individual plants. Clearly, without an accurate estimate of diet composition, it is not possible to predict an animal's response to different components within the sward and the associated impact upon a given vegetation community.

A number of recent studies have suggested that the use of long-chain saturated hydrocarbon (n-alkane) markers has potential as a noninvasive approach for determining diet composition. This methodology relies on the n-alkane profiles of epicuticular waxes derived from different plant species being sufficiently distinct to allow assessment of the proportions of different dietary components (Bugalho et al. 2002). Previous studies have demonstrated that this technique can be used to distinguish different plant species in two-component mixtures including perennial ryegrass/white clover, heather/hill grass, and rush/perennial ryegrass (Dove

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1992, Dove and Mayes 1996). Although evidence indicates that the reliability of estimates based only on n-alkane profiles will decline as the number of dietary components increases, it has been suggested that analysis of additional compounds may improve discrimination (Dove and Mayes 1996, Bugalho et al. 2002, Ali et al. 2004). We hypothesized that the profile of a combination of n-alkanes and long-chain fatty alcohols in feces can be used to estimate the relative proportions of a range of different plant types in a ruminant's diet. We tested this using a zero-grazing approach in order to allow diet composition to be measured precisely. The objectives of our study were to quantify the accuracy of estimating the diet composition of animals grazing complex plant mixtures from fecal n-alkane and long-chain fatty alcohol profiles and to identify which combination of individual n-alkane and long-chain fatty alcohols gave the estimate that was most similar to the actual diet consumed.

Methods

Experimental procedure

The experiment was conducted during September 2002 on the west coast of the United Kingdom at the Institute of Grassland and Environmental Research at Aberystwyth, Ceredigion, Wales (52° 25′ N, 4° 05′ W, annual rainfall ~1100 mm) using material from two indigenous vegetation communities: a *Molinia caerulea*-dominated grassland (hill grass mix) and a *Calluna vulgaris*-dominated dwarf-shrub community (heather mix). Both mixtures were obtained from local sites managed as part of a commercial hill sheep farm, with the hill grass mix cut from swards at Lle'r Neuaddau, Nantymoch, Ceredigion, and the heather mix cut from a stand at Ceiro, Plynlumon, Ceredigion. The mixtures were harvested separately at a cutting height of 100 mm and 200 mm, respectively, using a tractor-mounted rotary mower, and stored at 4° C until feeding. A higher cutting height was used for the heather mix to ensure that the *Calluna vulgaris* within the diets offered consisted predominantly of shoots and thin wood, making it more representative of what animals would consume if free-ranging on this type of vegetation community (Grant et al. 1987, Fraser and Gordon 1997).

During a zero-grazing experiment, the mixtures were offered to sheep in three different mass ratios: (1) 10% heather mix : 90% hill grass mix; (2) 20% heather mix : 80% hill grass mix; and (3) 30% heather mix : 70% hill grass mix. All ratios were prepared using the plants as cut, i.e., on a fresh matter (FM) basis. The proportions of heather in the experimental diets were chosen to reflect those likely to be consumed by free-ranging sheep grazing *Calluna*-dominated heathland during the latter part of the growing season (Grant et al. 1987). The diets were offered to 12 mature Welsh Mountain ewes, with a mass of 38.5 ± 0.89 kg (mean ± SE; $n = 4$ per mixture). All the animals had grazed hill vegetation during the preceding summer and were familiar with the plant species included in the experimental diets.

Table 1. Botanical composition of the hill grass mix and heather mix as harvested (dry mass proportion × 100).

Category	Hill grass mix	Heather mix
Molinia caerulea	46.6	0.1
Festuca spp.	1.7	0.4
Dead grass	25.3	0
Juncus effusus	13.0	0.9
Carex spp.	4.2	0
Calluna vulgaris	0	94.9
Erica tetralix	0	0.5
Vaccinium myrtillus	0	1.2
Moss	8.0	1.9

Note: The experiment was conducted during September 2002 on the west coast of the United Kingdom at the Institute of Grassland and Environmental Research at Aberystwyth, Ceredigion, Wales.

After a 22-day adaptation period, data were collected over a 7-day measurement period. For the first 13 days of the adaptation period, all the sheep were group-penned and offered a 20% heather mix : 80% hill grass mix diet. During this time, subsamples from six separate cuts each of the heather mix and hill grass mix were oven-dried to determine dry matter (DM) content. This information was then used to estimate the fresh masses of forage required to achieve an allowance of 700 g DM·head^{-1}·d^{-1} of each experimental diet. The quantity of feed offered was restricted to this amount in order to minimize selective feeding by the sheep and ensure the entire range of plant species was consumed. On day 14 of the adaptation period the animals were randomly assigned to experimental diets and transferred to individual metabolism pens for a further 8-day adaptation. Once in the pens, the sheep were offered 700 g DM·head^{-1}·d^{-1} of their allocated experimental diet as a single feed given at 09:30 hours. Water was freely available throughout.

During the measurement period, replicate samples of the heather and hill grass mixtures were taken daily as the experimental diets were prepared and oven-dried for 24 hours to determine DM content. An additional daily subsample of each forage type was stored at −20° C. At the end of the measurement week these daily subsamples were bulked according to forage type (heather and hill grass mixtures) and thoroughly mixed. A representative subsample of this bulked material was then taken for each forage type and separated into categories based on botanical classification, dietary significance, and state (live or dead). The separated material was then oven-dried for 24 hours and weighed to determine proportional botanical composition. Once the botanical composition of the grass and heather mixes had been established, individual samples of each of the plant groups (Table 1) were separated out from the bulked sample. This approach is standard in feeding experiments to ensure that the herbage analyzed is representative of what was offered to the animals and that any influence of feed storage during the experiment on

chemical composition is accounted for. Once separated, the samples were freeze-dried and milled in preparation for chemical analysis.

The amount and DM content of the feed refused by each animal were recorded daily. The remaining refusals were then bulked on an individual animal basis, before being separated to determine botanical composition as described above. This data, together with the botanical composition of the diet as offered, were used to calculate the composition of the diet consumed by each animal. Total fecal output was recorded daily and a subsample from each animal was retained and stored at −20° C. At the end of the measurement week, the daily fecal samples were bulked on an individual animal basis, thoroughly mixed and subsampled. This sample was then freeze-dried and milled to pass through a 1-mm sieve in preparation for chemical analyses.

Determining n-alkane and long-chain fatty alcohol concentrations and recoveries

The n-alkane and long-chain fatty alcohol concentrations of the dietary components and the fecal samples were determined according to the gas chromatographic methodologies described by Duncan et al. (1999) and Ali et al. (2004), respectively. The n-alkanes are saturated aliphatic hydrocarbons and are hereafter referred to by the number of carbons in the chain-length, e.g., C29 is the alkane *n*-nonacosane ($C_{29}H_{60}$). Long-chain fatty alcohols are aliphatic compounds with a chain length >10 carbons that possesses a terminal CH_2OH group and are hereafter referred to by the number of carbons in the chain length, e.g., 1-C30-ol is the fatty alcohol 1-triacontanol ($C_{30}H_{62}O$).

The fecal recovery of each n-alkane and long-chain fatty alcohol was calculated using the following equation: [fecal output of the compound (in grams per day)]/ total intake of the compound (in grams per day). If no breakdown occurred during digestion, the fecal recovery would be 1 (i.e., 100% of the amount consumed), whereas if all of the compound consumed was broken down, the fecal recovery would be 0.

Data analysis

Our experiment was designed to test whether it is possible to determine the botanical composition of the diet of free-ranging ruminants from the profile of n-alkanes and fatty alcohols present in their feces. To estimate the diet consumed, we used MATLAB version 7 (MathWorks 2004) to minimize a function designed to compare alkane and fatty-alcohol profiles in feces with that of the presumed diet. The comparison function calculated the total sums of squares of differences between diet and feces profiles on a proportional basis, together with Lin's concordance correlation coefficient (r_c) of the natural logarithms of the diet and feces profiles (Lin 1989, 2000). In computing the estimated diet composition, the "*fgoalattain*" function of the MATLAB optimization toolbox was set to maximize r_c toward a value of 1, while minimizing the total sums of squares of differences and maintaining the sum of the relative proportions of the dietary components (between the values of 0 and 1) at a value of 1 (i.e., 100%). The "*fgoalattain*" function of MATLAB uses a sequential quadratic programming multi-objective optimization method (MathWorks 2004). In sum, the analysis estimated the similarity between the presumed diet and the fecal content, assessing the proportions of each element in the diet.

In order to investigate whether particular combinations of n-alkanes and long-chain fatty alcohols gave more accurate estimations of diet composition, we tested the effect of including or omitting different n-alkanes and fatty alcohols in calculations starting with a set of the three alkanes C29, C31, and C33. This was done by repeated computation of potential diet compositions using all possible combinations ($n = 1024$) of the remaining odd-chain alkanes C23, C25, C27, and C35 and the even-chain fatty alcohols 1-C20-ol to 1-C30-ol. Fecal recoveries were assumed to be 100% of consumed alkanes and alcohols for all calculations.

Having used different combinations of n-alkane and long-chain fatty alcohols to estimate diet composition, we compared the range of diets estimated from fecal analysis with the actual diets consumed by the animals. We did this by calculating the r_c for each estimated diet against the actual diets selected, and the different models (comprising presence or absence of particular alkanes or alcohols) were ranked in descending order of r_c value. The top model was judged to be the one that had the highest r_c, in other words, that which generated the most accurate prediction of the composition of the actual diets consumed. The r_c of individual plant categories in the diet were also calculated and again ranked in descending order of r_c.

The diet composition estimation routine required initial values to be supplied, and we used the composition of the diets offered to each sheep as starting values during the selection of the best models. However, some optimization routines generate results that are dependent on the starting values, because the routine identifies a so-called local minimum value of the function rather than the true or global minimum value. To ensure that the diet estimates did not result from local minima in the optimization routine, we generated a range of starting values for each animal by systematically varying the relative proportions of the constituent plant categories to create a number of different starting values for the optimization routine. If a dietary category accounted for <10% of the total diet, its starting value was varied from 0 to 20% in 2% increments; if it accounted for between 10% and 20%, its starting value was varied by −10% to +10% of the actual value in 1% increments; and if it accounted for >20%, its starting value was varied by −20% to +20% of the actual value in 2% increments. Each plant category was thus changed in turn, with the remaining plant categories being adjusted in proportion

TABLE 2. Concentrations of individual n-alkanes and total of measured values within each dietary category (mg/kg dry mass).

Dietary category	C21	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	C33	C35	C36	Total
Molinia caerulea	0	4	8	17	13	34	33	76	8	36	2	9	1	2	242
Festuca spp.	0	2	4	8	6	34	19	450	20	928	10	191	4	2	1678
Dead grass	0	2	6	12	9	33	28	129	12	111	4	33	2	3	384
Juncus effusus	0	2	2	3	3	14	17	290	16	113	3	9	0	0	472
Carex spp.	0	2	5	7	4	13	8	89	10	411	12	192	2	2	757
Calluna vulgaris	12	6	7	9	7	31	12	97	20	534	41	440	12	1	1229
Vaccinium myrtillus	0	2	4	13	10	45	42	151	33	201	11	46	1	1	562
Erica tetralix	1	3	3	7	5	50	18	926	45	1838	46	687	4	2	3637
Moss	1	2	4	4	3	11	6	30	4	46	2	19	1	1	133

to their relative contribution to the diet to maintain a total of 100%. Since the experiment was based on nine plant categories, a total of 181 starting diet compositions was generated and tested, including the original values of the diets offered. The median value for each plant category was used as the final estimate of that category's contribution to the complete diet, which reduced the effect that outliers or skewed distributions of estimates would have on a mean value.

Having established a ranking of the different possible models based on the r_c, we carried out a paired t test on the actual vs. estimated diet composition values for all animals for each model. This was done to identify which, if any, of the estimated diets were statistically similar to the diet actually consumed and thus which model(s) might be the best for estimating diet composition in free-ranging animals. Data were subjected to a log transformation before analysis.

RESULTS

Botanical composition of the mixtures as fed

There were three grass categories that accounted for >0.5% of the forage mixes: *Molinia caerulea* (purple moor grass), *Festuca* spp. (fescues), and dead grass (Table 1). The *Molinia caerulea* and *Festuca* spp. categories consisted of green leaves, stems, and inflorescences of these grasses, while the dead grass category consisted of dead leaves and stems from all grass species present. Although leaf shatter had made accurate identification of this dead material difficult, it was estimated that >95% of it was dead *Molinia caerulea*. The remaining six categories consisted of two further monocot groups, three dwarf-shrub groups, and moss. The additional monocot categories were *Juncus effusus* (soft rush) and *Carex* spp. (sedges), and the three dwarf-shrub categories were *Calluna vulgaris* (ling heather), *Erica tetralix* (cross-leaved heather), and *Vaccinium myrtillus* (bilberry). Each of these six categories included both live and dead plant parts, although in all cases the material was predominately (>75%) living. Three additional plant groups, *Nardus stricta* (mat grass), *Agrostis* spp. (bents), and *Galium saxatile* (heath bed-straw), were identified as being present within the grass mix, but accounted for <0.5% of the total and so were excluded from further analyses.

Herbage concentrations of n-alkanes and long-chain fatty alcohols and their fecal recoveries

The concentrations of odd-chain n-alkanes (Table 2) were substantially higher than those of the even-chain alkanes, and subsequent calculations used only these values. The mean proportional fecal recoveries of the odd-chain alkanes were 0.87, 1.12, 1.01, 1.07, 1.06, 1.18, 1.06, and 1.27 for C21 to C35, respectively, indicating that the majority were essentially indigestible. The concentrations of even-chain long-chain fatty alcohols (Table 3) were higher than those of the odd-chain alcohols, and subsequent calculations only used the even-chain values. The mean proportional fecal recoveries of the even-chain long-chain fatty alcohols were also high at 0.93, 0.92, 0.99, 1.39, 1.36, and 1.56 for 1-C20-ol to 1-C30-ol, respectively.

Estimated vs. actual diet composition

Lin's concordance correlation coefficient (r_c) was used to identify the top 30 highest ranking models for estimating diet composition (Table 4). This coefficient can take a value of +1 (absolute correlation) to −1 (absolute inverse correlation), and the highest ranking model had an r_c of 0.982. The alkanes C29, C31, and C33 were chosen for use in all models as they were present in the greatest concentrations of all odd-chain alkanes and varied the most between different plant categories (the standard deviations were greater than the mean values). These alkanes also tend to have fecal recovery values close to 100%. However, the model that used only these three core alkanes gave very poor estimates of diet composition, with an r_c of 0.000. The alkane C21 was excluded from subsequent calculations because of its complete absence from some of the fecal samples, which was assumed to be an analytical artifact, although use of all of the other odd-chain alkanes improved the diet composition estimates dramatically. The model based on only n-alkanes (C23–C35 odd-chain alkanes) had an r_c of 0.734, while the model that used all available information (C23–C35 odd-chain alkanes and all even-chain alcohols) had a similar r_c of 0.725. Selecting only key alkanes and alcohols improved the r_c values further, and all but one of the top nine models were based on concentrations of the alkanes C25, C29, C31, and C33, and nearly all included data for only two long-chain fatty alcohols.

TABLE 3. Concentration of individual long-chain fatty alcohols and total of measured values within each dietary category (mg/kg dry mass).

Dietary category	1-C20-ol	1-C21-ol	1-C22-ol	1-C23-ol	1-C24-ol	1-C25-ol	1-C26-ol	1-C28-ol	1-C29-ol	1-C30-ol	Total
Molinia caerulea	38	0	25	6	34	11	90	2382	55	269	2953
Festuca spp.	48	19	47	16	71	39	1117	1086	68	359	2902
Dead grass	56	16	68	23	86	65	281	2041	86	312	3072
Juncus effusus	10	0	7	0	8	17	58	88	31	401	625
Carex spp.	17	6	28	17	122	18	109	260	8	477	1069
Calluna vulgaris	221	21	1190	27	474	35	203	450	509	829	4056
Vaccinium myrtillus	271	19	511	27	362	26	334	383	104	931	3046
Erica tetralix	62	11	560	34	1124	48	1015	1496	152	4465	9174
Moss	38	12	174	16	216	22	170	471	45	726	1907

The correlations between estimated and actual diet compositions were higher for models that used combinations of selected alkanes and alcohols. None of the top 30 models included the use of C23 or C35, while most included either C25 or C27 but never both. Of the long-chain fatty-alcohols, 1-C28-ol was included in 18 of the top 30 models, and this alcohol tended to be present in greatest concentrations among the plant categories. Alcohols 1-C24-ol, 1-C26-ol, and 1-C30-ol were included in the top 30 models a similar number of times, either individually, in pairs, or all together, but the alcohol 1-C22-ol was included only twice.

The relative diet proportions of some plant categories, e.g., *Calluna vulgaris* and *Vaccinium myrtillus*, could be independently predicted with excellent precision by a number of different models (Fig. 1), whereas the diet proportion of other plant categories, e.g., dead grass and moss, could not be independently predicted well by any model. Likewise, although the overall correlation between actual and estimated diet may be high for some models, the accuracy of estimation of certain plant categories is not necessarily good in all situations (Table 5). For example, the high proportion of moss and low proportion of dead grass in the average diet estimated using one of the models was caused by a nonsensically high estimate of moss in the diet of one animal, leading to an abnormally high mean value for all animals. Such variability with regard to the results for individual animals led to a statistically significant difference

TABLE 4. Top 30 prediction models, ranked in descending order of Lin's concordance correlation coefficient (r_c), indicating presence (X) or absence of specific alkanes and alcohols in the model.

	Alkanes							Long-chain fatty alcohols						
Model	C23	C25	C27	C29	C31	C33	C35	C20	C22	C24	C26	C28	C30	r_c
1		X		X	X	X				X		X	X	0.982
2				X	X	X				X	X			0.981
3		X		X	X	X						X	X	0.981
4		X		X	X	X			X			X		0.981
5		X		X	X	X				X		X		0.981
6		X		X	X	X					X	X		0.981
7		X		X	X	X					X			0.981
8		X		X	X	X				X	X		X	0.981
9		X		X	X	X						X		0.981
10				X	X	X				X		X	X	0.981
11		X		X	X	X					X		X	0.981
12				X	X	X				X	X		X	0.981
13		X		X	X	X				X	X			0.981
14			X	X	X	X						X		0.976
15			X	X	X	X					X			0.976
16			X	X	X	X						X	X	0.976
17			X	X	X	X					X	X		0.976
18			X	X	X	X				X		X		0.976
19			X	X	X	X			X			X		0.976
20		X		X	X	X		X				X		0.976
21				X	X	X				X				0.976
22			X	X	X	X					X	X	X	0.976
23			X	X	X	X					X		X	0.976
24				X	X	X				X			X	0.976
25				X	X	X					X	X	X	0.976
26				X	X	X		X				X		0.976
27			X	X	X	X				X			X	0.975
28				X	X	X						X		0.974
29			X	X	X	X				X				0.974
30				X	X	X				X		X		0.974

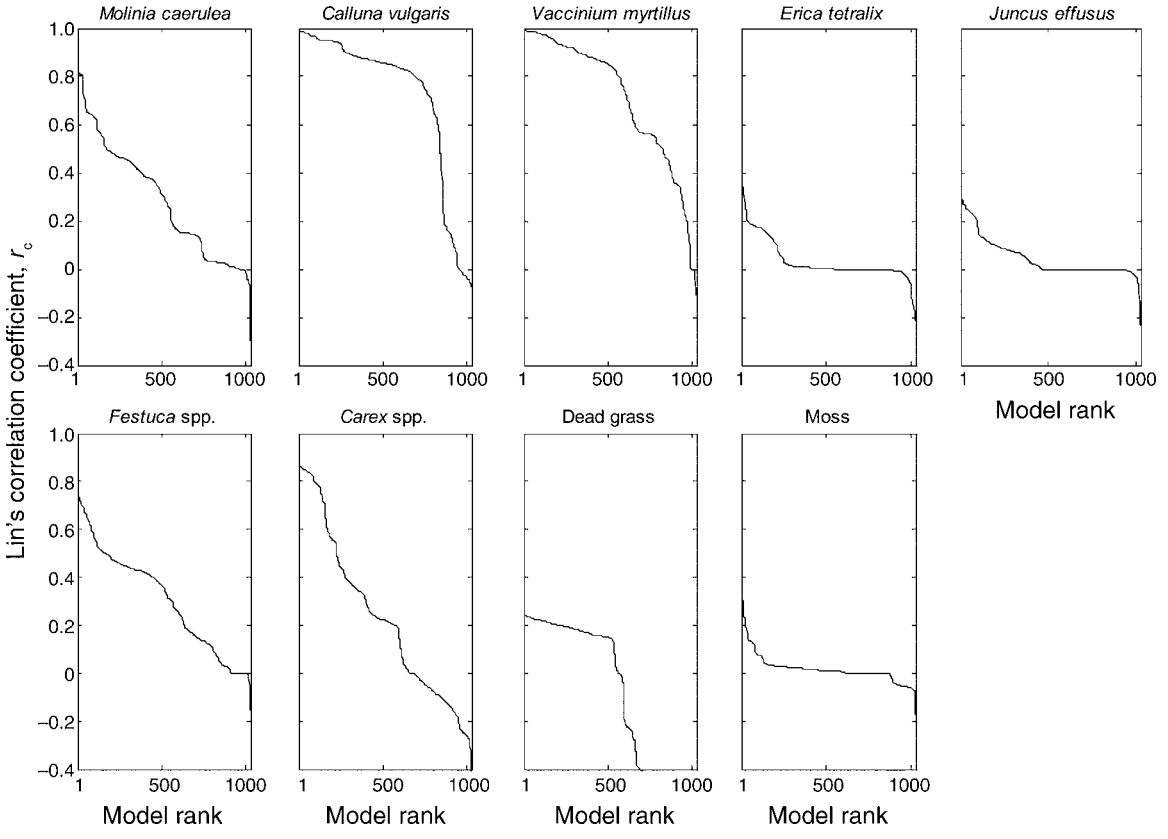

FIG. 1. Lin's concordance coefficients (r_c) for each of 1024 test models ranked in descending order of goodness of fit for each dietary category. A high r_c value indicates a good correlation between actual and predicted dietary values. The r_c for each model was derived using the data from 12 animals. The experiment was conducted during September 2002 on the west coast of the United Kingdom at the Institute of Grassland and Environmental Research at Aberystwyth, Ceredigion, Wales.

between the estimated and actual diets for several of the models.

DISCUSSION

The aim of our study was to evaluate the potential of using n-alkanes and long-chain fatty alcohols to categorize a range of different components within a complex mixed diet. The use of n-alkanes to determine diet composition has been investigated in a number of different animal species, including dairy cattle (Hameleers and Mayes 1998), sheep (Duncan et al. 1999, Valiente et al. 2003), mountain hares (Hulbert et al. 1996), and rabbits (Martins et al. 2002). However, to date the number of dietary categories defined within a given diet has been small, and in general there has been no way of validating the results obtained. A major potential advantage of the use of n-alkanes and long-chain fatty alcohols is that a range of possible markers are available for estimating diet composition, thus making it feasible to characterize complex diets (Dove

TABLE 5. Mean observed diet composition vs. median estimated diet composition for the top 10 models as defined in Table 4 (M1–M10; all values dry mass proportion × 100).

Dietary component	Consumed	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
Molinia caerulea	41.4	38.9	35.4	38.9	38.9	39.0	35.3	38.8	35.2	38.8	38.9
Calluna vulgaris	18.2	16.6	19.3	16.5	16.1	16.6	17.4	16.6	17.5	16.6	16.5
Vaccinium myrtillus	0.2	2.3	0.4	2.4	0.2	2.4	9.5	2.4	9.2	2.4	2.4
Erica tetralix	0.2	0.9	2.3	0.9	0.4	0.9	0.7	0.8	0.6	0.8	0.9
Juncus effusus	7.0	2.7	7.4	2.6	4.7	2.7	2.0	2.8	2.0	2.8	2.7
Festuca spp.	1.7	3.6	3.6	3.5	5.0	3.6	3.4	3.7	3.6	3.7	3.6
Carex spp.	3.9	5.8	5.8	5.8	3.0	5.8	3.8	5.8	3.8	5.9	5.8
Dead grass	21.1	20.8	18.6	20.7	6.0	20.8	20.8	20.8	21.2	20.8	20.8
Moss	6.4	7.1	5.9	7.2	25.2	7.2	6.3	7.1	6.3	7.1	7.1
Paired *t* test probability	···	0.639	0.758	0.089	0.006	0.518	0.621	0.002	0.044	0.002	0.949

Note: Paired *t* test compared observed diets vs. predicted diets of all animals.

PLATE 1. Sheep grazing mosaics of heather and hill grass similar to the vegetation cut and fed during the experiment. Photo credit: J. M. Moorby.

and Mayes 1996). However, previous studies have focused on validating the use of n-alkanes to determine diet composition in two-component dietary mixes such as *Calluna vulgaris* and an *Agrostis*/*Festuca* grass mix and *Juncus effusus* and ryegrass (Dove and Mayes 1996).

During our experiment the animals were zero-grazed to allow the diet offered and refused to be accurately quantified, but the forage fed was cut directly from heterogeneous vegetation communities (see Plate 1). Consequently, the hill grass and heather mixes that formed the basis of the diets offered contained a range of plant species commonly found within indigenous vegetation communities in the hills and uplands of the United Kingdom (Grant et al. 1985, 1987, Fraser and Gordon 1997, Fraser 1998). As the DM of the heather and hill grass mixes were broadly similar to one another (450 and 563 g/kg FM for the grass and heather mixes, respectively), the ratios of amount of heather mix : grass mix expressed on a DM basis were similar to the FM ratios given in the *Methods* section.

Differences in n-alkane and fatty alcohol profiles

According to Dove and Mayes (1991), for greatest sensitivity when determining botanical composition, the total alkane concentrations of the component species should be similar but their patterns markedly different. However, during the current study, the total n-alkane concentrations recorded ranged from 133 mg/kg DM for moss to 3637 mg/kg DM for *Erica tetralix*. These findings concur with those from an earlier study of n-alkane profiles of plant species indigenous to the hills and uplands of the United Kingdom, which found a relatively low mean total alkane concentration for *Molinia caerulea* and relatively high values for *Erica tetralix* and *Calluna vulgaris* (Hyslop 2001). However, as indicated above, it is the pattern of alkanes that is key to discriminating between dietary components, and the more extreme the differences in the n-alkane profile between different plant species consumed, the better the estimates of diet composition will be.

The alkanes in plant cuticular wax have carbon-chain lengths principally in the range C21–C37, and in pasture plants the range of alkanes most commonly encountered is C25–C37 (Dove and Mayes 1996). In our study, odd-chain n-alkanes were present in much greater amounts than even-chain n-alkanes, which concurs with previous reports (Dove and Mayes 1991, Hyslop 2001, Bugalho et al. 2002). More importantly, there were substantial differences in the pattern of odd-chain n-alkane (C27–C33) concentrations between the different dietary categories, again agreeing with results reported by Hyslop (2001) for a range of upland plant species.

The calculation of diet composition from n-alkane concentrations in feces is based on two assumptions. The first is that the n-alkane profiles differ markedly among the diet components, as has been demonstrated for the plant species fed in this experiment. The second is that the proportions of the n-alkanes in the forage eaten are not significantly different from those in the feces. Brosh et al. (2003) quantified recovery rates for goats and cattle and found that the recovery of odd-chain length n-alkanes increased linearly with n-alkane chain length, which is in agreement with the results of our study. Although Dove and Mayes (1996) indicate that plant alkanes are substantially indigestible, other studies have suggested that they are differentially digestible

(Newman et al. 1998). To further confound the differential digestibility problem, it has been pointed out that digestibility may differ substantially between individual animals (Dove and Mayes 1996). As with previous studies, recovery values >1 were calculated in our experiment (Brosh et al. 2003). The high but incomplete fecal recoveries recorded for the long-chain fatty alcohols also agree with previous findings (Ali et al. 2004).

Brosh et al. (2003) found that without recovery corrections being applied to fecal n-alkane concentrations, the estimated proportions of diet components were significantly different from the proportions actually consumed, and the best estimates were achieved when individual animal corrections were applied. However, in grazing experiments with free-ranging animals fecal recovery values will not be available for all diet components or for individual animals, and so we assumed recovery values to be 100% of consumed alkanes and alcohols during subsequent calculations. By doing this, comparison of the different models used (inclusion or exclusion of different n-alkanes and long-chain fatty alcohols) did not require correction by recovery values, making the results more applicable to other diets that may contain components not included in the current study.

Determining diet composition

Previous techniques for computing diet composition have included various forms of non-negative least-squares estimations (Dove and Moore 1995, Newman et al. 1995, Duncan et al. 1999, Martins et al. 2002, Valiente et al. 2003). Although a common approach with these methods is to minimize the squared differences between the diet and fecal marker profiles, none of the previous reports had an additional constraint on the minimization routine of profile equivalence (i.e., fitting to a line of equality). In this study, we used Lin's concordance correlation coefficient for this purpose and to establish equivalence between different models on the basis of their ability to estimate diet composition. Like Pearson's correlation coefficient (r), r_c can take a value of +1 to −1. Unlike r, however, r_c also gives a measure of reproducibility.

The optimization process proved to be robust in terms of the starting parameters required, as shown by the estimates of diet composition being similar for a range of starting values, varied in a systematic way from the diets offered. The median value from the range of estimates generated was chosen as this was theoretically less likely to be affected by outliers than the mean, although in practice the two values were almost exactly the same. This means that despite the composition of a diet consumed by an animal differing markedly from the composition of the sward from which it is grazed, the sward composition values could be used as a set of starting parameters for the diet estimation process.

Any optimization software should be able to compute diet composition estimates using the approach outlined in *Methods: Data analysis*. Indeed the original methods for the work reported here were developed using the Solver tool of Microsoft Excel, and this gave very similar results to the MATLAB programs. Hameleers and Mayes (1998) compared three least-squares optimization methods, including the use of the Excel Solver tool, and found each gave a similar prediction of the white clover and perennial ryegrass content of dairy cows diets.

Accuracy of estimates

Some models were better at estimating the proportions of different dietary components than others, and the best models differed for each component (Fig. 1). *Calluna vulgaris* and *Vaccinium myrtillus* could both be estimated individually with a high degree of accuracy by a number of different models, despite being present in very different proportions in the diets. On the other hand, moss, dead grass, *Erica tetralix*, and *Juncus effuses* were not well estimated individually by any model. Some of this discrepancy may be caused by errors in the measurement of diet composition by botanical separation, particularly for those components present in small proportions. In addition to this, during the current experiment the sheep selected plant leaf material in preference to stem and shoot material in preference to wood, as would be expected under free-ranging conditions (Grant et al. 1987, Fraser and Gordon 1997). As there can be significant differences in alkane concentration between plant parts (Dove et al. 1996), this could also be a potential source of error, yet good estimates of *Calluna vulgaris* consumption were obtained, despite the herbage sample analyzed being a mixture of shoots and woody material. However, if selection of plant parts from particular plant species was of interest, then any differences in n-alkane profiles could be exploited and the methodology described here adjusted to allow consumption of different plant parts to be estimated.

The best overall models were chosen on the basis of the ranking of the r_c for complete diets, rather than individual components. Although the top 10 models (as defined in Table 4), had very similar values for r_c, paired t tests revealed that five of these, M3, M4, M7, M8, and M9, generated figures for diet composition that were significantly different from the actual diet consumed. The other five models all ranked the major and minor components well, and for many of the components the different models produced values that were broadly similar. However, there were differences between the models in the accuracy of certain components. These results indicate that the best model for predicting individual components can differ, and consequently an alternative and possibly more accurate overall prediction may be obtained by using different models to predict the different components independently. How-

ever, it is worth noting that even the best r_c for some of the botanical components was relatively low, as illustrated in Fig. 1. In terms of overall diet composition, there were only relatively minor differences in the models that were based on combinations of the n-alkanes C25, C29, C31, and C33 and the long-chain fatty alcohols 1-C24-ol, 1-C28-ol, and 1-C30-ol.

It should be noted that accurate estimates of diet composition were obtained by a number of different models despite the presence within the herbage mixes of a small number of additional plant species that were not included in the analyses, but that were still contributing to the fecal profile of n-alkanes and long-chain fatty alcohols, a scenario that is likely to be repeated during grazing studies.

Application of the technique

Our experiment has demonstrated that n-alkanes and long-chain fatty alcohol markers could provide a viable alternative to current methods of estimating diet composition, all of which have limitations. For example, nonobtrusive observations or measurements of the utilization of forage plants have been commonly employed when studying wild animals (Parker et al. 1993), but such methods are subject to observer error, particularly where an assessment of the composition of a diet selected on structurally complex vegetation is required. Microhistological analysis of feces is an alternative approach that identifies cellular structures by comparing the subject epidermal material patterns with those of known plant reference material, allowing fecal particles to be identified to species or family level. However, this method does not take into account variations in rate of digestion of different plant species or plant parts (Slater and Jones 1971), and there is typically a high proportion of unidentified cuticle and other plant remains occurring in the feces (McInnis et al. 1983). Identification of plant fragments in extrusa collected from esophageal fistulates is another approach that has been used to quantify the composition of the diet consumed by animals grazing heterogenous vegetation communities (Grant et al. 1987, Fraser and Gordon 1997). However, the accuracy of this method has also been questioned (Coates et al. 1987, Jones and Lascano 1992), as the sample collected represents only a small portion of the total diet that may be selected by the animal. This methodology also raises ethical issues and is of limited use in wild herbivores. In contrast, the approach investigated during the current study is noninvasive, would have minimal impact on the grazing behavior of the study animal, and could be used for both domesticated and wild animals. Furthermore, given that many laboratories offer n-alkane analysis as a commercial service and that the data analysis required can be carried out using standard software such as Microsoft Excel, this methodology could be used by land managers and conservationists as well as researchers.

The diet composition information obtained using this methodology has wide-ranging applications in terms of the assessment of the impact of grazing animals on particular vegetation communities or the quantification of nutrient supply to the animal from different diets. The diversity and species richness within a plant community are influenced by interactions between the diet preferences of grazing animals and the competitive abilities of the plants. With greater emphasis now being placed on environmental aspects of land management, much more information is needed regarding the impact of grazing by different types of animal, in order to develop policies that balance environmental as well as production objectives. The methodology outlined above would allow diet selection by different species or breeds of grazing animal from a given community type to be directly compared, in order to determine the most appropriate grazing management strategies for environmentally sensitive vegetation communities. This information is vital for the development of optimal grazing management guidelines that achieve an appropriate balance between maintaining or enhancing biodiversity and ensuring acceptable levels of animal welfare and performance.

Conclusions

Our results demonstrate that n-alkanes and long-chain fatty alcohols can be used to estimate several components within the diet of animals grazing complex swards and that a number of different combinations of specific markers will give good approximations of the diet consumed. The most appropriate model for predicting diet composition depends on which plant types are likely to be the main components within the diet.

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Literature Cited

Ali, H. A. M., R. W. Mayes, C. S. Lamb, B. L. Hector, A. K. Verma, and E. R. Ørskov. 2004. The potential of long-chain fatty alcohols and long-chain fatty acids as diet composition markers: development of methods for quantitative analysis and faecal recoveries of these compounds in sheep fed mixed diets. Journal of Agricultural Science **142**:71–78.

Arnold, G. W., J. Ball, W. R. McManus, and I. R. Bush. 1966. Studies on the diet of the grazing animal. I. Seasonal changes in the diet of sheep grazing on pastures of different availability and composition. Australian Journal of Agricultural Research **17**:543–556.

Brosh, A., Z. Henkin, S. J. Rothman, Y. Aharoni, A. Orlov, and A. Arieli. 2003. Effects of faecal n-alkane recovery in

estimates of diet composition. Journal of Agricultural Science **140**:93–100.

Bugalho, M. N., R. W. Mayes, and J. A. Milne. 2002. The effects of feeding selectivity on the estimation of diet composition using the n-alkane technique. Grass and Forage Science **57**:224–231.

Coates, D. B., P. Schachenmann, and R. J. Jones. 1987. Reliability of extrusa samples from steers fistulated at the oesophagus to estimate the diet of resident animals in grazing experiments. Australian Journal of Experimental Agriculture **27**:739–745.

Dove, H. 1992. Using the n-alkanes of plant cuticular wax to estimate the species composition of herbage mixtures. Australian Journal of Agricultural Research **43**:1711–1724.

Dove, H., and R. W. Mayes. 1991. The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. Australian Journal of Agriculture Research **42**:913–952.

Dove, H., and R. W. Mayes. 1996. Plant wax components: a new approach to estimating intake and diet composition in herbivores. Journal of Nutrition **126**:13–26.

Dove, H., R. W. Mayes, and M. Freer. 1996. Effects of species, plant part, and plant age on the n-alkane concentrations in the cuticular wax of pasture plants. Australian Journal of Agricultural Research **47**:1333–1347.

Dove, H., and A. D. Moore. 1995. Using a least-squares optimisation procedure to estimate diet composition based on the alkanes of plant cuticular wax. Australian Journal of Agricultural Research **46**:1535–1544.

Duncan, A. J., R. W. Mayes, C. S. Lamb, S. A. Young, and I. Castillo. 1999. The use of naturally occurring and artificially applied n-alkanes as markers for estimation of short-term diet compositon and intake in sheep. Journal of Agricultural Science **132**:233–246.

Fraser, M. D. 1998. Diet composition of guanacos (*Lama guanicoe*) and sheep (*Ovis aries*) grazing in grassland communities typical of UK uplands. Small Ruminant Research **29**:201–212.

Fraser, M. D., and I. J. Gordon. 1997. The diet of goats, red deer and South American camelids feeding on three contrasting Scottish upland vegetation communities. Journal of Applied Ecology **34**:668–686.

Grant, S. A., and T. J. Maxwell. 1988. Hill vegetation and grazing animals: the biology and definition of management options. Pages 201–214 *in* M. B. Usher and D. B. A. Thompson, editors. Ecological change in the Uplands. Blackwell Scientific, Oxford, UK.

Grant, S. A., D. E. Suckling, H. K. Smith, L. F. Torvell, and J. Hodgson. 1985. Comparative studies of diet selection by sheep and cattle grazing individual hill plant communities as influenced by season of the year. 1. The indigenous grasslands. Journal of Ecology **73**:987–1004.

Grant, S. A., L. Torvell, H. K. Smith, D. E. Suckling, and J. Hodgson. 1987. Comparative studies of diet selection by sheep and cattle: blanket bog and heather moor. Journal of Ecology **75**:947–960.

Hameleers, A., and R. W. Mayes. 1998. The use of n-alkanes to estimate herbage intake and diet composition by dairy cows offered a perennial ryegrass white clover mixture. Grass and Forage Science **53**:164–169.

Hulbert, I. A. R., G. R. Iason, and P. A. Racey. 1996. Habitat utilization in a stratified upland landscape by two lagomorphs with different feeding strategies. Journal of Applied Ecology **33**:315–324.

Hunter, R. F. 1962. Hill sheep and their pasture: a study of sheep-grazing in south east Scotland. Journal of Ecology **50**: 651–680.

Hyslop, J. J. 2001. Adaptation of the n-alkane technique for use within extensively managed cattle and sheep grazing studies. Final Project Report. Ministry of Agriculture, Fisheries, and Food, London, UK.

Jarman, P. J., and A. R. E. Sinclair. 1979. Feeding strategy and the pattern of resource partitioning in ungulates. Pages 130–163 *in* A. R. E. Sinclair and M. Norton-Griffiths, editors. Serengeti, dynamics of an ecosystem. University of Chicago Press, Chicago, Illinois, USA.

Jones, R. J., and C. E. Lascano. 1992. Oesophageal fistulated cattle can give unreliable estimates of the proportion of legume in the diets of resident cattle grazing tropical pastures. Grass and Forage Science **47**:128–132.

Lin, L. I.-K. 1989. A concordance correlation coefficient to evaluate reproducibility. Biometrics **45**:255–268.

Lin, L. I.-K. 2000. A note on the concordance correlation coefficient. Biometrics **56**:324–325.

Lundberg, P., and M. Astrom. 1990. Functional response of optimally foraging herbivores. Journal of Theoretical Biology **144**:367–377.

Martins, H., D. A. Elston, R. W. Mayes, and J. A. Milne. 2002. Assessment of the use of n-alkanes as markers to describe the complex diets of herbivores. Journal of Agricultural Science **138**:425–434.

MathWorks. 2004. Optimization toolbox user's guide. Version 3. MathWorks, Natick, Massachusetts, USA.

McInnis, M. L., M. Vavra, and W. C. Krueger. 1983. A comparison of four methods used to determine the diets of large herbivores. Journal of Range Management **36**:302–306.

Newman, J. A., F. Cribari-Neto, and M. J. Jensen. 1998. The sensitivity of n-alkane analysis to measurement error: implications for use in the study of diet composition. Journal of Agricultural Science **131**:465–476.

Newman, J. A., W. A. Thompson, P. D. Penning, and R. W. Mayes. 1995. Least-squares estimation of diet composition from n-alkanes in herbage and faeces using matrix mathematics. Australian Journal of Agricultural Research **46**:793–805.

Parker, K. L., M. P. Gillingham, and T. A. Hanley. 1993. An accurate technique for estimating forage intake of tractable animals. Canadian Journal of Zoology **71**:1462–1465.

Senft, R. L., M. B. Coughenour, D. W. Bailey, L. R. Rittenhouse, O. E. Sala, and D. M. Swift. 1987. Large herbivore foraging and ecological hierarchies. Bioscience **37**: 789–799.

Slater, J., and R. J. Jones. 1971. Estimation of the diets selected by grazing animals from microscopic analysis of the faeces—a warning. Journal of the Australian Institute of Agricultural Science **37**:238–240.

Spalinger, D. E., T. A. Hanley, and C. T. Robbins. 1988. Analysis of the functional response in foraging in the Sitka black-tailed deer. Ecology **69**:1166–1175.

Valiente, O. L., P. Delgado, A. de Vega, and J. A. Guada. 2003. Validation of the n-alkane technique to estimate intake, digestibility, and diet composition in sheep consuming mixed grain: roughage diets. Australian Journal of Agricultural Research **54**:693–702.