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Plant wax alkanes and alcohols as herbivore diet composition markers

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Abstract

The n-alkanes in plant cuticular wax have been used as markers for estimating the species composition of herbivore diets, but the long-chain fatty alcohols (LCOH) of plant wax may also be useful. The objective of this research was to assess if LCOH contributed extra information to differentiate plant species, compared with n-alkanes only. We used 3 data sets consisting of n-alkane and LCOH concentrations of plant species occurring in pastures of New South Wales, Australia. We used Principal Component Analysis (PCA) to summarise the data for n-alkane and LCOH concentrations obtained for the species in these data sets. The first 3 principal components explained 86 to 93% and 75 to 99% of the variance in n-alkane and LCOH concentrations, respectively. Orthogonal Procrustes Rotation (OPR) was then used to compare the results of PCA conducted with n-alkane and LCOH data, with a view to establishing whether LCOH provided discriminatory information in addition to that provided by the n-alkanes. Results of OPR indicated that this was so for all 3 data sets, and suggested that the LCOH would be useful additional markers for discriminating between plant species. We tested this by using Discriminant Analysis and cross-validation procedures in 2 data sets to distinguish between defined species groups of C_3 grasses, C_4 grasses, clovers and *Lotus* spp. The discrimination between these categories and the proportion of plant species correctly classified into the defined categories was better when using n-alkanes and LCOH together, compared with alkanes alone. Our results indicate that LCOH provided additional information that could be used for distinguishing plant species as part of estimating the species composition of herbivore diets.

Key Words: n-alkanes, long-chain fatty alcohols, markers, diet composition, herbivores

Resumen

Los n-alcanos de la cera cuticular de las plantas han sido usados como marcadores para determinar la composición de especies en las dietas de los herbívoros, pero los alcoholes grasos de cadena larga (LCOH) también pueden ser útiles. El objetivo de esta investigación fue evaluar si los LCOH aportan información extra para diferenciar las especies de plantas, comparados con los n-alcanos solos. Usamos 3 juegos de datos consistentes de concentraciones de n-alcanos y LCOH de especies de plantas que están presentes en los potreros de Nueva Gales del Sur, Australia. Usamos el análisis de componentes principales (PCA) para resumir los datos de las concentraciones de n-alcanos y LCOH obtenidos para las especies de plantas de los juegos de datos utilizados. Los tres primeros componentes principales explicaron del 86 al 93% y del 75 al 99% de la variación en las concentraciones de n-alcanos y LCOH respectivamente. Entonces se uso la Rotación de Procrustes Ortogonales (OPR) para comparar los resultados de los componentes principales realizados con los datos de n-alcanos y LCOH, con la mira de establecer si los LCOH proveyeron información discriminatoria adicional a la proporcionada por los n-alcanos. Los resultados de OPR indicaron que esto si fue así para los 3 juegos de datos y sugirieron que los LCOH pudieran ser marcadores adicionales útiles para discriminar entre especies de plantas. Probamos esto usando procedimientos de Análisis Discriminante y validación cruzada en dos juegos de datos para distinguir entre grupos de especies definidas de zacates C_3, zacates, C_4, tréboles y Lotus spp. La discriminación entre estas categorías y la proporción de especies de plantas correctamente clasificadas dentro de las categorías definidas fue mejor cuando se uso n-alcanos y LCOH juntos en comparación con los alcanos solos. Nuestros resultados indican que LCOH proveyeron información adicional que pudiera ser utilizada para distinguir especies de plantas como parte de la estimación de la composición de especies de las dietas de los herbívoros.

The species composition of herbivore diets can be estimated by exploiting differences among plant species in their cuticular wax compounds, especially n-alkanes (Dove and Mayes 1991). The n-alkanes are relatively inert, long-chain saturated hydrocarbons (C21 to C37) that, in ruminants, can be recovered in feces to a degree dependent on their carbon-chain length (Dove and Mayes 1991, 1996). Diet composition is estimated by relating the n-alkane content of feces to the n-alkane content of the plants available to herbivores, after correcting for n-alkane fecal recovery (e.g. Dove and Moore 1995). Using plant wax n-alkanes or other wax components as diet markers offers several advantages over other nutritional markers (Dove and Mayes 1999, Mayes and Dove 2000, Sandberg et al. 2000). An important constraint, however, is

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that the number of species that can be differentiated in the diet cannot exceed the number of alkane markers available; this limits differentiation to usually lower than 15 species (Mayes and Dove 2000). When dealing with complex plant communities such as rangelands, the number of plant species available to the herbivore may thus exceed the number of n-alkane markers available. One means of overcoming this constraint is to include more classes of plant wax components as markers for discriminating between plant species. A prerequisite of this approach is that the additional markers must provide discriminatory information additional to that provided by the n-alkanes.

Long-chain fatty alcohols (LCOH) are widespread in the plant species consumed by herbivores and frequently occur at higher concentrations than n-alkanes (Kolattukudy 1976, Dove and Mayes 1999, Mayes and Dove 2000, Kelman et al.2003). Furthermore, the LCOH can be separated and quantified by an extension (unpublished data, R.W. Mayes) of the existing procedure used to analyse n-alkanes. This suggests that LCOH could be useful diet composition markers in addition to the n-alkanes.

Our objectives in the present study were:

1. To quantify n-alkane and LCOH concentrations in the cuticular wax of pasture species commonly found in New South Wales, Australia;
2. To assess if the LCOH provided additional discriminatory information, compared with n-alkanes alone, to distinguish between plant species;
3. To compare the effectiveness of using n-alkanes alone or in combination with LCOH, to describe and differentiate between species or defined groupings of plant species.

Methods

Plant data sets

Three sets of samples were used to obtain information on the n-alkane and long-chain fatty alcohol (LCOH) content of plant species commonly found in a variety of pastures in Australia. Samples were collected at: Ginninderra Experiment Station, Australian Capital Territory, latitude 35° 12' S, longitude 149° 12' E (Data set 1); near Goulburn, New South Wales, latitude 34° 39' S, longitude 149° 31' E (Data set 2) and near Grafton, New South Wales, latitude 29° 37' S, longitude 152° 57' E (Data set 3). Differences in the method of sample collection and the nature of the material collected suggest that these may be regarded as 3 independent data sets, although combinations of data sets were also examined to explore the trends in between-species differences in the patterns of alkane or LCOH concentrations.

Data set 1 consisted of shoot material from 5 grass species (*Austrodanthonia richardsonii* (Cashmore) H.P. Linder cv. Taranna, *Phalaris aquatica* L., *Vulpia myuros* (L.) Gmel., *Bromus catharticus* Vahl. and *Festuca arundinacea* Schreb.) and 7 legumes (*Trifolium striatum* L., *T. glomeratum* L., *T. repens* L., *Lotus corniculatus* L. "Prostrate", *L. corniculatus* L. cv. Goldie, *L. pedunculatus* Cav. cv. Sharnae, *L. pedunculatus* Cav. cv. Grasslands Maku). Individual plants of each species were cut from a grazed sward (Kelman et al. 2003) using electric clippers, and were then composited within species to provide sufficient material for plant wax analyses.

The second data set consisted of leaf tissue from 7 grass species (*Austrodanthonia racemosa* (R. Br.) H.P. Linder, *A. pilosa* (R. Br.) H.P. Linder, *Elymus scaber* (R. Br.) A. Love, *Bothriochloa macra* (Steud.) S.T. Blake, *Chloris gayana* Kunth, *Themeda australis* (R. Br.) Stapf., *Microlaena stipoides* (Labill.) R. Br.) with the last species coming from 2 sampling locations at the site (designated *Microlaena stipoides* 1 and 2). The leaf tissue was obtained by hand separation of material harvested from a sward of spaced plants (Norton et al. 2001), using an electric sheep-shearing handpiece.

The third data set consisted of shoot material of 10 tropical grasses (*Paspalum notatum* Flugge, *Paspalum dilatatum* Flugge, *Axonopus fissifolius* (Raddi) Kulhm, *Imperata cylindrica* (Nees) C.E.Hubbard, *Setaria anceps* Stapff ex Massey, *Pennisetum clandestinum* Hoscht. ex Chiov., *Cynodon dactylon* (L.) Pers., *Digitaria didactyla* (Willd.), *Chloris gayana* Kunth, *Sporobolus indicus* (Buse) Baijens var. *major*) similarly harvested from pastures in sub-tropical northern New South Wales, Australia, within a larger study of the biology of the weed species *Sporobolus indicus* var. *major* (see Hoebee et al. 1998).

Following collection, all plant samples were stored at −18° C, before being freeze-dried and ground (1 mm screen) for n-alkane and LCOH analysis.

N-alkane and LCOH extraction and quantification

The n-alkanes in data sets 1 and 2 were extracted and quantified as described by Mayes et al. (1986), with modifications indicated by Salt et al. (1992); internal standards used were docosane (C22 alkane) and tetratriacontane (C34 alkane). In addition, 1-pentacosanol (C25 normal alcohol) was used as an internal standard to quantify the LCOH. It should be noted that the alkaline extraction conditions used for the n-alkanes (Mayes et al. 1986) extract not only the free LCOH of cuticular wax, but also the LCOH bound as wax esters. Extracted LCOH were eluted from silica gel columns using 1:1 heptane:ethanol immediately following alkane elution. The LCOH eluates were dried, redissolved in 1:1 heptane:ethanol and injected into a 30m x 0.53mm ID BPX35 bonded-phase silica column (0.5mm film thickness; SGE Pty Ltd Australia) in a PU4550 gas chromatograph fitted with a splitless injector and flame ionization detector. The carrier gas was helium.

Because of difficulties encountered in separating alcohols from sterols under the chromatography conditions used, it is possible that the compounds described as 1-C27-OL and 1-C29-OL could in fact be sterols. However, as this does not affect subsequent data analysis or the arguments presented in this paper, these components are assumed hereafter to be primary alcohols.

Essentially the same methods were used to extract and quantify the alkanes and LCOH in data set 3 except that C24 alkane (tetracosane) was used as the first internal standard for alkanes. Both n-alkanes and LCOH were quantified through a 15m x 0.53 mm DB1 column fitted in a Varian 3400 gas chromatograph, using helium as the carrier gas and flame ionization detection.

Statistical analysis

The n-alkane and LCOH analyses were based on bulk samples of individual plant species, and thus do not provide an estimate of the variability in the concentration of these compounds between individual plants or plant parts, within a species. However, as we describe in more detail below, earlier studies with similar plant species suggest that most of the variance in alkane or LCOH concentration can be ascribed to plant species (e.g. Dove et al. 1996, Mayes and Dove 2000).

Alkanes C21 to C24 were excluded from the statistical analyses because their estimated concentrations were very low and of the same order of magnitude as the analytical error (3 to 4 ppm). Alkane C35

was excluded from the statistical analyses of data set 1, for the same reason. Similarly, in data set 3 (Table 2) data were available only for the even-chain LCOH between C24OH and C30OH. Before statistical analysis, concentrations of n-alkanes and LCOH in each data set were normalized by log transformation (log (x +1)), to accommodate the fact that the untransformed concentrations of the 2 classes of compound differed by orders of magnitude.

Principal Component Analyses (PCA; GENSTAT, Lawes Agricultural Trust) were used to summarize the information on the pattern of alkane or LCOH concentrations within a data set or combinations of data sets. The overall significance of principal components of a given dimension was assessed using chi-square tests and where required, mean PCA scores for groups of species were compared by analysis of variance. Most of the original variability in the pattern of marker content in a plant species could be reduced to the first 2 to 3 principal components (PC1 to PC3).

For each data set, 2 PCA outputs were obtained, one based on the n-alkane content and the other based on the long-chain fatty alcohols (LCOH) content of the plant species in the set. These pairs of PCA outputs were then compared by Orthogonal Procrustes Rotation (OPR) to assess if the 2 classes of marker had simply identified the same variation, or whether the LCOH provided extra information of potential use for differentiating plant species. To achieve this, OPR rotates the axes of each PCA output in an attempt to minimize the residual sum of squares between the PCA scores based on n-alkanes and those based on LCOH. In the present study the PCA scores based on n-alkanes and on LCOH were used as the fixed and the fitted values, respectively. Large values of unexplained residual variance after OPR imply that there are large differences between the spatial configurations resulting from PCA on the n-alkanes or on the LCOH. This, in turn, indicates that n-alkanes and LCOH are providing different types of discriminatory information which could be used to differentiate between the plant species involved.

Discriminant Analysis (DA; SPSS Inc., Chicago) was then applied to the n-alkane and LCOH concentration data in the first 2 data sets in which logical groupings of species were possible (the combined data set (1+2), and data set 1 alone—see below), to assess how effectively defined groups of species could be differentiated using either the n-alkanes alone, or together with the LCOH.

Although the species groupings for the combined data set (1+2) are logical from the point of view of likely sward compositions, they do not take into account that plant wax marker concentrations are for leaf blade only in data set 2 and are based on samples collected from spaced plants at a different location from the shoot samples in data set 1. The conduct of DA with data set 1 alone thus allows a more valid assessment of the capacity to distinguish the defined groups of *Lotus* spp., clovers and grasses, in shoot samples collected contemporaneously from the same sward.

To perform DA analyses, species/cultivars within a defined group were regarded as replicates, the stepwise method was used and the Mahalanobis distance applied to assess differences between dietary categories (SPSS Inc., Chicago). At each step of the analysis, the variable that maximized the Mahalanobis distance between the 2 closest categories was entered. The minimum partial value of F for the variable to be entered was 3.84 and the maximum partial value of F for the variable to be removed was 2.71. The Jack-knife cross-validation procedure was used to assess the proportion of plant species correctly classified into the defined dietary categories (SPSS Inc., Chicago).

Differences among species groupings in DA scores were tested with a multi-way analysis of variance, followed by Scheffé multi-way tests, using the species groupings as the fixed factor and the discriminant scores as the independent variables (Zar 1996).

Results

Concentrations of n-alkanes and long-chain fatty alcohols

Patterns of alkane concentrations in the species in data sets 1 to 3 (Table 1) were, in general, similar to those in previous reports (Dove and Mayes 1996, Dove et al. 1996), in that odd-chain alkanes were present in higher concentration than even-chain alkanes.

Even-chain long-chain fatty alcohols (LCOH) were detected in higher concentrations than odd-chain LCOH and in addition, were often an order of magnitude higher than n-alkane concentrations. The LCOH 10-C29-OL was only detected in data set 1 and 1-C27-OL and 1-C28-OL were not detected in data set 3 (Table 2). Legumes tended to have higher concentrations of 1-C30-OL than grasses and, within legumes, 1-C30-OL was detected at high concentrations in clovers and 1-C26-OL and 1-C28-OL tended to be higher in *Lotus* spp. (Table 2, data set 1). Concentrations of LCOH in data set 3 tended to be lower than in data sets 1 and 2. Overall there were large variations in concentrations of even-chain LCOH among plant species in each data set which may indicate the value of LCOH as species markers.

Principal Component Analyses and Orthogonal Procrustes Rotation

Principal Component Analyses, based either on n-alkanes or on LCOH, were conducted for all data sets separately and for all possible combinations of data sets (Table 3). In all of these cases, most of the observed variance in the pattern of plant wax marker concentrations was explained by the first 3 principal components (PC1 to PC3). When analyses were based on n-alkanes, PC1 to PC3 explained 86 to 93% of the variance; PC1 to PC3 values for analyses based on the LCOH were 75 to 99% (Table 3).

When the 2 sets of PC scores were compared by OPR, 48 to 82% of the total sums of squares were left unexplained (64 to 82% within the individual data sets 1 to 3; Table 3). This indicates that in all analyses, the LCOH were contributing discriminatory information additional to that provided by the n-alkanes.

This is examined further in Figures 1 to 3, for the samples of individual plant species in data sets 1 to 3, respectively. These figures present the plots of PC1 versus PC2 in each data set, and indicate 2 things:

(a) the degree of rotation of these axes effected by OPR, in attempting to minimize the residual variation between PC scores based on n-alkanes or on LCOH (solid axes v. dotted axes, respectively) and,

(b) the nature of the scatter of points representing the samples of individual plant species, for both n-alkanes (upper case letters) and LCOH (lower case italic letters).

For all 3 data sets, it is clear that, despite substantial rotation of the PC1 and PC2 axes, the spatial distribution of the points representing the samples of the different species remains distinct.

Differentiation of groups of species using Discriminant Analyses

The results of the Principal Component Analysis (PCA) described above identified differences in the patterns of both n-alkane and long-chain fatty alcohol (LCOH) concentrations. Of greater significance for the

Table 1. N-alkane concentrations (mg/kg DM) in a group of grass and legume species occurring in temperate pastures (Data sets 1 and 2) and in sub-tropical grasses (Data set 3) in New South Wales, Australia.

	n-Alkanes C25	C26	C27	C28	C29	C30	C31	C32	C33	C35
Data set 1										
Plant Groups										
Lotus spp.										
Lotus corniculatus "Prostrate"	14.7	5.0	44.4	6.3	38.8	3.5	40.1	6.5	42.4	2.0
Lotus corniculatus cv. Goldie	14.4	4.1	37.9	5.5	37.5	2.8	34.3	5.0	33.3	0.0
Lotus pedunculatus cv. Sharnae	23.8	6.5	237.2	15.9	384.7	5.6	49.2	3.5	19.7	0.0
Lotus pedunculatus cv. Maku	15.0	4.5	150.9	9.7	211.7	3.6	55.0	4.1	37.3	1.2
Average	17.0	5.0	117.6	9.4	168.2	3.9	44.6	4.7	33.2	0.8
Grasses										
Austrodanthonia richardsonii	18.6	3.0	26.4	6.6	58.5	7.3	89.6	15.0	11.0	0.0
Phalaris aquatica	26.7	2.6	16.9	3.7	20.8	2.6	15.8	3.5	7.3	2.3
Vulpia myuros	27.7	3.4	40.6	9.4	175.5	19.9	183.8	9.4	37.3	2.9
Bromus catharticus	5.8	2.8	14.6	4.4	115.9	4.4	59.6	3.8	33.6	4.3
Festuca arundinacea	23.6	3.2	42.3	7.6	129.3	12.1	215.7	6.8	58.7	2.3
Average	*20.5*	*3.0*	*28.1*	*6.3*	*100.0*	*9.3*	*112.9*	*7.7*	*29.6*	*2.4*
Clovers										
Trifolium striatum	10.0	4.0	48.2	30.0	989.9	22.5	68.1	5.1	7.9	0.0
Trifolium glomeratum	10.7	3.1	34.9	16.3	313.5	29.7	267.0	20.5	35.9	0.0
Trifolium repens	8.8	3.9	34.9	8.7	107.9	11.8	123.9	8.8	14.8	3.9
Average	*9.8*	*3.6*	*39.3*	*18.3*	*470.4*	*21.3*	*153.0*	*11.5*	*19.6*	*1.3*
Data set 2										
Austrodanthonia racemosa	7.6	5.7	17.0	10.5	73.3	22.0	613.4	38.3	625.0	13.0
Austrodanthonia pilosa	5.6	5.5	11.8	10.5	50.6	11.7	71.8	7.6	11.5	2.0
Microlaena stipoides 1	4.6	5.3	12.2	16.5	69.6	20.4	216.2	21.7	156.2	8.0
Microlaena stipoides 2	4.4	5.9	10.5	23.4	89.0	24.6	219.8	18.9	151.6	5.1
Elymus scaber	6.2	3.0	11.0	4.9	51.5	5.8	95.9	5.9	36.7	4.2
Bothriochloa macra	22.3	15.0	132.2	11.5	64.6	14.0	111.0	10.6	33.8	4.2
Chloris gayana	11.6	12.1	88.6	23.8	180.1	22.8	243.3	14.7	137.5	31.8
Themeda australis	*6.9*	*5.6*	*20.4*	*12.1*	*58.9*	*28.1*	*277.8*	*27.6*	*254.9*	*40.9*
Data set 3										
Paspalum notatum	0	0	0	0	4.8	5	35	8.1	168.4	307.8
Paspalum dilatatum	0	0	8	0	12	5.1	55.8	0	36.4	13.2
Axonopus fissifolius	0	0	8.5	0	25.9	7.9	118.5	15.3	222.1	93.5
Imperata cylindrica	0	0	11.4	14	48.9	47.9	206.8	35.9	153.9	36.3
Setaria anceps	32.2	12.6	82.2	10.1	61.9	5.8	73.5	0	24.7	4.8
Pennisetum clandestinum	0	0	6.7	0	11.5	3.9	78.5	6.7	194.5	204.3
Cynodon dactylon	0	0	11.4	7.6	29.9	11.1	65.5	8.1	90.9	57.6
Digitaria didactyla	5.3	5.1	24.3	9.7	55.3	13.1	96.1	10.6	125.7	42.1
Chloris gayana	4.4	0	20.6	6.3	31	11.9	122.8	19.3	251.7	87
Sporobolus indicus cv. major	0	0	5.8	0	11.3	11	101.5	14.1	118.2	34.6

use of cuticular wax components as markers is the fact that the Orthogonal Procrustes Rotation (OPR) established that the discriminatory information provided by the LCOH was additional to that provided by the n-alkanes. The Discriminant Analyses (DA) were conducted to establish whether the observed differences in the concentration patterns of n-alkanes alone, or n-alkanes with LCOH, could be used to differentiate defined groups of species. The grouping of species provided a means of generating replicates within the DA, and a major criterion for defining species groups was that livestock would be likely to encounter them together within a sward. Since it would be most unlikely that animals would encounter the sub-tropical grasses in data set 3, growing together with the temperate species in data sets 1 and 2, discriminant analyses were not conducted for data set 3 or any combinations involving it. The DA were thus restricted to the temperate species in data sets 1 and 2.

Combined data set (1 + 2): The defined species groupings when data set 1 and 2 were combined were *Lotus* spp., clovers, C_3 grasses and C_4 grasses (*Themeda australis, Chloris gayana, Bothriochloa macra*). When based on n-alkanes alone (Fig 4-a), the first 2 discriminant scores explained 68.9% and 23.2%, respectively, of the variance between the defined groups, which were significantly differentiated on both the first (DS1; $P < 0.001$) and second (DS2; $P = 0.008$) discriminant axes. *Lotus* spp. were discriminated from the other species groupings in DS1 ($P < 0.05$) and C_4 grasses similarly differed in DS1 from C_3 grasses and clovers ($P < 0.05$). The mean DS1 of clovers and C_3 grasses did not differ significantly. On DS2, *Lotus* spp. differed from the C_3 and C_4 grasses ($P < 0.05$) but other groups could not be distinguished. Mean scores on the third discriminant axis (DS3) did not differ significantly. Nevertheless, cross-validation procedures indicated that 80% of individual species were correctly classified, although *Lotus corniculatus* cv. Goldie, *Trifolium repens* and *Themeda australis* were all misclassified as C_3 grasses, while *Bothriochoa macra* was misclassified as a *Lotus* sp..

When n-alkanes and LCOH were used together, DS1 and DS2 explained 87.1%

Table 2. Long-chain fatty alcohol concentrations (mg/kg DM) in a group of grass and legume species occurring in temperate pastures (Data sets 1 and 2) and in subtropical grasses (Data set 3) in New South Wales, Australia.

	Long-chain fatty alcohols						
	1-C24-OL	1-C26-OL	1-C27-OL	10-C29-OL	1-C28-OL	1-C29-OL	1-C30-OL
Data set 1							
Plant groups							
Lotus spp.							
Lotus corniculatus "Prostrate"	20.3	1999.5	94.4	0.0	843.5	27.5	1005.4
Lotus corniculatus cv. Goldie	12.9	2154.4	84.0	0.0	905.0	41.1	1014.9
Lotus pedunculatus cv. Sharnae	18.6	1378.8	81.2	13.9	844.1	63.7	785.2
Lotus pedunculatus cv. Maku	27.8	2463.1	104.1	0.0	1327.5	42.2	1284.8
Average	*19.9*	*1999.0*	*90.9*	*3.5*	*980.0*	*43.6*	*1022.6*
Grasses							
Austrodanthonia richardsonii	65.6	435.2	24.0	0.0	170.7	42.5	130.8
Phalaris aquatica	18.6	3726.1	15.0	22.7	44.7	0.0	472.0
Vulpia myuros	79.9	900.8	17.1	12.7	42.3	30.7	64.7
Bromus catharticus	12.7	84.9	25.3	17.1	4052.2	36.1	83.7
Festuca arundinacea	26.8	638.8	21.2	10.4	100.5	17.1	58.0
Average	*40.7*	*1157.2*	*20.5*	*12.6*	*882.1*	*25.3*	*161.8*
Clovers							
Trifolium striatum	37.0	214.4	28.0	0.0	443.5	75.4	1259.3
Trifolium glomeratum	47.7	124.4	21.0	0.0	72.9	36.8	1199.0
Trifolium repens	18.0	142.6	18.6	0.0	61.0	27.3	1297.3
Average	*34.2*	*160.5*	*22.5*	*0.0*	*192.5*	*46.5*	*1251.9*
Data set 2							
Austrodanthonia racemosa	45.0	65.6	22.6	n.d.	126.1	102.5	455.4
Austrodanthonia pilosa	32.6	34.9	16.6	n.d.	24.0	88.5	556.1
Microlaena stipoides 1	11.4	17.0	25.7	n.d.	35.5	169.1	1660.3
Microlaena stipoides 2	10.0	17.0	22.7	n.d.	50.7	233.1	3225.9
Elymus scaber	53.5	1868.3	19.6	n.d.	201.0	101.7	116.0
Bothriochloa macra	60.6	43.9	22.7	n.d.	285.4	239.8	221.0
Chloris gayana	64.1	56.4	39.7	n.d.	186.8	190.1	148.5
Themeda australis	26.4	33.2	23.3	n.d.	14.9	112.2	68.9
Data set 3							
Paspalum notatum	31	9.2	n.d.	n.d.	n.d.	5.1	0
Paspalum dilatatum	41.2	15.3	n.d.	n.d.	n.d.	268.9	20.1
Axonopus fissifolius	28.4	15.5	n.d.	n.d.	n.d.	16.7	24.2
Imperata cylindrica	10.8	0	n.d.	n.d.	n.d.	0	0
Setaria anceps	28	20	n.d.	n.d.	n.d.	60	24.3
Pennisetum clandestinum	29.7	8.7	n.d.	n.d.	n.d.	11.4	0
Cynodon dactylon	31.1	24.9	n.d.	n.d.	n.d.	55.7	0
Digitaria didactyla	88	75.6	n.d.	n.d.	n.d.	88	0
Chloris gayana	83.9	27.9	n.d.	n.d.	n.d.	70.9	17.3
Sporobolus indicus cv. major	126.5	99.6	n.d.	n.d.	n.d.	32.9	0

n.d.=not determined

and 10.9% of the between-group variance, respectively, and species groups were significantly discriminated by DS1 ($P < 0.0001$) and DS2 ($P < 0.001$). Discrimination on DS3 approached significance ($P < 0.10$). All pairs of species groups were distinguishable on DS1 ($P < 0.05$) except clovers and C_3 grasses. On DS2, C_4 grasses differed significantly from *Lotus* spp., C_3 grasses and clovers ($P < 0.05$). By contrast with DA based on n-alkanes alone, C_3 grasses and clovers could be distinguished on DS3 ($P < 0.05$; Fig. 4-b). Cross-validation procedures indicated that 85% of individual species were correctly classified. *Vulpia myuros* was misclassified as a clover and *Trifolium repens* and *Themeda australis* as C_3 grasses.

Data set 1 alone:

In this data set, using n-alkanes significantly differentiated the defined dietary categories along DS1 ($P = 0.01$) but not DS2, although separation approached significance in this axis ($P = 0.054$). *Lotus* spp. were significantly differentiated from grasses ($P = 0.002$) and clovers ($P = 0.003$) on DS1, but there was no significant difference between grasses and clovers on this axis (Fig. 5-a). The jackknife cross-validation procedures indicated that, based on alkane patterns alone, white clover was misclassified as belonging to the *Lotus* group (Fig. 5-a).

When n-alkanes were used in conjunction with LCOH, all defined groups were clearly separated with highly significantly differences along both DS1 ($P < 0.0001$) and DS2 ($P < 0.0001$) (Fig. 5-b). All plant species were correctly classified by cross-validation procedures.

Discussion

Plant wax n-alkanes have been used successfully for estimating the composition of the herbivore diet in a number of studies (Salt et al. 1994, Fraser and Gordon 1997, Dove et al. 1999, Duncan et al. 1999), but most of these were conducted in field or controlled experiments in which the animals had a limited number of plant species from which to choose. A higher number of markers will be required if there are many plant species to be differentiated in the

Table 3. Percentage of the variance in the pattern of cuticular wax marker concentration explained by the first 3 principal component scores (PC1 to PC3), following Principal Component Analyses based either on n-alkanes or on long-chain fatty alcohols (LCOH), together with the residual variance left after comparison of the 2 sets of principal component scores by Orthogonal Procrustes Rotation (OPR). Analyses were conducted for each of data sets 1 to 3 and for all possible combinations of data sets.

Data set	Wax marker	% Variance explained by:			Residual variation remaining after OPR
		PC1	PC2	PC3	
(1+2+3)	Alkanes	58.7	21.5	5.9	
	LCOH	70.0	19.6	8.2	52.0
(1+2)	Alkanes	51.1	22.9	11.6	
	LCOH	41.2	20.7	13.2	65.9
(1+3)	Alkanes	68.6	14.6	5.6	
	LCOH	79.4	10.5	8.2	48.2
(2+3)	Alkanes	57.4	24.1	7.9	
	LCOH	69.0	25.0	5.0	64.0
1	Alkanes	51.9	23.2	13.8	
	LCOH	40.3	27.6	17.4	64.0
2	Alkanes	59.0	27.7	5.9	
	LCOH	49.3	22.5	15.4	74.7
3	Alkanes	56.0	26.4	9.0	
	LCOH	57.9	31.8	8.8	81.7

diet. This is frequently the case in studies dealing with free-grazing or wild herbivores feeding on rangelands or more complex plant communities. Theoretically, if the number of components to be differentiated in the diet can be reduced, then a lower number of markers will also be required (Mayes and Dove 2000). Thus, by pooling species into a small number of dietary categories (e.g. grasses, legumes, browse) the number of required markers will be reduced. Such categories, however, must themselves be distinctly characterized by a set of markers. For instance, Dove and Mayes (1991) suggested that legumes and grasses could be differentiated using the C29 and C31 alkanes and, more recently, n-alkanes were successfully used to differentiate the broad categories of browse and grasses in the diet of herbivores (e.g. Pérez-Barbería et al. 1997, Bugalho et al. 2001).

The possibility of differentiating either single plant species or groups of plants in the diet increases if additional markers are available, especially if the additional markers provide different or complementary information about the plant species concerned. This possible differentiation of a wider range of plant species in the diet of the grazing animal requires 2 things in sequence:

(a) the demonstration that, using a marker or markers in the plant wax, individual plant species or groups of species can be distinguished as such,

(b) the demonstration that, after correcting for any loss of marker during transit through the gut of the grazing animal ('fecal recovery'), the fecal marker patterns can be resolved into the intake of individual plant species, using the marker patterns of the plant species.

The present study concentrated on the first of these issues, that is, the potential

Table 4. Contribution of plant species to the variance in estimated concentrations of long-chain alkanes and long-chain fatty alcohols (LCOH) in plant cuticular wax.

Source	Plant species type	Alkane/LCOH range	%variance in alkane concentration due to species
Alkanes			
Herbin and Robins (1968) (recalculated)	*Agave* spp	C26 to C33	81 ± 9.76 (all alkanes) 94.9 ± 2.68 (odd-chain alkanes only)
Laredo et al. (1991) (recalculated)	Tropical pasture species	C27 to C35	80.7 ± 5.50 (all alkanes)
Dove and Mayes (1996) (recalculated)	Temperate pasture species	C27 to C35	81.6 ± 3.28 (all alkanes)
Dove et al. (1996)	Temperate pasture species	C25 to C35	85.7 ± 3.44 (all alkanes)
Chen et al. (1998)	Temperate pasture species	C25 to C35	87.0 to 93.0 (odd-chain alkanes only)
H.Dove and T.P Bolger (unpublished)	Temperate pasture species	C25 to C33	87.3 ± 4.11 (all alkanes) 95.5 ± 1.01 (odd-chain alkanes only)
LCOH			
R.W Mayes et al. (unpublished)	*Lolium perenne, Trifolium repens, Calluna vulgaris, Vaccinium myrtillus, Betula pendula, Juncus effusus*	C20OH to C34OH (even-chain only)	62.0± 6.03 67.1 ± 6.16 (C20OH to C30OH only) (Plant part = < 0.1 to 21.5% of variance)
H.Dove and T.P.Bolger (unpublished)	*Temperate pasture species*	C20OH to C30OH (even-chain only)	82.4 ± 7.46

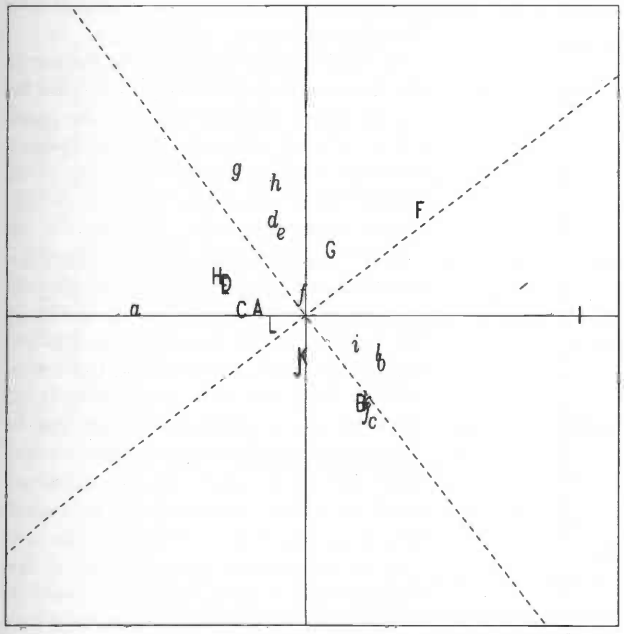


Fig. 1. Graphical representation of the results of Orthogonal Procrustes Rotation (OPR) based on the cuticular wax marker content of the plant species in data set 1. Upper case letters and solid axes refer to the n-alkanes in the species as listed below, while lower case italic letters and dotted axes refer to long-chain fatty alcohols (LCOH). The residual variance after OPR on the first 3 Principal Component scores following Principal Components Analysis on the alkane and LCOH data is given in Table 4.

A, *a* *Bromus catharticus*
B, *b* *Trifolium glomeratum*
C, *c* *Austrodanthonia richardsonii*
D, *d* *Lotus corniculatus cv.* **Goldie**
E, *e* *L. corniculatus* **'Prostrate'**
F, *f* *L. pedunculatus* **cv. Sharnae**
G, *g* *L. pedunculatus* **cv. Maku**
H, *h* *Phalaris aquatica*
I, *i* *T. striatum*
J, *j* *Festuca arundinacea*
K, *k* *Vulpia myuros*
L, *l* *T. repens*

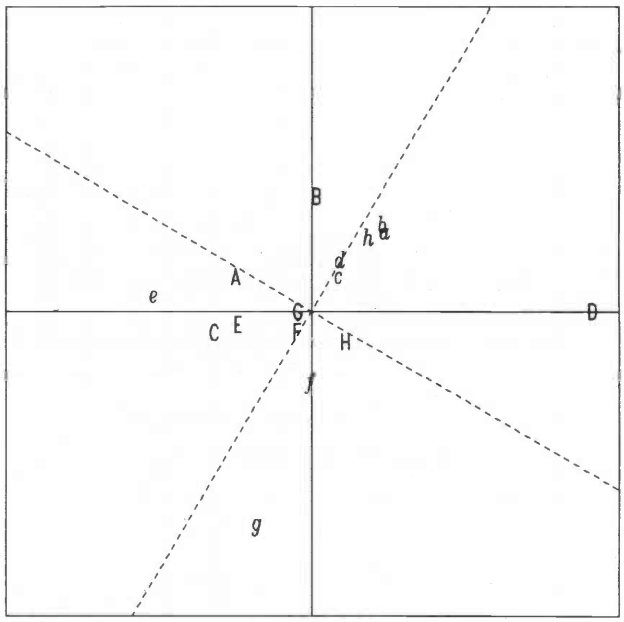


Fig. 2. Graphical representation of the results of Orthogonal Procrustes Rotation (OPR) based on the cuticular wax marker content of the plant species in data set 2. Upper case letters and solid axes refer to the n-alkanes in the species as listed below, while lower case italic letters and dotted axes refer to long-chain fatty alcohols (LCOH). The residual variance after OPR on the first 3 Principal Component scores following Principal Components Analysis on the alkane and LCOH data is given in Table 4.

A, *a* *Bothriochloa macra*
B, *b* *Chloris gayana*
C, *c* *Austrodanthonia pilosa*
D, *d* *Austrodanthonia racemosa*
E, *e* *Elymus scaber*
F, f *Microlaena stipoides 1*
G, *g* *Microlaena stipoides 2*
H, *h* *Themeda australis*

for differentiation of the species themselves, if more markers are available. In our 3 data sets, the Orthogonal Procrustes Rotation (OPR) results indicated that each class of marker (n-alkanes, LCOH) contributed different discriminatory information. This result is particularly important for it suggests that the combination of the 2 types of marker can, on theoretical grounds, permit discrimination between a greater number of plant species.

Although the results of our Principal Component Analyses (PCA) and OPR have demonstrated that n-alkanes and LCOH provide different information, they do not in themselves quantify the between-species differences in the patterns of concentrations of these markers, since our results were obtained with bulked samples of the species. However, other published reports and unpublished data provide strong evidence that the differentiation evident in Table 3 and Figs 1 to 3 do indeed represent between-species variance. In the data collated and summarised in Table 4, it is clear that in a series of published reports and 1 recent unpublished study, 80 to 95% of the variance observed in cuticular n-alkane concentrations is attributable to the variance between species. There are no equivalent published data for the LCOH, but in data obtained by 2 of the authors (unpublished data, R.W. Mayes and H. Dove), approximately 65 to 80% of the observed variance in LCOH concentrations could be attributable to species. We therefore suggest that the data presented in Table 3 and Figs 1 to 3 of this report do represent between-species differences in marker concentration.

Our results thus suggest that the possibility of differentiating a particular plant species or groups of species can be increased if long-chain fatty alcohols (LCOH) are used as additional markers. Several important issues, however, must be addressed before using more than one class of marker for estimating the composition of the diet of herbivores.

First, if there are large differences in concentration either within or between different classes of marker, such as those obvious in Tables 1 and 2, it is possible that those markers with the highest con-

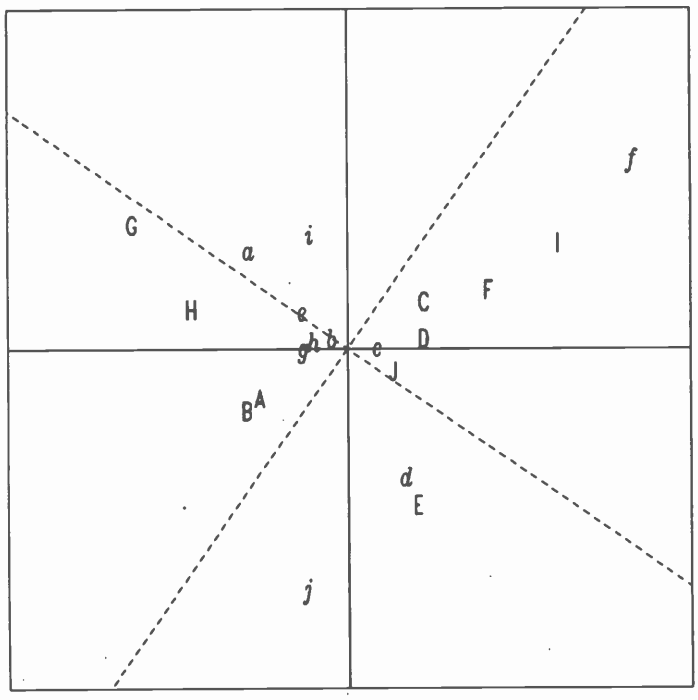


Fig. 3. Graphical representation of the results of Orthogonal Procrustes Rotation (OPR) based on the cuticular wax marker content of the plant species in data set 3. Upper case letters and solid axes refer to the n-alkanes in the species as listed below, while lower case italic letters and dotted axes refer to long-chain fatty alcohols (LCOH). The residual variance after OPR on the first 3 Principal Component scores following Principal Components Analysis on the alkane and LCOH data is given in Table 4.

A, *a* *Axonopus fissifolius*
B, *b* *Chloris gayana*
C, c *Cynodon dactylon*
D, *d* *Digitaria didactyla*
E, *e* *Imperata cylindrica*
F, *f* *Paspalum dilatatum*
G, *g* *Paspalum notatum*
H, *h* *Pennisetum clandestinum*
I, *i* *Setaria anceps*
J, j *Sporobolus indicus v. major*

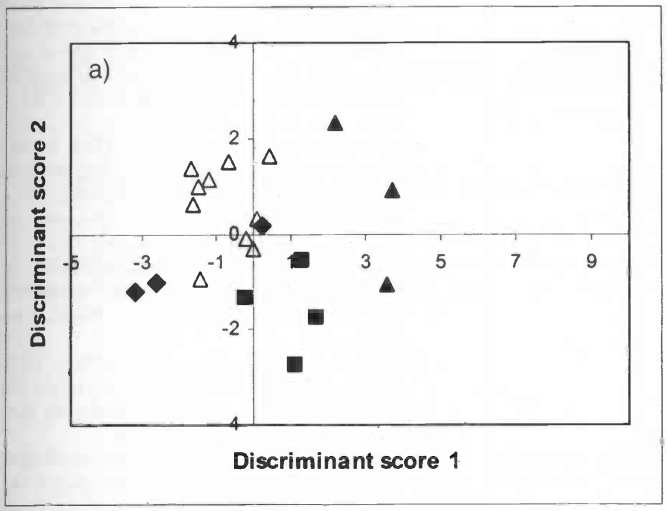
centrations will exert undue influence when the markers are used to estimate diet composition. In such cases, particularly if minimization algorithms (Dove and Moore 1995) are used, some form of data scaling or weighting may be required before concentration data are entered into the algorithm. Further work is needed to identify the best scaling procedures to use, in relation to the error structures inherent in the concentration data for the different markers and the nature of the algorithm used to estimate diet composition.

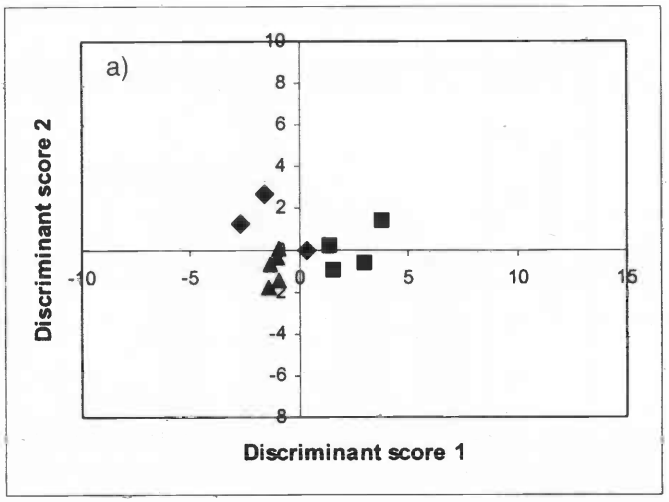
Second, in moving from the differentiation of the plant species themselves to the estimation of the diet composition of the grazing animal, fecal marker concentrations must be adjusted for the possible loss of marker during gut transit. In the case of n-alkanes, it is now well established that fecal recovery of consumed n-alkane is not complete, but increases in curvilinear fashion with increasing carbon-chain length (see Dove and Mayes 1996, Dove and Oliván 1998). For alkane C35 and above, fecal recovery approaches 100%. Data are therefore available for use in correcting fecal n-alkane concentrations for incomplete recovery. The possible use of LCOH as diet composition markers is a very recent development and there are no published estimates of fecal LCOH recovery. However, recent data obtained with sheep fed mixed diets (Hafiz Ali, R.W. Mayes and E.R. Ørskov, unpublished data) indicate that, as with the n-alkanes, the fecal recovery of LCOH is incomplete but increases with carbon-chain length to be essentially complete for 1-C30-OL. By contrast with the n-alkanes, the response appears linear rather than curvilinear. To realize the potential of LCOH as diet composition markers, there is an urgent need for more such data.

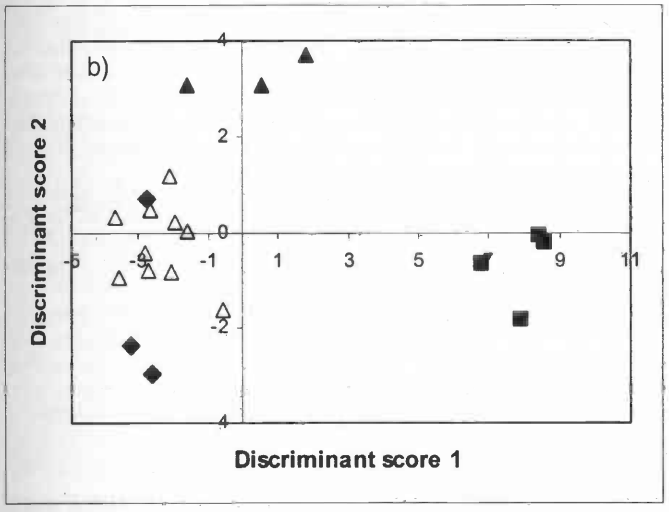
A further point relates to the number of markers used in the analysis. Whilst the use of more markers increases the possibility of differentiating plant species (Mayes and Dove 2000), using more markers does not always imply better diet composition estimates. This may be the case when using markers that are not distinct for species or groups of plants and, consequently, do not correlate well with the differentiation between possible diet components (see, for example, Dove et al. 1999). To overcome this difficulty, a reduced set of effective markers can be selected by employing multivariate statistical methods such as Discriminant Analysis (DA). Selecting markers using DA, however, requires that replicate samples of the species be available. If diet composition is to be estimated based on individual plant species, then replicate samples of the marker content of each individual species will be required. If diet composition is to be estimated based on defined groups of species, then a number of plant species must be available to function as 'replicates' within each group.

Multivariate statistical tools such as DA are a promising approach for classifying dietary groups. It should be noted that 'statistically meaningful' groups (i.e., formed by plant species similar in their marker content) may not always correspond to taxonomically or nutritionally relevant groups, though this is more likely if just 1 class of marker is used. For instance, when using n-alkanes alone, white clover was grouped with *Lotus* spp.. Conversely, if diet composition is to be estimated based on defined dietary categories, nutritionally meaningful groups should only be composed of species statistically similar in their marker content. If that condition is not met, then the herbivore's potential feeding selectivity within the dietary group may influence the estimate of diet composition. For instance, this will occur if the herbivore feeds selectively on a particular species in which the pattern of marker concentration deviates substantially from the remaining species within the dietary group (Bugalho et al. 2002). In either case, using both LCOH and n-alkanes will increase the possibility of defining groups of plants that are relevant both nutritionally and statistically.

In a recent report, Kelman et al. (2003) used some of the plant wax marker concentrations in data set 1, together with equivalent fecal marker concentrations (adjusted for incomplete fecal recovery),







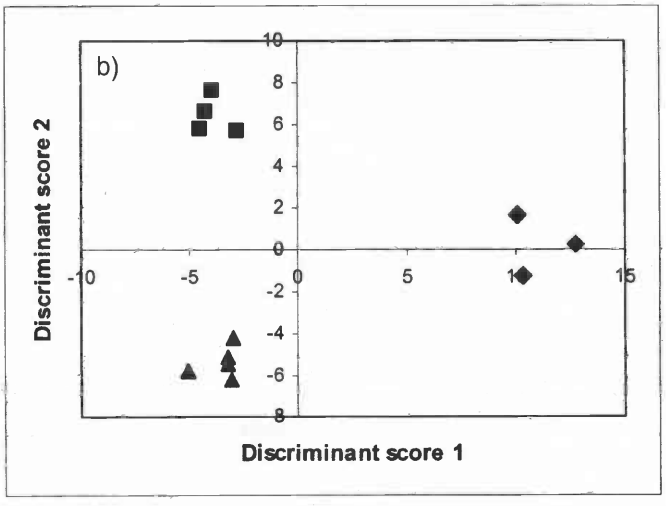


Fig. 4. Discriminant analysis based on the defined categories formed from the plant species in the combined data set (1+2), when using a) n-alkanes alone (variation explained: discriminant score (DS)1 = 68.9%, DS2 = 23.2% or b) n-alkanes and long-chain fatty alcohols (variation explained: DS1 = 87.1%, DS2 = 10.9%). Diamonds represent clovers, squares Lotus spp., open triangles C3 grasses and closed triangles C4 grasses.

Fig. 5. Discriminant analysis based on the defined categories formed from the plant species in data set 1, when using a) n-alkanes alone (variation explained: DS1 = 81.0%, DS2 = 19% or b) n-alkanes and long-chain fatty alcohols (variation explained: DS1 = 61.4%, DS2 = 38.6%). Diamonds represent clovers, triangles grasses and squares Lotus spp.; in Fig. 5a, the diamond closest to the squares represents white clover.

to estimate the species composition of the diet of sheep grazing the sward from which the samples in data set 1 were obtained. When based on n-alkane concentrations alone, estimated diet composition indicated a higher proportion of clover in the consumed diet than in the sward (Fig.6). However, this result was possibly compromised by the fact that, based on n-alkanes alone, white clover was often misclassified as a *Lotus*, in a manner similar to the misclassification described above, for the differentiation of the plant species themselves (Fig. 5-a). Diet compositions were therefore re-estimated using n-alkanes and LCOH. This confirmed that dietary clover content was much higher than in the sward (Fig. 6), and also indicated that over 90% of the *Lotus* consumed was *L. corniculatus*, the species with lower condensed tannin content. The authors considered these to be reliable estimates of diet composition, because all herbage species were statistically well discriminated by the combination of n-alkanes and LCOH.

Conclusions

Long-chain fatty alcohols (LCOH) in plant cuticular wax, used together with n-alkanes, allowed a better discrimination between plant species or groups of species than using n-alkanes alone, which shows the potential of using both classes of compounds for estimating the species composition of the diet of herbivores. The LCOH are widespread in plant cuticles and can be extracted and quantified by an extension of the procedure used for n-alkanes. Using such markers increases the possibility of

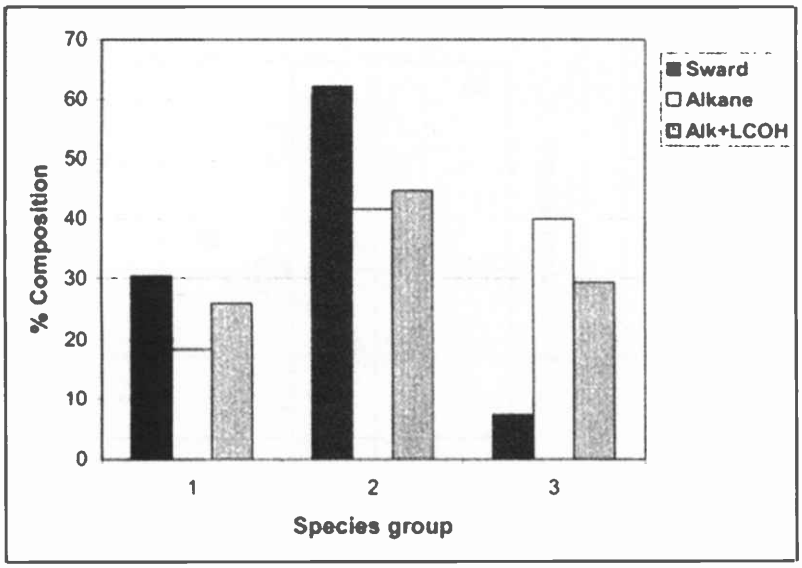


Fig. 6. Comparison of the content of the defined species groups (data set 1) in the sward (solid bars), with the species composition of the diet of sheep grazing the sward, estimated either from herbage and fecal n-alkane concentrations (open bars), or these plus herbage and fecal long-chain fatty alcohol (LCOH) concentrations (stippled bars). Species group 1 = Lotus; species group 2 = grasses; species group 3 = clovers. Re-calculated from tabulated data in Kelman et al. (2003).

differentiating species or groups of species in an herbivore's diet, because they contribute discriminatory information additional to the n-alkanes. The combination of n-alkanes and LCOH was recently used successfully to differentiate grass, clover and *Lotus* spp. in the diet of grazing sheep (Kelman et al. 2003). Although further validation work is needed, particularly in relation to the estimation of rates of recovery of LCOH in feces, LCOH appear to have great potential as additional diet composition markers in studies of herbivore nutrition. Plant wax alkanes and alcohols may complement or be advantageously used with other techniques for estimating diet composition of herbivores (Holecheck et al. 1982).

Literature Cited

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

Bugalho, M.N., J.A. Milne, and P.A. Racey. 2001. The foraging ecology of red deer (*Cervus elaphus*) in a Mediterranean environment: is a larger body size advantageous? J. Zool. (London). 255:285–289.

Chen, W., R.D.B. Lefroy, J.M. Scott, and G.J. Blair. 1998. Field variations in n-alkane signatures among plant species in "degraded" and perennial pastures on the Northern Tablelands of New South Wales. Aust. J. Agr. Res. 49:263-268.

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. Aust. J. Agric. Res. 42:913-952.

Dove, H. and R.W. Mayes. 1996. Plant wax components: A new approach to estimating intake and diet composition in herbivores. J. Nutr. 126:13-26.

Dove, H. and R.W. Mayes. 1999. Developments in the use of plant wax markers for estimating diet selection in herbivores. *In*: H. Dove and S.W. Coleman (Eds.) Emerging Techniques for Studying the Nutritional Status of Free-ranging Herbivores. Satellite Meeting of the Vth International Symposium on the Nutrition of Herbivores, San Antonio, Texas (CD-ROM).

Dove, H. and A.D. Moore. 1995. Using a least-squares optimization procedure to estimate botanical composition based on the n-alkanes of plant cuticular wax. Aust. J. Agric. Res. 46:1535–1544.

Dove, H. and M. Oliván. 1998. Using synthetic or beeswax alkanes for estimating supplement intake in sheep. Anim. Prod. Aust. 22: 189–192.

Dove, H., R.W. Mayes and M. Freer. 1996. Effects of species, plant part and age on the n-alkane concentrations in the cuticular wax of pasture plants. Aust. J. Agr. Res. 47: 1333-1347.

Dove, H., J.T. Wood, R.J. Simpson, B.J. Leury, K.L. Gatford, C. Siever-Kelly and T.A. Ciavarella. 1999. Spray topping annual grass pasture with glyphosate to delay loss of feeding value during summer. III. Quantitative basis of the alkane-based procedures for estimating diet selection and herbage intake by grazing sheep. Aust. J. Agr. Res. 50:475–485.

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 composition and intake in sheep. J. Agr. Sci. 132:233–246.

Fraser, M. D. and I.J. Gordon. 1997. Organic matter intake, diet digestibility and feeding behaviour of goats, red deer and South American camelids feeding on three contrasting Scottish upland vegetation communities. J. Appl. Ecol. 34:677–698.

Herbin, G.A. and P.A. Robins. 1968. Studies on plant cuticular waxes – II. Alkanes from members of the genus *Agave* (Agavaceae), the genera *Kalanchoe*, *Echeveria*, *Crassula* and *Sedum* (Crassulaceae) and the genus *Eucalyptus* (Myrtaceae) with an examination of Hutchinson's sub-division of the Angiosperms into Herbaceae and Lignosae. Phytochem. 7:257–268.

Hoebee, S.E., H. Dove, and D.I. Officer. 1998. Using plant wax alkanes to estimate the species composition of sub-tropical grass mixtures. Anim. Prod. Aust. 22: 364.

Holechek, J.L., M. Vavra, and R.D. Pieper. 1982. Botanical composition determination of range herbivore diets: A review. J. Range Manage. 35:309–315.

Kelman, W., M. Bugalho, and H. Dove. 2003. Cuticular wax alkanes and alcohols used as markers to estimate diet composition of sheep (*Ovis aries*). Biochem. Syst. Ecol. 31: 919–927.

Kolattukudy, P.E. 1976. Introduction to natural waxes, p. 1-15 *In*: P.E. Kolattukudy (Ed.) Chemistry and Biochemistry of Natural Waxes. Elsevier. Amsterdam.

Laredo, M.A., G.D. Simpson, D.J. Minson and C.G. Orpin. 1991. The potential for using n-alkanes in tropical forages as a marker for the determination of dry-matter intake by grazing ruminants. J. Agr. Res. 177:355–361

Mayes, R.W. and H. Dove. 2000. Measurement of dietary nutrient intake in free-ranging mammalian herbivores. Nutr. Res. Rev. 13:107–138.

Mayes, R.W., C.S. Lamb, and P.M. Colgrove. 1986. The use of dosed and herbage n-alkanes as markers for the determination of herbage intake. J. Agr. Sci. 107: 161–170.

Norton, M.R., D. Garden, C. Waters, R.D.B. Whalley, D. Friend, M. Mitchell, E. Kobelt, G. Auricht, and P. Sanford. 2001. On-farm native and low input grasses network; multi-site testing. Final Report on Project TR045, Meat and Livestock Australia. Australia.

Pérez-Barbería, F.J., M. Oliván, K. Osoro, and C. Nores. 1997. Sex, seasonal and spatial differences in the diet of Cantabrian chamois *Rupicapra pyrenaica parva*. Acta Ther. 42:37–46.

Salt, C.A., R.W. Mayes, and D.A. Elston. 1992. Effects of season, grazing intensity and diet composition on the radiocaesium intake by sheep on re-seeded hill pasture. J. Appl. Ecol. 29:378–387.

Salt, C.A., R.W. Mayes, P.M. Colgrove, and C.S. Lamb. 1994. The effects of season and diet composition on the radiocaesium intake by sheep grazing on heather moorland. J. Appl. Ecol. 31:125–136.

Sandberg, R.E., D.C. Adams, T.J. Klopfenstein, and R.J. Grant. 2000. N-alkane as an internal marker for predicting digestibility of forages. J. Range Manage. 53:159–163

Zar, J.H. 1996. Biostatistical Analysis. Prentice Hall International, Inc. New Jersey.