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n-Alkanes for grazing studies with ruminants: where can we go?

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Abstract. An attempt was made to estimate the amount and quality of pasture consumed by sheep, using simultaneously faecal microhistology procedures and n-alkanes markers as markers. Cuticles of field-surveyed plants were characterized and n-alkane profiles of those consumed by the animals (cuticles found in faeces) determined to estimate diet composition and then dry matter intake and digestibility. Since in grazing conditions faecal recoveries of the different n-alkanes cannot be calculated directly, they were estimated by dosing through that of dotriacontane (C_{32}), an artificial artificially dosed alkane and quantifying its recovery in . In the present work large differences were observed when diet composition was estimated using different methods of calculation. No method was able to detect all species identified by faecal microhistology, except in the case of six out of the 24 animals in which the composition of the diet was estimated. It can be concluded from the results that good estimates of diet composition and intake are obtained in grazing animals, using n-alkanes as markers, including only the discriminant hydrocarbons in the calculations. Also better results of diet composition (more compatible with microhistological findings in the faeces) and intake (more compatible with live weight changes of the grazing animals) are obtained applying faecal recovery to diet components instead to faeces. Besides, it seems necessary to estimate accurately the faecal recoveries of *n*-alkanes, even in grazing conditions, as they largely influence the digestibility results. Furthermore, it is important to use a large population of experimental animals as in our case only 20 % presented consistent results of diet composition and intake.

Keywords. *n*-Alkanes – Diet selection – Intake – Sheep.

Les n-alcanes pour l'étude des ruminants sur les pâturages : jusqu'à où peut-on aller ?

Résumé. *Une tentative a été faite pour estimer la quantité et la qualité des pâturages consommés par les ovins, en utilisant conjointement la microhistologie fécale et la technique des n-alcanes. Les cuticules des plantes du terrain étudié ont été caractérisées et les profils des n-alcanes des plantes consommées par les animaux (cuticules trouvées dans les fèces) déterminés afin d'estimer la composition de la diète et l'ingestion de la matière sèche et sa digestibilité. Puisque, dans les conditions de pâturage, les récupérations fécales des différents n-alcanes ne peuvent être calculées directement, ils ont été estimés à partir d'un alcane dosé (dotriacontane-C_{32}) qui a montré une haute concentration dans les fèces. Dans le présent travail, il a été démontré que de grandes différences apparaissent lorsque la composition du régime est estimée en utilisant différentes méthodes de calcul. Aucune méthode n'était capable de détecter toutes les espèces identifiées par la microhistologie fécale, sauf dans le cas de six des 24 animaux dans lesquels la composition du régime a été estimée. À partir des résultats obtenus, il peut être conclu que de bonnes estimations de la composition de la diète et d'ingestion en utilisant la technique des n-alcanes chez les animaux aux pâturages sont obtenues notamment lorsque les hydrocarbures discriminants sont inclus dans les calculs. Également, de meilleures estimations de la composition de la diète (plus compatibles avec les résultats de la microhistologie fécale) et d'ingestion (plus compatibles avec les changements de poids vif des animaux aux pâturages) sont obtenues lorsque la correction pour la récupération fécale est appliquée aux composants de la diète plutôt qu'aux échantillons de selles. Il est également nécessaire d'évaluer avec précision les récupérations fécales des n-alcanes, même en pâturage, car elles influencent largement sur les résultats de digestibilité. En outre, il est important d'utiliser une large population animale dans ce genre d'estimations vu que dans notre cas, seulement 20% ont présenté des résultats cohérents en ce qui concerne la composition du régime alimentaire et l'ingestion.*

Mots-clés. *n-Alcanes – Sélection du régime – Quantités ingérées – Ovins.*

I – Introduction

Small ruminants provide cheap animal protein in many areas of the Mediterranean basin, being their production mainly based on extensive pasture grazing. In these conditions, animal performance depends primarily on intake and diet quality, which are both extremely difficult to accurately estimate in sheep and goats grazing heterogeneous (multispecies) dryland pastures. The *n*-alkane methodology (Mayes *et al*., 1986) is currently one of the most used for this purpose, although it requires a correct sampling and identification of the plant species (and/or parts) actually consumed by the herd (Dove and Mayes, 2005). Microhistological examination of cuticles from oesophageal, ruminal or faecal samples has been used to this purpose for a long time (Holechek and Gross, 1982), although it presents the disadvantage that a huge number of reference images are needed. These techniques are also time consuming and give poor estimates of the proportion of an individual diet component (Dove and Mayes, 2006).

In the last twenty-five years more than fifty annual or biannual species have been identified in the grazed lucerne fields of the Ebro valley (North-East Spain), although no characterization of their cuticles has been performed. On the other hand, no information exists about their *n*-alkane profiles which knowledge is compulsory for intake and diet composition estimations.

In this work cuticles and *n*-alkanes profiles of the cited species were characterized, and then diet composition and dry matter intake and digestibility were estimated in sheep. To this purpose, different methods (Mayes *et al.*, 1994; Dove and Moore, 1995) were compared.

II – Materials and methods

The study was carried out in 2003, and a survey aimed at identifying as many plant species as possible was conducted in a 1-ha paddock of dryland lucerne. Specimens of each identified plant kind were taken, and two subsamples obtained: one for cuticle characterization (Stewart, 1967) and one for *n*-alkane analysis. Then a grazing trial was performed with 24 non-pregnant, non-lactating Rasa Aragonesa ewes (average live weigth 48.5 ± 1,36 kg) during 28 consecutive days. Barley straw was available *ad libitum* in the field, its cuticle characterized and its n-alkane concentrations analyzed. Animals' live weight was registered weekly.

During the whole grazing period a once-daily dose of 1.5 g of paper pellets containing equal amounts of tetracosane (C_{24}), dotriacontane (C_{32}) and hexatriacontane (C_{36}) was given to each animal with a dosing gun at 09:00 h. About 5% of the pellets were sampled and analysed for alkane concentration. Average concentration (± SEM) of C_{24}, C_{32} and C_{36} in the dosed pellets was 76.2±4.07, 76.8±4.25 and 74.4±4.29 mg/pellet. Spot faecal samples were collected daily, directly from the rectum, at the same time as alkane dosing, freeze-dried and pooled, on a DM basis, to a single sample per animal for analysis.

Estimates of diet selection were obtained using different mathematical approaches. First, concentrations of individual alkanes in the consumed plant species (those which cuticles were present in the faeces, including barley straw) were expressed as proportion of the total amount, and the ratios were arcsin transformed in order to satisfy normality. A discriminant analysis was then performed to obtain centroids for every group (species) using subsequent functions. Only discriminant functions increasing at least 10% of accumulated variability were analysed (Dove *et al.*, 1996). Results obtained were subjected to a one-way analysis of variance and then validated using the cross-validation method. The statistical package SPSS 19.0 was used.

Estimates of diet composition were obtained with either all alkanes (C_{21} to C_{36}, except dosed alkanes and C_{22} and C_{24} which were used as internal standards) or only discriminant alkanes (different depending on the animal considered) using the 'Solver' routine of the 'Microsoft Excel' programme with non-negative restrictions (Mayes *et al.*, 1994) or the 'EatWhat' programme (Dove and Moore, 1995). In the first case, diet composition was estimated by minimisation of the sum of the squared discrepancies between the measured faecal proportions of individual

alkanes (recovery-corrected and expressed relative to the total faecal alkane (R), and diet alkane proportions (of the total alkane) calculated from alkane profiles of dietary components (E), as follows:

$$\sum [R-E]^2_{alk:1...n} = \sum \left[\frac{H_i}{H_t} - \frac{xA_i + yB_i + zC_i}{xA_t + yB_t + zC_t} \right]^2_{alk:1...n} \quad \text{[Eq. 1]}$$

where x, y and z represent the proportions of components A, B and C in the diet; Hi, Ai, Bi and Ci the concentrations of alkane i in faeces (recovery-corrected) and components A, B and C, and Ht, At, Bt and Ct total alkane concentrations (recovery-corrected in the case of the faeces). Since in grazing conditions faecal recoveries of the different n-alkanes cannot be calculated directly, they were estimated relative to the dosed alkane C_{32} (which showed the highest faecal concentration/dose ratio) as suggested by Dove *et al.* (1999).

In the present work differences between the two methods of calculation appeared and for the sake of understanding reasons for that the following equation was used with the 'Solver' routine:

$$\sum [R-E]^2_{alk:1...n} = \sum \left[\frac{H_i}{H_t} - \frac{(xA_i + yB_i + zC_i) * FR_i}{x(A_i * FR_i + A_j * FR_j + ...) + y(B_i * FR_i + B_j * FR_j + ...) + z(C_i * FR_i + C_j * FR_j + ...)} \right]^2_{alk:1...n} \quad \text{[Eq. 2]}$$

In this equation *i, j,* … represent different n-alkanes, and FR_i, FR_j, … their faecal recoveries. This is the form data must be introduced in the 'EatWhat' programme. As a third option, the expression:

$$\sum [R-E]^2_{alk:1...n} = \sum \left[\frac{H_i}{H_t} - (x * \frac{A_i}{A_t} + y * \frac{B_i}{B_t} + z * \frac{C_i}{C_t}) \right]^2_{alk:1...n} \quad \text{[Eq. 3]}$$

was used, recovery-correcting either faecal (Equation 3.1) or diet components (Equation 3.2) *n*-alkane concentrations.

Intake estimates were performed in animals where diet composition was accurately obtained [$\Sigma (R-E)^2$ equal or very close to zero] using the n-alkane pair C_{31}/C_{32} (Mayes *et al*., 1986).

Dry matter digestibility was estimated, in the same animals as intake, using the 'EatWhat' programme or hentriacontane (C_{31}) as internal marker.

III – Results and discussion

Thirty-two plant species were identified in the field (including barley straw-*Hordeum vulgare*), although only eleven were found in the faeces of sheep (Table 1).

A great variability between animals was noted in diet selection, and the most appreciated species were *Medicago sativa* (selected by 92% of the animals), *Poa* ssp. (83%) and *Marrubium vulgare* (50%). Cuticle fragments of barley straw (*Hordeum vulgare*) were identified only in 67% of the animals' faeces. It might be argued that some cuticles could have not been identified because of digestion processes, but faecal samples were taken along 28 consecutive days, and hence the probability of identifying some relatively intact structures was supposed to be high.

Table 2 shows the sum of the squared discrepancies between the measured faecal proportions of individual alkanes (R), and diet alkane proportions (of the total alkane) calculated from alkane profiles of dietary components (E). Values were obtained using the 'Solver' routine of the 'Microsoft Excel' programme and Equation 1, Equation 2, Equation 3.1 and Equation 3.2.

Table 1. Plant species identified in the faeces of sheep grazing a dryland lucerne field

	Animal number																	
	4	3	2	1	6, 9, 14	7	5, 16, 20	8, 23	11	10	12	13, 24	15	18	19	17	21	22
Medicago sativa	x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x
Poa ssp.	x	x			x	x	x	x	x	x	x	x	x		x	x		x
Hordeum vulgare	x		x	x	x	x		x	x		x	x		x			x	x
Marrubium vulgare						x	x	x		x				x	x	x	x	x
Anacyclus clavatus		x		x		x				x	x	x						
Dactylis hispanica	x		x	x										x				x
Carduus tenuiflorus		x							x		x		x					
Erodium cicutarium	x	x																
Salvia verbinaca															x	x		
Descurainia sophia																x		
Plantago lanceolatus						x												

Table 2. Sum of the squared discrepancies (±SEM) between the measured faecal proportions of individual alkanes (expressed relative to the total faecal alkane), and diet alkane proportions (of the total alkane) calculated from alkane profiles of dietary components (n = 24). Values were obtained using the 'Solver' routine of the 'Microsoft Excel' programme, and Equations 1, 2, 3.1 and 3.2 are defined in the Materials and Methods section

	Equation 1	Equation 2	Equation 3.1	Equation 3.2
Including all alkanes	0.008±0.0132	0.007±0.0128	0.008±0.0132	0.007±0.0128
n	0	0	0	0
Including only discriminant alkanes	0.002±0.0235	0.001±0.0211	0.002±0.0235	0.001±0.0211
n	6	6	6	6

n: number of sheep in which all plant species detected in faeces by microhistology were included in the estimates of diet composition.

Including only discriminant alkanes in the different equations improved the accuracy of diet composition estimates compared to when using all alkanes, although even in that case only in 6 out of 24 sheep all species identified in faeces by microhistology appeared in the estimated diet composition. In addition, Equation 1 and Equation 2, and Equation 3.1 and Equation 3.2, provided identical results only when $\sum (R\text{-}E)^2 = 0$ or very close to zero (individual data not presented). On the other hand, equations Equation 2 and Equation 3.2 gave more accurate estimates of diet composition than equations Eqn.1 and Eqn.3.1. Values of $\sum (R\text{-}E)^2$ were identical regardless the mathematical models used were Equation 1 or Equation 3.1, or Equation 2 or Equation 3.2.

A comparison between diet composition estimates obtained using the 'Solver' routine of the 'Microsoft Excel' programme or the 'EatWhat' programme (only with Eqn.2) was carried out in order to try and understand the reasons for the different estimates of diet composition obtained using the different mathematical models. Results of this comparison are given in Table 3. The 'S' values, which are a measure of the goodness of fit of the solution, with units of alkane concentration (Dove and Moore, 1995), were much lower when only the discriminant alkanes were used. Hence, only these results were included in Table 3. Only when 'S' values were = 0 or very close to zero estimates of diet composition obtained with the two programs were

practically identical (animals 5, 6, 9, 10, 16 and 20). In these cases, also the $\sum (R-E)^2$ values obtained with the 'Solver' were = 0 or very close to zero (data not presented). In other cases, a lower value of 'S' did not mean more accuracy, and vice versa (e.g. O1 vs O15 in Table 3).

Table 4 shows different estimates of intake and dry matter digestibility (DMD) for the six animals in which diet composition was assumed to have been accurately obtained.

Table 3. Estimates of diet composition (as proportions) obtained using the 'Solver' routine of the 'Microsoft Excel' programme or the 'EatWhat' programme, and Equation 2. Only discriminant alkanes were employed, and data from selected animals (O) are given

		Solver	EatWhat	S			Solver	EatWhat	S
O1	*Hordeum*	0.6856	0.8640	9.52	O10	*Medicago*	0.0867	0.0860	0.00
	Anacyclus	0.0000	0.0000			*Poa*	0.2933	0.2920	
	Dactylis	0.3144	0.1360			*Marrubium*	0.0622	0.0630	
						Anacyclus	0.5578	0.5590	
O5	*Medicago*	0.7943	0.7940	0.00	O15	*Medicago*	0.4352	0.4050	77.67
	Poa	0.0249	0.0250			*Poa*	0.5648	0.5950	
	Marrubium	0.1809	0.1810			*Carduus*	0.0000	0.0000	
O6	*Medicago*	0.1087	0.1090	0.00	O16	*Medicago*	0.8965	08960	0.00
	Poa	0.1982	0.2010			*Poa*	0.0026	0.0030	
	Hordeum	0.6931	0.6890			*Marrubium*	0.1009	0.1010	
O9	*Medicago*	0.0875	0.0870	0.00	O20	*Medicago*	0.8972	0.8970	0.00
	Poa	0.1279	0.1230			*Poa*	0.0048	0.0050	
	Hordeum	0.7846	0.7910			*Marrubium*	0.0980	0.0980	

Table 4. Intake (g/day) and dry matter digestibility (DMD; %) in animals (O) where diet composition estimate was considered accurate

	O5	O6	O9	O10	O16	O20
Intake Equation 1- Equation 2	731	2161	1748	1588	846	457
Intake Equation 3.1	739	1282	1056	1073	846	449
Intake Equation 3.2	729	1276	959	1067	844	444
DMD EatWhat	58.68	79.76	84.94	76.8	60.63	60.16
DMD C_{31} Equation 1- Equation 2	35.74	79.78	83.56	76.77	34.96	38.49
DMD C_{31} Equation 3.2	33.32	65.35	72.17	62.77	32.00	35.94
DMD C_{31} Equation 3.1	30.86	65.19	69.51	62.54	30.72	34.46

Intake Equation 1- Equation 2: estimates obtained using diet composition values derived from Equation 1 or Equation 2. Intake Equation 3.1: estimates obtained using diet composition values derived from Equation 3.1. Intake Equation 3.2: estimates obtained using diet composition values derived from Ec.3.2. DMD EatWhat: estimates obtained using the 'EatWhat' programme. DMD C_{31} Equation 1- Equation 2: estimates obtained using the C_{31} alkane as internal marker and equations Equation 1 or Equation 2. DMD C_{31} Equation 1: estimates obtained using the C_{31} alkane as internal marker and equation Equation 1. DMd C_{31} Equation 2: estimates obtained using the C_{31} alkane as internal marker and equation Equation 2.

In the case of intake, values obtained from diet composition derived from equations Equation 3.1 or Equation 3.2 agreed much more with changes in body weight for all six animals included in Table 4. On the contrary, estimates of DMD were not considered satisfactory in any case. As an example, estimates of straw intake in animals O6 and O9 were high (34% and 45% of the

total dry matter intake), whereas DMD obtained was also abnormally high. It is speculated that an accurate estimation of faecal recovery of n-alkanes (relative to that of C_{32} in the present work) might play an important role.

IV – Conclusions

From the results obtained, it can be concluded that good estimates of diet composition and intake using the n-alkanes technique in grazing animals can be obtained including only the discriminant hydrocarbons in the calculations. Also better results of diet composition (more compatible with microhistological findings in the faeces) and intake (more compatible with live weight changes of the grazing animals) were obtained applying faecal recovery to diet components instead to faeces. For intake estimates, the use of equation Equation 3 gave the best results and hence is proposed as an alternative to the classical approaches of Mayes *et al.* (1994) or Dove and Moore (1995). It seems also necessary to estimate accurately the faecal recovery, as it largely influences the digestibility results. A large population of experimental animals should be used as in our case only 20% presented sound results.

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