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Evaluation of n-alkanes and their carbon isotope enrichments ($\delta^{13}C$) as diet composition markers

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Plant cuticular n-alkanes have been successfully used as markers to estimate diet composition and intake of grazing herbivores. However, additional markers may be required under grazing conditions in botanically diverse vegetation. This study was conducted to describe the n-alkane profiles and the carbon isotope enrichment of n-alkanes of common plant species from the Mid Rift Valley rangelands of Ethiopia, and evaluate their potential use as nutritional markers. A total of 23 plant species were collected and analysed for long-chain n-alkanes ranging from heptacosane to hexatriacontane (C_{27} to C_{36}), as well as their carbon isotopic ratio ($^{13}C/^{12}C$). The analysis was conducted by gas chromatography/combustion isotope ratio mass spectrometry following saponification, extraction and purification. The isotopic composition of the n-alkanes is reported in the delta notation ($\delta^{13}C$) relative to the Vienna Pee Dee Belemnite standard. The dominant n-alkanes in the species were C_{31} (mean $\pm$ s.d., 283 $\pm$ 246 mg/kg dry matter) and C_{33} (149 $\pm$ 98 mg/kg dry matter). The carbon isotopic enrichment of the n-alkanes ranged from -19.37‰ to -37.40‰. Principal component analysis was used to examine interspecies differences based on n-alkane profiles and the carbon isotopic enrichments of individual n-alkanes. Large variability among the pasture species was observed. The first three principal components explained most of the interspecies variances. Comparison of the principal component scores using orthogonal procrustes rotation indicated that about 0.84 of the interspecies variances explained by the two types of data sets were independent of each other, suggesting that the use of a combination of the two markers can improve diet composition estimations. It was concluded that, while the n-alkane profile of the pasture species remains a useful marker for use in the study region, the $\delta^{13}C$ values of n-alkanes can provide additional information in discriminating diet components of grazing animals.

Keywords: n-alkanes, carbon isotope, marker, diet composition, feed intake

Implications

Ruminant livestock production in tropical regions is mainly based on grazing on natural grasslands. Sustainable production in such areas requires that the nutritional status of animals is adequately monitored, thus providing an objective basis for various management decisions such as optimum allocation of forage to different types of animals, selection of animals compatible with the forage resource, selection of species for reseeding deteriorated ranges, as well as designing appropriate supplementation strategies. The importance of the present findings is that n-alkanes and their carbon isotopic composition can be used as markers for the indirect estimation of feed intake, diet composition and nutrient digestibility of grazing animals.

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Introduction

In extensive agro-pastoral systems rangelands are the main sources of nutrition for domestic and wild herbivores. In Ethiopia, about 62% of the total land mass is classified as arid and semi-arid and mainly used for livestock production based on grazing (Kassahun *et al*., 2008). Proper management of grazing animals is important to maintain sustainable range resource utilisation in such areas (Bailey *et al*., 1998).

Naturally, free-ranging herbivores grazing on diversified plant communities exert different levels of selection to optimise their nutrient intake (Prache *et al*., 1998). Understanding the type of plant species selected by the animal and the contribution of each species to the total intake could give an insight into the nutritional status of the animal and offer a feasible range management strategy to optimise resource utilisation (Dumont *et al*., 2002). However, measurement of feed intake, diet composition and nutrient digestibility in free-ranging animals remains a challenge in nutritional studies

because of the inherent errors associated with the methods that are used at present (Dove and Mayes, 1991; Mayes and Dove, 2000).

The use of plant wax components, mainly n-alkanes, as markers for the estimation of intake and diet composition of herbivores has evolved in the last two decades (Dove and Mayes, 2005; Ferreira *et al.*, 2007). N-alkane profiles of plants show distinct differences between species and, to some extent, between plant parts of the same species. In addition, they have high recovery rates in the faeces of herbivores (Ferreira *et al.*, 2009), offering an opportunity to reconstitute the diet of the herbivore from the faecal patterns of these compounds (Dove and Mayes, 1996; Bugalho *et al.*, 2004). Validation experiments revealed the potential of using n-alkanes to estimate intake, diet composition and nutrient digestibility of individual animals (Monks *et al.*, 2005; Oliván *et al.*, 2007a).

Although validation experiments using n-alkanes produced good estimates with less complex vegetations, grouping of species or use of other markers in addition to n-alkanes was necessary for the correct estimation of diet composition of herbivores grazing botanically diverse vegetation (Oliván *et al.*, 2007b). Stable carbon isotopic (^{13}C) composition of plants has been used to estimate the proportion of C_3 and C_4 plants in the diet of herbivores (Bennett *et al.*, 1999). Garcia *et al.* (2000) reported that the use of a combination of the n-alkanes and ^{13}C composition of the organic matter of feeds could increase the accuracy of estimation of diet compositions in cows. However, so far, the ^{13}C of n-alkanes has not been evaluated as an additional marker together with the alkane profiles themselves. Currently, the possibility of separating organic compounds of interest prior to isotope ratio analysis using gas chromatography (GC)–combustion isotope ratio mass spectrometry provides an opportunity to consider the ^{13}C of n-alkanes rather than the whole organic matter. The latter would improve the reliability of isotopic techniques, as plant compounds that are stable both in herbage and in faeces can be specifically targeted for isotope analysis.

There is little information about the plant wax profiles of native pasture species in Ethiopia for application in nutritional assessments of grazing animals. The aims of this study were: (1) to describe the n-alkane profiles of pasture species commonly available in the Mid Rift Valley rangelands of Ethiopia, (2) to determine the stable carbon isotope enrichment of individual n-alkanes for each pasture species and (3) to evaluate the potential for using the two markers to estimate the diet composition of free-ranging herbivores.

Material and methods

Description of study site

The research area lies in the Mid Rift Valley region of Ethiopia extending from 7°30′N to 8°00′N and from 38°35′E to 38°45′E. The area is classified as semi-arid with an annual rainfall ranging from 500 to 700 mm/annum (Ministry of Agriculture (MoA), 2000). The rainfall pattern is bimodal with short rains from March to May, followed by the main wet season from July to October. The mean annual minimum and maximum daily temperature ranges between 11.4°C and 26°C. The grazing lands exhibit typical savannah woodland vegetation with a scattered population of acacia trees and broadleaved shrubs. Cattle are the dominant livestock in the area followed by goats. Natural pasture is the main source of feed, supplemented by agricultural crop residues (Central Statistical Agency (CSA), 2007).

Plant sampling and processing

A total of 23 commonly available pasture species were collected from the study area during the months of July and August, 2008. For the collection of samples, several transect walks covering 15 km of length were conducted across both enclosed and communal grazing lands. Whole-plant pasture species were sampled from various locations along the transects by cutting at a height of 5 cm from the ground. The sampling was done at the time of the flowering stage for all the species. After harvesting, the biomass sample of the individual species was coded and stored in a pollen bag, while a specimen for each species was placed in a plant press for species confirmation in a herbarium. On average, a species was sampled from about 20 sites. Biomass samples of the same species collected from different sites were pooled to a sample before drying. The samples were dried in a forced air oven at 60°C for 48 h. The dried samples were ground to pass through a 1 mm sieve size (Thomas Wiley Lab mill, Model 4, Philadelphia, USA), and afterwards pulverised using a bullet mill (MM 2000; 4 min at 80 Hz; Retsch Technology GmbH, Haan, Germany) before analysis of n-alkane concentrations and ^{13}C enrichment of alkanes.

Chemical analysis

The chemical analysis was conducted at the laboratory of the Animal Nutrition Group of Wageningen University, the Netherlands. N-alkane extraction and analysis were carried out as described by Mayes *et al.* (1986), with modifications by Salt *et al.* (1992) and tetratriacontane (C_{34}) was used as an internal standard. The extracted samples were analysed for n-alkanes (C_{27} to C_{36}) using a GC (Carlo Erba HRGC Mega 2 series, CE Instruments, Milan, Italy) fitted to a flame-ionising detector, using helium as the carrier gas. The column was a 40 m $\times$ 0.32 mm (i.d) fused silica capillary (SPB-1) with 0.10 μm film thickness. A split-type injector was used, with a split ratio of 1 : 10. The temperature for both the detector and injector was 340°C (temperature program: 1 min at 210°C, increase at 7.2°C/min to a temperature of 300°C, 6 min at 300°C). Chrom Card Data System 2.2 (Thermo Finnigan, Waltham, MA, USA) software was used to calculate peak areas. The data were transferred to an Excel spread sheet to calculate alkane concentrations according to the following formula:

$$\text{Alkane}_i\,(\text{mg/kg DM}) = [10 \times \text{area}\,\%\,\text{alkane}_i \times \text{IS wt (mg)}]/\text{SDW} \times \text{SRF}_i$$

where IS wt is the weight of the internal standard, SDW is sample dry weight and SRF_i is the standard response factor for

alkane$_i$, calculated as area % alkane$_i$ in the mixed standard divided by weight % alkane$_i$ in the mixed standard.

Using the same alkane extracts, the carbon isotope composition of the alkanes was determined by fitting a GC (Finnigan_MAT, TraceGC Ultra, Milan, Italy) with a split/splitless injector operated in split mode (split ratio 1 : 10), to a combustion interface (Finnigan_MAT Combustion interface III, Bremen, Germany), which was connected to an elemental analyser isotope ratio mass spectrometer (Finnigan_MAT CN, Bremen, Germany). Full-base line separation of all individual alkanes was achieved by fitting the TraceGC with a capillary column as described earlier and using helium as carrier gas. The temperature setting of the column was identical to that described earlier. The isotope ratio of the alkanes was calculated in terms of conventional delta values ($\delta^{13}C$) as follows:

$$\delta^{13}C = 1000\,(R_{sample} - R_{standard})/R_{standard}$$

where, R_{sample} is the abundance ratio of ^{13}C to ^{12}C in the plant sample, and $R_{standard}$ is the abundance ratio of ^{13}C to ^{12}C in the standard sample (Vienna Pee Dee Belemnite, PDB).

Data analysis

Principal component analysis (PCA) was used to explore the pattern of n-alkane profiles and ^{13}C enrichments of alkanes across the species. The correlation matrix was used for the calculation, after the data was mean-centred and standardised. The first two principal components (PC1 and PC2) were plotted graphically where points on the graph represent plant species. The distance between species in the scatter plots is an indication of the difference in marker profile between the species. The species that are positioned close together in the scatter plots are the ones with a similar marker profile. On the other hand, the species that are placed far apart are expected to have large differences in their marker profiles.

The principal components for the two groups of markers were compared by orthogonal procrustes rotation (OPR) to assess the similarity between the two data sets in describing the species identities. The PCA coordinates based on n-alkanes were used as fixed values, and those based on ^{13}C were used as fitted values. The OPR procedure rotates the fitted PCA axes to match the fixed axes, minimising the residual sum of squares between the two PCA configurations. The magnitude of unexplained residual variance after OPR indicates the extent to which the two PCA configurations differ from one another. The two data sets were again examined together by employing redundancy analysis (RDA), in which the PCA based on the alkane profile was constrained by ^{13}C enrichments of n-alkanes and then the dispersion of species was presented in a two-dimensional space. Data were analysed using GenStat for Windows (11th edition).

Results

Alkane concentrations

The n-alkane concentrations (C_{27} to C_{35}) in the pasture species collected from the grazing lands are shown in Table 1. The odd-chain alkanes were found in much higher concentrations than the even-chain alkanes. The even-chain alkane C_{36} was excluded from the results as the values for most of the species were within the range of the analytical error of the GC. In most species, C_{31} was the most abundant odd-chain alkane, ranging from 33 mg/kg dry matter (DM) in *Eragrostis aspera* to 1265 mg/kg DM in *Zaleya pentandra* with a mean concentration of 283 ± 246 mg/kg (mean $\pm$ s.d.) DM across the species. This was followed by C_{33}, which ranged from 13 mg/kg DM in *Pennisetum polysachion* to 363 mg/kg DM in *Rhynchelytrum repens* with a mean concentration of 149 ± 98 mg/kg DM for all the species. While alkane C_{35} was abundant in some species (e.g. *Cenchrus ciliaris*, *Cynodon dactaylon*), it was found in very low concentration in *Indigofera spicata, Cymbopogon pospischilii* and *Pennisetum polystachion*. The sum of C_{27} to C_{35} concentrations showed large between-species variation, ranging from 169 mg/kg DM in *E. aspera* to 1398 mg/kg DM in *Z. pentandra*.

Carbon stable isotope composition of alkanes

The stable isotope enrichment of carbon ($\delta^{13}C$) for individual n-alkanes (Table 2) showed a wide variation, ranging from -19.37‰ (*Digitaria abyssinica*) to -37.40‰ (*I. spicata*). The lowest level of enrichment was observed in *I. spicata*, which was the only legume species in the collected samples, followed by that of *Z. pentandra* (a non-legume forb). The other grass species showed higher levels of enrichment, but differences could be observed between species. Regarding the delta values of individual alkanes, the odd-chain alkanes had a higher level of ^{13}C enrichment by at least one delta unit than the subsequent even-chain alkanes (Table 2). The level of enrichment tended to decrease, in both even- and odd-chain alkanes with increasing carbon number.

Principal component and RDA

The results of the PCA are shown in Table 3, and Figures 1 and 2. When the PCA was based on n-alkane concentrations, about 91% of the variance between species was explained by the first three principal components (PC1 to PC3). Similarly, when the analysis was based on $\delta^{13}C$ values of n-alkanes, 74% of the variance was explained by the first three principal components (Table 3).

The scatter plot based on n-alkanes (Figure 1) shows good species separation. For example, *Brachiaria marlothi*, *I. spicata*, *Z. pentandra* and *E. aspera* scattered widely from the rest of the species. *Brachiaria lachnantha, Heteropogon contortus, P. polystachion, C. pospischilii, C. ciliaris* and *C. dactylon* were also separated along the two principal axes. On the other hand, clustering between some of the species, like *Chloris gayana* and *Cynodon ethiopicus* was observed. The scatter plot based on the $\delta^{13}C$ values of n-alkanes (Figure 2) showed that the species were scattered along the two axes in a different way. On one hand, those species that clustered closer when the analysis was based on n-alkanes (Figure 1), showed wider separation when the analysis was based on $\delta^{13}C$ values of n-alkanes (Figure 2). On the other

Table 1 *n-Alkane concentration for pasture species collected from the Mid Rift Valley rangelands of Ethiopia*

	n-Alkane concentration (mg/kg dry matter)								
Species	C27	C28	C29	C30	C31	C32	C33	C35	Total
Cynodon dactaylon	64	11	67	13	153	13	186	198	705
Pennisetum straminium	40	7	130	15	596	9	126	64	987
Cenchrus ciliaris	39	8	88	13	391	14	282	210	1043
Cymbopogon pospischilii	86	16	132	8	158	2	40	9	451
Indigofera spicata	23	24	76	7	202	6	20	6	363
Heteropogon contorus	68	5	46	10	266	11	238	98	742
Zaleya pentandra	14	33	35	8	1265	6	29	10	1398
Chloris gayana	116	18	125	11	318	11	258	165	1022
Eragrostis aspera	37	19	38	5	33	2	17	18	169
Eragrostis cilianensis	58	6	75	9	185	14	192	75	613
Cynodon ethiopicus	58	17	103	7	196	6	190	132	709
Eleusine mutiflora	59	11	75	9	186	12	170	51	574
Brachiaria lachnantha	30	17	45	6	129	11	171	158	569
Aristida odscensionis	31	8	81	7	225	5	103	32	493
Bracheria marlothi	429	31	281	18	174	6	72	23	1034
Sporobolus pellucisus	262	27	227	24	397	20	300	120	1378
Dactyloctenium aegyptium	84	7	86	11	313	12	208	33	754
Digitaria abyssinica	74	12	159	12	278	11	117	32	694
Pennisetum polystachion	40	6	36	5	73	3	13	7	182
Hyparrhenia anamesa	48	9	57	10	233	6	62	25	449
Snowdenia petitiana	28	39	81	10	245	11	160	84	657
Rhynchelytrum repens	91	30	76	21	356	22	363	91	1049
Melinis repens	78	24	54	11	127	7	104	53	457
Mean $\pm$ s.d.	81 $\pm$ 91	17 $\pm$ 10	95 $\pm$ 61	11 $\pm$ 5	283 $\pm$ 246	9 $\pm$ 5	149 $\pm$ 99	74 $\pm$ 63	
Pooled s.e.[a]	1.27	1.07	1.52	0.60	1.84	0.54	0.94	1.65	

[a]Pooled s.e. of measurement.

hand, some of the species (e.g. *C. dactaylon* and *Z. pentandra*; *C. ciliaris* and *B. lachnantha*), which were better separated with the n-alkane data set, showed aggregation with the isotope data set.

The comparison of the species ordinations by OPR revealed that the residual variance remaining after fitting the two PCA scores was 84.7% (Table 3). This indicated little similarity between the two PCA scores and that the majority of the variance explained by $\delta^{13}C$ values of n-alkanes was additional to that explained by the n-alkane profile of species.

Figure 3 shows a tri-plot of species points and the direction of the steepest increase for n-alkanes profile and $\delta^{13}C$ values of n-alkanes in the space of the first two principal axes derived from RDA. As shown in the graph, overlapping or clustering of species points was not observed. Those species that were clustered together in Figure 1 (PCA based on n-alkane) and Figure 2 (PCA based on $\delta^{13}C$ values of n-alkanes) appeared separated in Figure 3. On the other hand, those species that had distinct coordinate points in either Figures 1 or 2 remained distinct in Figure 3.

Discussion

n-Alkane concentrations

The presence of significant variability in the n-alkane profile between plant species has long been documented and this variability is increasingly being used for the indirect estimation of the diet composition of free-ranging herbivores (Dove and Mayes, 2005; Ferreira *et al*., 2007). To make effective use of these markers in nutritional studies, however, it is important to document location-specific information on the n-alkane profiles of available herbage species (Ali *et al*., 2005). This is because environmental conditions and geographical locations could influence the pattern of the cuticular wax profile of plant species growing in different places (Samuels *et al*., 2008).

In this report, the general trend that odd-chain alkanes exist in higher concentration than even-chain alkanes, conforms with previous findings (Dove and Mayes, 1996). The dominance of C_{31} in the pasture species was also consistent with previous reports (Ali *et al*., 2005). This makes it easier to quantify odd-chain herbage alkanes more accurately than even-chain alkanes, and hence their role as a diet composition marker appears indispensable. Although found in small quantities some of the even-chain alkanes, like C_{30} and C_{32}, have shown high discriminatory potentials (Pueyo *et al*., 2005).

Feed intake estimation using the ratio of a natural odd-chain to dosed even-chain n-alkane in the faeces is another important advantage of the n-alkane method (Mayes *et al*., 1986). One of the requirements for estimating intake accurately using this approach is that the faecal recovery rates of the dosed and herbage n-alkanes should be similar

Table 2 *Carbon stable isotope (^{13}C) enrichment of n-alkanes for pasture species collected from the Mid Rift Valley rangelands of Ethiopia*

	$\delta^{13}C$ values (‰) of n-alkanes							
Species	C27	C28	C29	C30	C31	C32	C33	C35
Cynodon dactaylon	−22.93	−27.36	−23.90	−25.79	−22.69	−27.84	−23.43	−23.66
Pennisetum straminium	−20.69	−23.09	−20.59	−22.26	−20.10	−24.50	−21.42	−21.71
Cenchrus ciliaris	−20.85	−23.41	−21.13	−23.01	−21.80	−23.65	−21.94	−22.87
Cymbopogon pospischilii	−20.61	−22.08	−20.39	−24.91	−20.81	−26.84	−22.55	−22.30
Indigofera spicata	−32.27	−34.00	−36.61	−36.68	−37.40	−35.67	−33.77	−34.58
Heteropogon contorus	−19.46	−23.47	−20.10	−23.17	−20.45	−22.51	−21.08	−21.94
Zaleya pentandra	−25.67	−27.21	−25.12	−25.47	−21.11	−27.83	−24.11	−25.22
Chloris gayana	−21.37	−22.83	−21.55	−23.07	−21.08	−24.75	−22.26	−22.91
Eragrostis aspera	−21.09	−23.46	−22.13	−25.73	−21.95	−23.15	−24.52	−23.15
Eragrostis cilianensis	−21.86	−24.67	−22.33	−24.61	−23.33	−25.18	−24.32	−25.72
Cynodon ethiopicus	−23.44	−25.20	−23.42	−25.11	−22.17	−25.09	−22.55	−23.71
Eleusine mutiflora	−22.25	−26.01	−23.11	−25.37	−23.65	−26.03	−24.97	−25.57
Brachiaria lachnantha	−20.81	−23.22	−21.88	−23.50	−21.34	−22.90	−22.94	−21.49
Aristida odscensionis	−22.49	−25.00	−22.68	−24.29	−22.69	−23.39	−23.31	−23.30
Bracheria marlothi	−20.72	−22.13	−21.41	−23.84	−22.45	−35.64	−26.36	−22.40
Sporobolus pellucisus	−21.05	−22.93	−21.50	−23.85	−22.50	−23.60	−24.22	−24.40
Dactyloctenium aegyptium	−21.94	−26.58	−23.12	−27.14	−22.48	−26.60	−23.55	−24.79
Digitaria abyssinica	−19.37	−20.87	−19.81	−20.56	−19.67	−22.88	−22.05	−22.46
Pennisetum polystachion	−22.52	−22.57	−23.04	−22.93	−23.80	−23.79	−27.21	−23.70
Hyparrhenia anamesa	−20.12	−25.02	−19.75	−23.93	−19.97	−22.03	−21.68	−22.42
Snowdenia petitiana	−21.97	−25.11	−23.08	−24.66	−23.68	−26.63	−25.18	−24.58
Rhynchelytrum repens	−21.70	−22.80	−22.55	−21.88	−22.97	−21.59	−23.54	−24.55
Melinis repens	−20.92	−22.94	−21.94	−22.37	−21.31	−22.09	−21.78	−23.34
Mean ± s.d.	−22.00 ± 2.62	−24.34 ± 2.69	−22.66 ± 3.33	−24.53 ± 3.04	−22.58 ± 3.45	−25.03 ± 3.72	−23.77 ± 2.67	−23.95 ± 2.62
Pooled s.e.[a]	.29	.52	.27	.42	.16	.78	.26	.32

[a]Pooled s.e. of measurement.

Table 3 *The variance (%) in the pattern of n-alkane concentration and $\delta^{13}C$ values of n-alkanes explained by the first three principal component axes (PC1, PC2 and PC3) for each data set, and the residual variance (%) remaining after comparison by Orthogonal Procrustes Rotation (OPR) of the two principal component scores*

	Variance explained (%)				
Marker	PC1	PC2	PC3	Total	Residual variance (%) remaining after OPR
n-Alkanes	54.6	22.1	14.1	90.8	84.7
δC^{13} of n-alkanes	35.7	22.3	16.0	74.0	

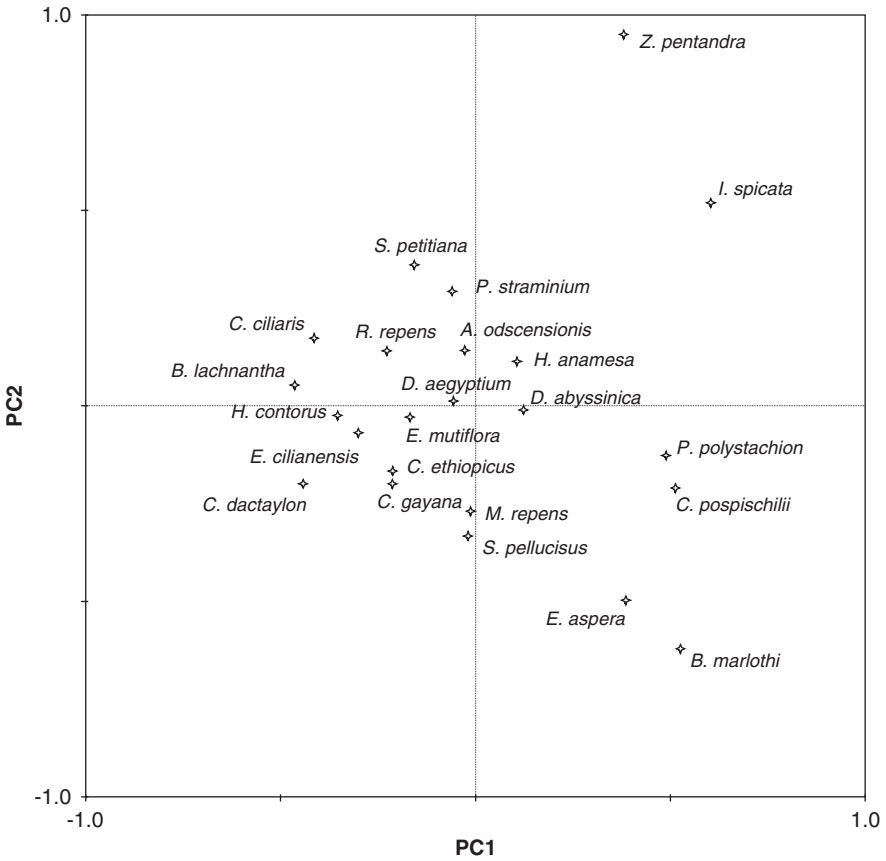

Figure 1 Scatter plot of pasture species on a two dimensional space using the first two principal components (PC1 and PC2) derived from PCA based on the n-alkane concentrations.

(Dove and Mayes, 1991). Generally, pairs of n-alkanes with consecutive carbon chain lengths are reported to have similar recovery rates (Mayes and Dove, 2000). As a result, a combination of either C_{31}/C_{32} or C_{33}/C_{32} has been used for this purpose. The present analysis also confirms herbage C_{31} and C_{33} alkanes as priority choices for intake estimation together with dosed C_{32}. The alkane C_{35} could also be used in combination with dosed $C_{36,}$ as it was found in considerable amounts in many of the species (Table 1), and is known to have high faecal recovery rates (Ferreira *et al*., 2009).

The $\delta^{13}C$ values of n-alkanes

It is known that all photosynthetic plants discriminate against the natural stable isotope ^{13}C during their CO_2 absorption and utilisation. This results in the depletion of ^{13}C in organic tissues, as well as in specific compounds, like n-alkanes, of plants in comparison with the natural abundance (Bendle *et al*., 2006). Plants that follow the C_3 photosynthetic pathway exhibit a higher level of carbon isotope fractionation than C_4 plants (Marshall and Zhang, 1994). The resulting difference in carbon isotope composition of the organic matter has been exploited to estimate the gross diet composition of herbivores in terms of the two plant groups (Coates *et al*., 1987; Norman *et al*., 2009).

Differences in carbon isotope fractionation between species that follow the same photosynthetic pathway have also been documented (Ehleringer, 1991). However, these differences have not been evaluated as an additional source of plant marker. The main reason for that may be the general assumption that the within-photosynthetic-group variations

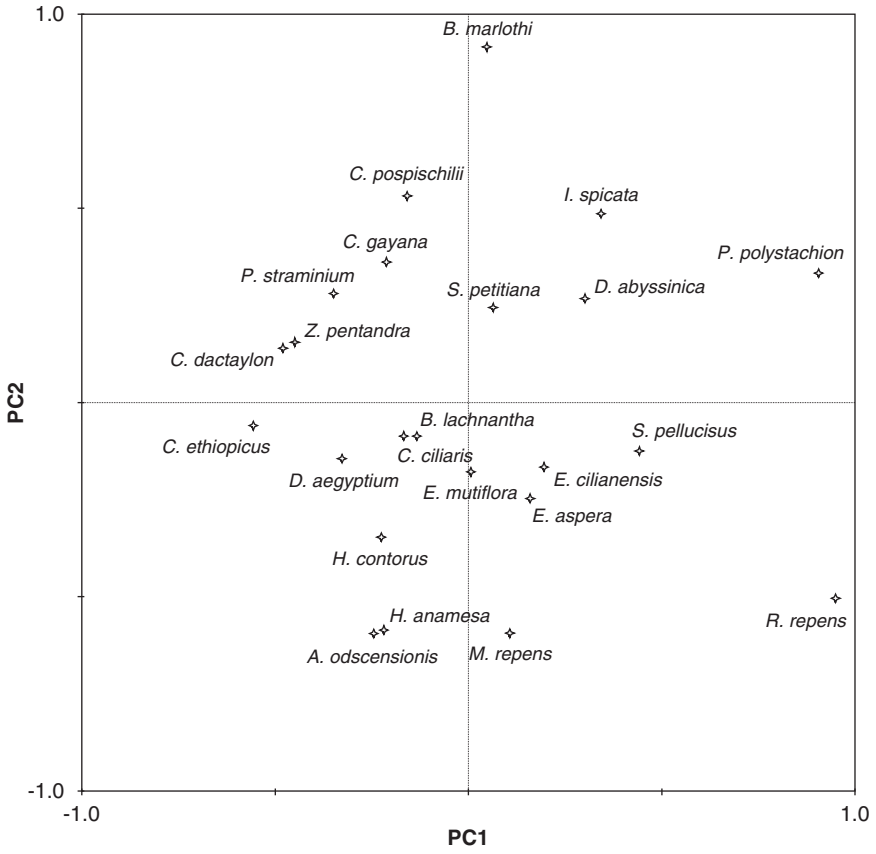

Figure 2 Scatter plot of pasture species on a two dimensional space using the first two principal components (PC1 and PC2) derived from PCA based on $\delta^{13}C$ values of n-alkanes.

in carbon isotopic ratio are too small to be used as an additional marker (Osmond *et al*., 1973).

From previous reports, the $\delta^{13}C$ values of n-alkanes range between −30% and −40% for C_3 plants and −17% to −24% for C_4 plants (Reddy *et al*., 2000). The results of the present analyses largely agree with these ranges of values. *I. spicata* was the only legume species analysed in this study, and the $\delta^{13}C$ values obtained for this species (−32.27% to −37.40%) fall within the range of values observed for C_3 plants. This conforms to the established knowledge that in tropical grasslands, legumes are represented by C_3 plants (Dove and Mayes, 2005). The other species that are analysed exhibited a range of carbon isotope enrichment, which is typical of C_4 plants (Bendle *et al*., 2006), although some of the species like *Z. pentandra* showed a lower level of enrichment. In this study, the general isotope enrichment level of even- and odd-chain alkanes agrees with the finding of Reddy *et al*. (2000), who reported that even-chain alkanes were depleted by about –1% compared to the neighbouring odd-chain alkanes. This may suggest that during the biosynthesis of n-alkanes (elongation of carbon skeletons) there is a differential carbon isotopic fractionation.

Multivariate analysis

A variety of multivariate statistical procedures are available to study the patterns of interspecies variability in n-alkanes and other plant markers (Dove *et al*., 1996; Dove *et al*., 1999). PCA was chosen here as the n-alkane and ^{13}C analyses were based on bulked samples and the data do not provide within-species variability in the two markers. The PCA carried out showed that most of the variance between species was explained by the first three principal components. This indicated the presence of high variability among the plants studied, which can be ascribed to the patterns of the two markers. The results obtained with regard to the n-alkanes is similar to several other investigations over the past decades for pasture and browse species (Ferreira *et al*., 2007). However, to our knowledge, there is no previous published report regarding interspecies variability in the ^{13}C enrichment of n-alkanes.

One of the constraints in using n-alkanes is that the concentrations of many of the lower-chain n-alkanes and the even-chain n-alkanes are too low for accurate measurement. As a result the number of n-alkanes that can be used as markers is limited. This, in turn, may limit the number of diet components that can effectively be estimated. There are, however, circumstances that the diet of animals grazing on botanically diverse vegetation could contain more diet components than the number of effective n-alkane markers available for diet composition estimation. Taking this limitation into account, research in the area has focused on evaluating other plant wax components, like long-chain fatty alcohols and fatty acids, for use as additional markers (Mayes, 1998). This has been supported by the development

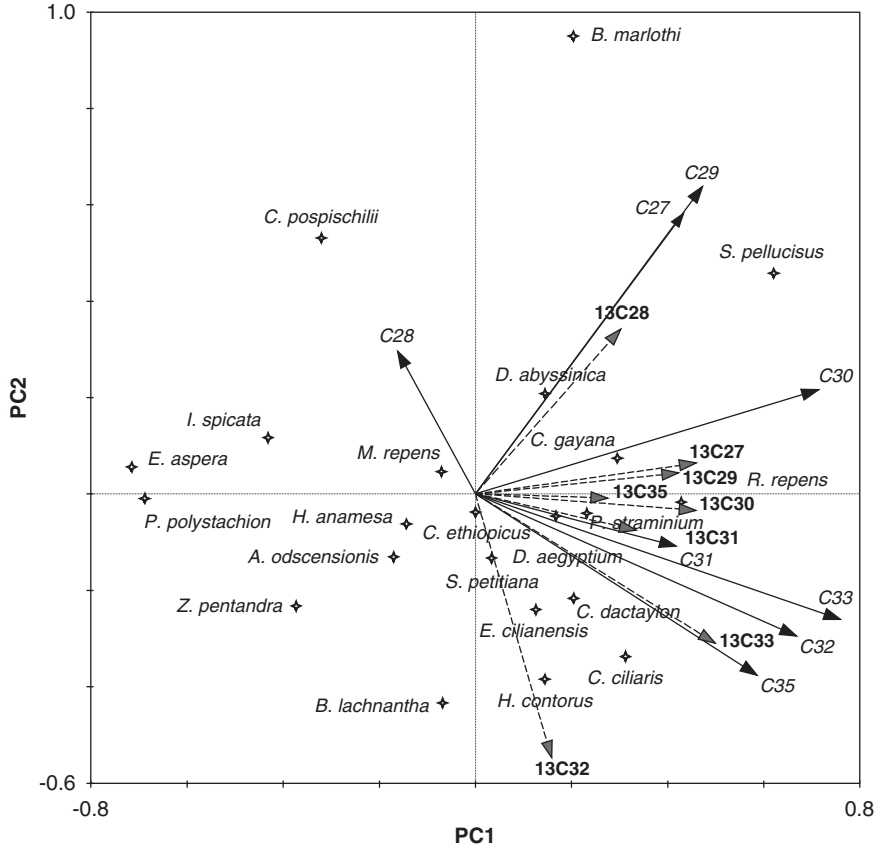

Figure 3 A triplot in the space of the first two principal axes derived from redundancy analysis of n-alkane concentration of species (solid arrows) as constrained by $\delta^{13}C$ values of n-alkanes (dashed arrows). The direction of the arrow points towards the steepest increase of the marker it represents.

of exhaustive analytical laboratory protocols (Dove and Mayes, 2006).

Generally, the use of a combination of plant wax component n-alkanes, long-chain fatty alcohols and acids, has provided increased accuracy and power in the estimation of diet composition (Kelman *et al*., 2003; Bugalho *et al*., 2004; Fraser *et al*., 2006). The present analysis also showed that interspecies variability in $\delta^{13}C$ values of n-alkanes could be used as an additional source of information to estimate diet components of herbivores. The scatter plot derived from RDA, by constraining the species dispersion based on the n-alkane profile by the isotope composition of n-alkanes, showed that those species that tended to cluster when the analysis was based on either n-alkanes or $\delta^{13}C$ values of n-alkanes, appeared to be separated. This graphical presentation supports the OPR result that the isotopic composition of the n-alkanes provides additional discriminatory power to species separation.

The increased analytical capacity to separate specific compounds prior to isotope composition analysis (Muccio and Jackson, 2009) provides enormous potential to study the isotopic ratio of not only n-alkanes but also long-chain fatty alcohols and fatty acids. The possibility of generating two different types of internal markers from a single set of compounds such as n-alkanes would be a desirable feature in terms of increasing the discriminatory power of wax components.

Estimation of diet composition

Estimation of diet composition using plant wax n-alkanes as markers is achieved by relating the marker patterns found in faeces (corrected for incomplete recovery) to those calculated from the marker patterns of individual diet components (plant species or plant parts). A number of mathematical algorithms and approaches including least squares optimisation procedures (Dove and Mayes, 2005) and linear programming (Barcia *et al*., 2007) have been developed and used for this purpose. Regarding the application of isotopic enrichment, Bugalho *et al*. (2008) demonstrated that the linear programming model of Barcia *et al*. (2007) can be effectively used to estimate the contribution of different sources to a mixture by relating the isotopic composition of carbon ($\delta^{13}C$) and sulphur ($\delta^{34}S$) in the sources and mixture. In relation to the present findings, it should also be possible to adopt a suitable mathematical model to use both the n-alkane profiles and their carbon isotopic compositions ($\delta^{13}C$) as inputs in the calculation of diet compositions.

It is now well established that the recovery of n-alkanes in faeces is incomplete with the recovery rate generally increasing in a curvilinear fashion with increasing carbon number. A suitable faecal recovery correction factor is therefore required to increase the accuracy of the calculations (Ferreira *et al*., 2009). However, little is known about the relative recoveries of ^{12}C and ^{13}C isotopes for a particular

n-alkane, which could potentially alter the carbon isotope enrichment values of the alkane in feed and faeces and influence diet composition estimation. Controlled *in vivo* experiments may be required to document the relative fates of the two carbon isotopes in the gut. Gut microorganisms are unable to synthesise long-chain n-alkanes (Keli *et al.*, 2008) indicating that there would be no bias in the estimation of diet composition due to endogenous n-alkane excretion into faeces.

Due to the difficult nature of the measurement, the presence of differential recoveries (if any) of the same n-alkane originating from different plants has not yet been established. In view of this study that the same n-alkane originating from different plants could have different isotopic compositions and that this could be used as an additional marker, it would be interesting to investigate the issue of differential recoveries in relation to the molecular weight of the alkanes.

Conclusion

The n-alkane profile as well as the isotopic enrichment of n-alkanes showed considerable variability among the species studied. The majority of the interspecies variances explained by the two types of data sets are independent of each other. Therefore, generating information on the n-alkane concentration of plant species in combination with their isotopic enrichment could be a valuable tool to improve the accuracy of estimating diet composition and quality of free-ranging animals. However, further validations need to be conducted with actual feeding experiments. Within-species variations in the $\delta^{13}C$ values of n-alkanes, as well as changes with physiological maturity are also topics that need to be addressed.

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