Comparison of long-chain fatty acids and alkanes as markers to estimate diet composition of equines and cattle consuming heathland vegetation species

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ABSTRACT

This study aimed to evaluate the application of long-chain fatty acids (LCFA) as an alternative or as a complement to alkanes to estimate diet composition in equines and cattle. Twelve mature crossbreed mares (385 ± 47 kg live weight – LW) and 6 adult non-lactating cows (499 ± 36 kg LW) of Asturiana de los Valles breed were divided in groups of 3 animals and housed in individual stalls. Animals received a daily total amount of 1.0 kg DM/100 kg LW of different diets composed of herbaceous (*Lolium perenne*) and woody species (*Ulex gallii* and heather). Diet composition was estimated from LCFA (i.e., C₂₂–C₃₄) and alkane (i.e., C₂₅–C₃₁ and C₃₃) concentrations in diet and faeces by least-squares procedures, using marker faecal concentrations uncorrected for incomplete faecal recovery (FC0), corrected using mean recovery rate of the dietary treatment that the animal belonged to (FC1), or corrected using mean recovery rate across all experimental diets (FC2). For all diet components, LCFA concentrations were higher than the alkane ones, with even-chain LCFA accounting for more than 0.80 of total LCFA. In general, faecal recovery (FR) of the markers was incomplete and related to their carbon-chain length. In equines, the FR of both markers tended to decrease with carbon-chain length in a curvilinear fashion ($P < 0.001$), whereas in cattle LCFA and alkane FR tended to increase with carbon-chain length in a curvilinear ($P < 0.001$) and linear ($P < 0.001$) fashion, respectively. Diet composition had an effect ($P < 0.001$) on the LCFA and alkane FR in both animal species, and, in equines, seemed to be related with diet digestibility, i.e., decrease of marker disappearance from the digestive tract with a decrease of diet digestibility. In general, diet composition estimates based on LCFA alone were more accurate than those obtained using alkanes alone. Combination of LCFA and alkane markers resulted in an increase ($P < 0.05$) in the accuracy of diet estimates in both animal species, indicating a higher discriminatory power among plant species. The use of FC0 and FC2 resulted in the worst ($P < 0.05$) estimates of diet composition, while high levels of accuracy were observed when using more accurate faecal corrections (FC1). Results showed that LCFA can be useful markers for studying diet selection of equines and cattle grazing on heathland vegetation communities, if a proper adjustment of their faecal concentrations prior to their application is applied. It is also concluded that use of LCFA in combination with alkanes can increase the accuracy of diet composition estimates.

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1. Introduction

Heathlands are shrubby vegetation communities widespread in the northwest of the Iberian Peninsula as a

consequence of the abandonment of agricultural and livestock managements initiated in the 1950s, and by frequent forest fires. Feral cattle and equines use these vegetation areas in free-ranging systems, generally in completely wild conditions with minimal management. In general, there is a lack of technical and scientific knowledge on how these herds behave (i.e., grazing paths, foraging behaviour, etc.) and their influence on the dynamics of the vegetation community.

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Although cattle and equines show clear morpho-physiological differences (e.g., food prehension, digestive strategies, etc.), it seems that they present a high degree of dietary overlap and, consequently, a high competition for the available biomass. In fact, overlap levels higher than 70% have been recorded between these animal species in very different ecosystems such as North American sagebrush steppe (Krysl et al., 1984), African thornbush savannah (Lechner-Doll et al., 1995) and European Pyrenean pastures (Aldezabal, 2001), with an intensive utilization of the grass biomass in all situations. Their co-existence in very diverse ecosystems results of different digestive strategies (Duncan et al., 1990; Lechner-Doll et al., 1995), conferring them alternative abilities to use the same feed resources (Menard et al., 2002; Loucugaray et al., 2004). Studies conducted in upland vegetation communities showed that in the beginning of the grazing season equines and cattle showed an intensive use of grasslands due to the high availability of herbage of better nutritive quality, increasing the selection of woody species as herbage availability decreases (Osoro et al., 2008). In fact, equines were able to include high amounts (up to 30%) of woody species in their diets at the end of the grazing season (Osoro et al., 2008). Cattle hardly select green shoots of shrubs such as heathers (*Erica* spp., *Calluna vulgaris*) and gorse (*Ulex gallii*) (Aldezabal, 2001; Celaya et al., 2007), due to their muzzle anatomy (Illius and Gordon, 1993).

Alkanes have been used as faecal markers for estimating diet composition of different domestic herbivore species in heathlands with or without associated improved pastures (Celaya et al., 2007; Osoro et al., 2007). However, some plant species that can be found in these complex vegetation communities (*Lolium perenne*, *Trifolium repens*, *Pseudarrhenatherum longifolium*, *Agrostis capillaris* and *U. gallii*) present very low alkane concentrations, making them more prone to measurement errors (Brosh et al., 2003; Dove and Mayes, 2006), and their discrimination more difficult to accomplish (Charmley and Dove, 2007). This problem is even more important in the end of the grazing season due to a maturity stage effect on alkane concentrations (Dove et al., 1996), which Smith et al. (2001) related to an increase of the stem fraction in the whole plant, characterized by lower alkane concentrations than the leaf fraction. Moreover, recent results suggest difficulties in distinguishing herbaceous species and/or herbaceous species and gorse (*U. gallii*) due to similarities in their alkane profile, leading to diet composition estimation more prone to bias (Oliván et al., 2007; Ferreira et al., 2009a). An increase in the discriminatory power between these plant species can be accomplished by combining the alkanes with other epicuticular wax components such as long-chain fatty acids (LCFA, Dove and Mayes, 2006).

The objective of the present work was to assess the utilization of LCFA or LCFA + alkanes to estimate the diet composition of equines and cattle fed on browse and herb feeds of the end of the grazing season, and to compare these estimates with those obtained using only the alkanes.

2. Materials and methods

This study was conducted in the end of the grazing and vegetation growth season of 2004 (i.e., November and December) at Carbayal Research Station, situated at 900–1000 masl, at San Isidro's Mountain, Asturias, Spain (longitude

–6° 53', latitude 43° 20'), established on natural heather–gorse shrubland vegetation with or without adjacent areas of improved pasture (*L. perenne* and *T. repens*).

2.1. Animals, experimental design and diets

Twelve mature crossbreed mares (385 ± 47 kg live weight, LW) and six adult non-lactating cows (499 ± 36 kg LW) of Asturiana de los Valles breed were used in a 11 day experiment. Groups of 3 animals were assigned at random to one of four (E1, E2, E3 and E4 for equines) and two (C1 and C2 for cattle) diets that aimed to reflect the grazing behaviour of these animal species on these vegetation communities in the end of the grazing season (Osoro et al., 2005). Diet 1 (E1 and C1) contained only ryegrass (*L. perenne*), diet 2 (E2 and C2) included ryegrass (0.70) and heather (0.30), diet 3 (E3) consisted of ryegrass (0.70) and gorse (*U. gallii*, 0.30), and diet 4 (E4) was composed of ryegrass (0.40), gorse (0.30) and heather (0.30). Heather represented the proportions of *Erica umbellata*, *Erica cinerea* and *C. vulgaris* found in heather–gorse vegetation communities in the end of the grazing season. Diet components were harvested daily from random sites within the experimental field and were offered twice daily in individual mangers a total daily amount of 1.0 kg DM/100 kg LW.

Animals were housed in individual pens for 7 day's adaptation to diets, followed by 4 days of collection of representative samples of each plant species and animal faeces. Prior to the trial, animals were grazing on the type of vegetation described for these marginal areas, which was used in the experimental diets. Daily total faecal output was recorded individually. One sample of total faeces was collected from each animal every day for DM determination (i.e., using a forced-air oven at 60 °C for 72 h) and another sample was immediately frozen at –20 °C and then freeze-dried and milled through a 1 mm screen. After alkane analysis (Ferreira et al., 2007a), all samples were vacuum-packed in plastic containers.

2.2. Long-chain fatty acid analysis

Long-chain fatty acid concentrations of diet components and faeces were analysed in duplicate according to the methods of Dove and Mayes (2006). The first stage involved treating 0.2 g of faeces or 0.5 g of diet components for 16 h with 3 or 4 mL ethanolic KOH (1 M) at 90 °C, respectively, in a dry-block heater (Techne DB-3, Techne Ltd., Duxford, Cambridge, UK). Afterwards, a hot extraction was performed with heptane and water at 60 °C to remove hydrocarbons (including alkanes) and long-chain fatty alcohols (LCOH). After the extraction, the aqueous residue from the LCOH extraction was acidified with 1.5 mL of 5.8 M HCl and extracted with heptane/diethyl ether (50:50, v/v). To purify the LCFA extract, 2 mL of heptane was added and 0.5 mL of the extract was passed through a silica-gel column (Alltech Associates, Deerfield, IL, USA). The silica-gel column was washed with heptane–ethyl acetate (92:8, v/v) and the elution of LCFA was accomplished with the addition of heptane/ethyl acetate (70:30, v/v). Samples eluted from the columns were evaporated to dryness. To convert the LCFA to their fatty acid methyl esters, 1 mL toluene and 2 mL of methanol, previously acidified with sulphuric acid, were added to the dried elute which was kept at 50 °C overnight. After cooling, 2 mL of an aqueous solution of 0.5 M K_2CO_3 and

2 mL of heptane were added and the top organic layer was removed and evaporated to dryness. Fatty acid methyl ester extracts were redissolved with 0.2 mL of heptane (kept at room temperature) for chromatographic analysis.

Analysis of the LCFA (C_{22} – C_{34}) was carried out by gas chromatography–mass spectrometry (GC–MS), using an Agilent 6890 N (Agilent Technologies, Santa Clara, CA, USA) GC equipped with a 5973 N mass spectrometer and an Agilent 7683 automatic liquid sampler injector. The target analytes were separated using a DB-1 capillary column, 30 m $\times$ 0.25 mm with 0.25 μ m film thickness (Agilent Technologies, USA). Injection volume was 1 μ L (splitless, 0.5 min) and the injector temperature was 280 °C. The column was maintained at 170 °C for 4 min after injection, ramped at 30 °C/min up to 215 °C, 1 min hold, and then ramped at 6 °C/min up to 300 °C, where it was held for 20 min. Helium was used as the carrier gas at 34 cm/s average linear velocity. Mass spectra were acquired in electron impact mode at 70 eV, using full scan with a scan range of 40–800 atomic mass units. All chromatographic analyses were carried out in duplicate for each extract, and a mixed standard was run every 7 sample vials to enable corrections for variation in detector response, as suggested by Dove and Mayes (2006). The mixed standard solution consisted of several fatty acid methyl esters (i.e., methyl behenate, methyl tetracosanoate, methyl hexacosanoate, methyl octacosanoate and methyl triacontanoate) dissolved in heptane. The LCFA concentrations were quantified relative to known amounts of the internal standard hentriacontanoic acid (C_{31}) dissolved in 50:50 (v/v) heptane/ethanol (1.3 mg/g) added at the beginning of the extraction procedure. When not in use both the internal standard and the mixed standard solutions were stored (2–8 °C) and kept protected from the light.

2.3. Calculations

LCFA and alkane faecal recoveries (FR) were calculated for each animal as the proportion of marker consumed in the diet which was recovered in the faeces.

Diet composition estimates were obtained for each animal using an optimization procedure which minimises the sum of squared discrepancies between the actual (A) LCFA, alkane and LCFA + alkane proportions in faeces (adjusted or not for the incomplete faecal recoveries) and the estimated (E) proportions (different combinations of diet components), according to Salt et al. (1994), based upon the individual marker concentrations expressed as proportions of the total for that marker.

Estimates were based on LCFA alone (C_{22} – C_{34}), alkanes alone (C_{25} – C_{31} and C_{33}) or a combination of LCFA with alkanes. The C_{32} alkane was not used for diet composition estimation as it was supplied to E3, E4 and C1 groups for feed intake estimation. For diet composition calculations, different LCFA and alkane FR corrections were applied, being: 1) LCFA and alkane faecal concentrations without any correction for incomplete faecal recovery (FC0); 2) LCFA and alkane faecal concentrations corrected using mean recovery rate of the dietary treatment that the animal belonged to (FC1); 3) LCFA and alkane faecal concentrations corrected using mean recovery rate across all experimental diets (FC2).

Apparent *in vivo* dry matter digestibility (DMD_{ap}) was calculated from total faecal collection using the equation

$DMD_{ap} = (I - F)/I$, where I is the total DM intake and F the total DM faecal output.

2.4. Statistical analysis

Statistical analyses were performed using GenStat (2008). Differences between plant species in LCFA, alkane and LCFA + alkane profiles were explored using Principal Components Analysis (PCA). To assess if the discriminatory information provided by the LCFA markers was additional to that provided by the alkane markers, PC scores obtained with the two different classes of marker were compared by Orthogonal Procrustes Rotation (OPR) as suggested by Bugalho et al. (2004) and Dove and Charmley (2008).

Effects of animal species, diet composition and carbon-chain length and their interactions on FR of each LCFA and alkane were examined by ANOVA. Orthogonal contrasts were applied to determine linear and quadratic effects of carbon-chain length and its interaction with diet composition effect.

Accuracy of diet composition estimates as a whole was assessed by the Kulczynski similarity index (KSI, Krebs, 1989), i.e., overlap in diet composition between known values and those estimated using different sets of FR correction, as:

$$KSI = \frac{\sum 2c_i}{\sum (a_i + b_i)} \quad (1)$$

where c_i is the lesser proportion of i component in the 2 diets (known vs. estimated) and $(a_i + b_i)$ is the sum of proportions of each plant component in both diets (known and estimated). Effects of animal species (AS), diet composition (D), faecal recovery correction (FC), marker (M) and their interactions on the KSI values were examined by ANOVA. For multiple comparisons among means Tukey's test was used and differences with a P -value of less than 0.05 were considered significant.

3. Results

3.1. LCFA profiles of diet components

The LCFA and alkane content of plant species offered to the animals in the experimental period are presented in Table 1. The concentrations of alkanes C_{22} and C_{34} and C_{31} –LCFA are not shown as they were used as internal standards in GC–MS analyses and C_{33} –LCFA was absent in all plant species. For all diet components, total LCFA concentrations were higher than the alkanes, with heather showing the highest total LCFA concentration (4455 mg/kg DM) and *U. gallii* the lowest one (2119 mg/kg DM). It should be noted that, in general, the individual LCFA concentrations exceeded 100 mg/kg DM, while the alkane ones rarely exceed 50 mg/kg DM.

Even-chain LCFA predominated over the odd-chain ones in all diet components, accounting for more than 0.80 of total LCFA, ranging between 0.83 (*U. gallii*) and 0.88 (*L. perenne*). For *L. perenne* and *U. gallii*, shorter LCFA (C_{22} – C_{27}) predominated over the longer ones (i.e., 0.65 and 0.55, respectively), while for the other woody species (i.e., heather) shorter and longer LCFA showed similar proportions.

The PCA results (data not shown) showed that the first two principal components (PC) explained 1.0 of the variance

Table 1Mean ($\pm$ standard deviation) long-chain fatty acid (LCFA) and alkane content (mg/kg DM) of the plant species used in the diets of equines and cattle.

	LCFA concentrations				Alkane concentrations		
	<i>L. perenne</i>	<i>U. gallii</i>	Heather		<i>L. perenne</i>	<i>U. gallii</i>	Heather
C ₂₂	570 $\pm$ 100.8	603 $\pm$ 68.6	801 $\pm$ 142.9	C ₂₅	11 $\pm$ 1.1	3 $\pm$ 0.6	10 $\pm$ 1.4
C ₂₃	148 $\pm$ 29.6	141 $\pm$ 20.6	103 $\pm$ 18.0	C ₂₆	3 $\pm$ 0.5	2 $\pm$ 0.3	6 $\pm$ 0.7
C ₂₄	447 $\pm$ 54.3	217 $\pm$ 25.3	530 $\pm$ 108.4	C ₂₇	19 $\pm$ 1.5	21 $\pm$ 2.5	33 $\pm$ 6.6
C ₂₅	143 $\pm$ 32.9	77 $\pm$ 15.4	249 $\pm$ 70.3	C ₂₈	7 $\pm$ 0.8	11 $\pm$ 1.4	17 $\pm$ 6.3
C ₂₆	1002 $\pm$ 132.1	92 $\pm$ 6.0	405 $\pm$ 57.7	C ₂₉	78 $\pm$ 6.4	55 $\pm$ 5.5	109 $\pm$ 19.7
C ₂₇	115 $\pm$ 37.7	30 $\pm$ 4.0	127 $\pm$ 52.9	C ₃₀	10 $\pm$ 1.5	8 $\pm$ 0.8	27 $\pm$ 4.4
C ₂₈	479 $\pm$ 68.1	287 $\pm$ 37.0	456 $\pm$ 149.2	C ₃₁	161 $\pm$ 10.7	106 $\pm$ 10.3	654 $\pm$ 123.7
C ₂₉	40 $\pm$ 7.3	102 $\pm$ 11.6	65 $\pm$ 8.7	C ₃₂	8 $\pm$ 1.2	3 $\pm$ 0.3	55 $\pm$ 5.5
C ₃₀	458 $\pm$ 98	531 $\pm$ 73.4	747 $\pm$ 86.7	C ₃₃	93 $\pm$ 9.7	4 $\pm$ 1.3	519 $\pm$ 84.7
C ₃₂	247 $\pm$ 59.8	38 $\pm$ 6.6	718 $\pm$ 147.7	Total	394 $\pm$ 24.1	220 $\pm$ 31.4	1430 $\pm$ 162.5
C ₃₄	96 $\pm$ 10.2	1 $\pm$ 0.6	254 $\pm$ 38.4	Total odd-chain	367 $\pm$ 21.8	195 $\pm$ 26.9	1325 $\pm$ 153.2
Total	3745 $\pm$ 366.3	2119 $\pm$ 209.1	4455 $\pm$ 456.5				
Total even-chain	3299 $\pm$ 269.6	1769 $\pm$ 175.3	3911 $\pm$ 451.3				

among plant components when using either data on LCFA, alkanes or LCFA combined with alkanes. Plant species were clearly discriminated when using both LCFA and LCFA combined with alkanes, while *L. perenne* and *U. gallii* presented some resemblances in the alkane data as they were located close to each other in a biplot of the first two PC. These results suggested that LCFA markers provided additional discriminatory information to that given by the alkane markers. The OPR results confirmed that this was the case, by demonstrating that when PCA scores based on alkanes or on LCFA were compared, 0.68 of the residual variance remained after rotation.

3.2. LCFA and alkane faecal recoveries

The FR results of the LCFA and alkane markers are shown in Tables 2 and 3, respectively. In general, FR of these markers was incomplete, although several alkanes and LCFA presented FR above 100%. It should be noted that FR of C₃₄–LCFA was unexpectedly low in E1, E2, C1 and C2 groups (i.e., 0.46, 0.52, 0.62 and 0.44, respectively) when compared with other equine groups and LCFA with similar carbon-chain length. Mean coefficient of variation (CV) of FR was higher ($P=0.0024$) in the LCFA than in the alkane markers (13.3 vs. 6.9%, respectively) both in equines (13.8 vs. 8.2%, $P=0.0643$) and cattle (12.8 vs. 5.6%, $P=0.0196$). Similar ($P>0.05$) CV was observed for the odd-chain and even-chain LCFA and alkanes, both in equines and cattle.

The comparison of equine and cattle FR data showed that LCFA behaved differently ($P<0.001$, Table 4) in the digestive tract of equines and cattle. Equines had higher mean FR for all LCFA, ranging between 0.68 (C₃₄) and 0.99 (C₂₄). Differences in LCFA faecal recovery between equines and cattle tended to decrease with carbon-chain length from a maximum of 0.52 (C₂₃) to a minimum of 0.09 (C₂₉). Although animal species had no effect on alkane FR (Table 5), equines had higher FR for the shorter alkanes (i.e., C₂₅ to C₂₉), while cattle had higher FR for the longer ones (i.e., C₃₀ to C₃₃).

The LCFA and alkane carbon-chain length had an effect ($P<0.001$) on the FR (Tables 4 and 5, respectively) although their relationship varied between animal species. In equines, LCFA and alkane FR tended to decrease with carbon-chain length (C) in a curvilinear fashion ($P<0.001$, $FR = -0.52 + 0.119 C - 0.0024 C^2$,

$R^2_{\text{adjusted}} = 0.176$; $P<0.001$, $FR = 4.03 - 0.193 C + 0.003 C^2$, $R^2_{\text{adjusted}} = 0.219$, respectively; Fig. 1) as the quadratic term was significant ($P<0.01$) for both markers, except for the even-chain alkanes (Table 5) where a linear association ($P<0.001$, $FR = 2.216 - 0.042 C$, $R^2_{\text{adjusted}} = 0.376$) was observed. It should be emphasized that the curvilinearity observed between the carbon-chain length and LCFA FR (Fig. 1) in equines was a result of the unexpectedly low FR of C₃₄–LCFA. In fact, when C₃₄–LCFA was excluded from the analysis the quadratic term was no longer significant ($P=0.200$) and the equation slope (-0.009 ± 0.005) did not differ ($P=0.0632$) from zero. By contrast, in cattle both markers FR tended to increase with carbon-chain length in a curvilinear ($P<0.001$, $FR = -2.44 + 0.212 C - 0.0036 C^2$, $R^2_{\text{adjusted}} = 0.120$) and linear fashion ($P<0.001$, $FR = 0.112 + 0.028 C$, $R^2_{\text{adjusted}} = 0.550$) for LCFA and alkanes, respectively. As a result, the interaction between animal species and carbon-chain length was highly significant ($P<0.001$) for the LCFA and alkane FR (Tables 4 and 5, respectively).

Results also showed an effect ($P<0.001$) of diet composition on the LCFA and alkane FR (Tables 4 and 5, respectively). In general, the inclusion of gorse in the equines diets (i.e., E3 and E4) resulted in an increase of the FR of both LCFA and alkanes, whereas the inclusion of the other woody species (i.e., heather) in the cattle diets resulted in an increase of LCFA FR (Table 2) and a decrease of the alkane FR (Table 3). Also, an interaction between diet composition and carbon-chain length of both markers was observed, indicating a different FR pattern between diets in both animal species. For the LCFA, the analysis for each individual marker indicated an effect of diet composition on FR, except for C₂₃ and C₂₉ in equines and C₂₆, C₂₇ and C₂₈ in cattle. This effect was not so clear in the alkane markers, being significant for C₂₅ and C₂₆ in cattle, C₃₁ in equines, and C₂₇ and C₃₃ in both animal species.

3.3. Diet composition estimates

Diet composition estimates obtained based on LCFA and alkanes alone, and the combination of both markers for equines and cattle are illustrated in Figs. 2 and 3, respectively. Similar ($P=0.439$) level of accuracy was observed in equines (94.8%) and cattle (95.3%). The use of different marker data influenced ($P<0.001$) the accuracy (assessed by the KSI values) of the diet composition estimates in equines ($P=0.0122$) and cattle

Table 2

Mean ($\pm$ standard deviation) long-chain fatty acid (LCFA) recovery in faeces of equines and cattle (3 animals per diet) according to carbon-chain length and apparent *in vivo* DM digestibility (DMDap, g/kg).

Species	Diet	LCFA carbon-chain length											DMDap
		C ₂₂	C ₂₃	C ₂₄	C ₂₅	C ₂₆	C ₂₇	C ₂₈	C ₂₉	C ₃₀	C ₃₂	C ₃₄	
Equines	E1	0.75 $\pm$ 0.055	0.86 $\pm$ 0.082	0.97 $\pm$ 0.040	0.95 $\pm$ 0.037	0.81 $\pm$ 0.073	0.57 $\pm$ 0.041	0.81 $\pm$ 0.057	1.00 $\pm$ 0.036	0.71 $\pm$ 0.056	0.70 $\pm$ 0.041	0.46 $\pm$ 0.054	575 $\pm$ 45.1
	E2	0.89 $\pm$ 0.050	0.92 $\pm$ 0.056	0.93 $\pm$ 0.081	1.02 $\pm$ 0.026	1.04 $\pm$ 0.030	0.65 $\pm$ 0.041	0.86 $\pm$ 0.048	1.01 $\pm$ 0.041	0.69 $\pm$ 0.025	0.68 $\pm$ 0.005	0.52 $\pm$ 0.019	553 $\pm$ 39.8
	E3	0.98 $\pm$ 0.043	0.95 $\pm$ 0.064	1.07 $\pm$ 0.027	0.85 $\pm$ 0.098	1.04 $\pm$ 0.042	0.88 $\pm$ 0.055	1.01 $\pm$ 0.045	0.94 $\pm$ 0.072	0.96 $\pm$ 0.092	1.02 $\pm$ 0.019	0.94 $\pm$ 0.025	420 $\pm$ 4.8
	E4	1.01 $\pm$ 0.012	0.98 $\pm$ 0.019	1.00 $\pm$ 0.022	0.94 $\pm$ 0.033	1.00 $\pm$ 0.019	0.87 $\pm$ 0.099	1.01 $\pm$ 0.005	0.94 $\pm$ 0.029	0.95 $\pm$ 0.033	1.03 $\pm$ 0.041	0.81 $\pm$ 0.068	394 $\pm$ 2.2
	Mean	0.91	0.93	0.99	0.94	0.97	0.74	0.92	0.97	0.83	0.86	0.68	485
Cattle	SEM	0.033	0.020	0.019	0.023	0.031	0.044	0.029	0.015	0.041	0.051	0.061	25.9
	CV	12.6	7.3	6.8	8.6	10.9	20.5	10.8	5.4	17.3	20.7	30.8	18.5
	C1	0.71 $\pm$ 0.022	0.46 $\pm$ 0.036	0.69 $\pm$ 0.039	0.54 $\pm$ 0.014	0.71 $\pm$ 0.026	0.42 $\pm$ 0.013	0.76 $\pm$ 0.021	0.99 $\pm$ 0.014	0.68 $\pm$ 0.009	0.80 $\pm$ 0.027	0.62 $\pm$ 0.036	592 $\pm$ 9.6
	C2	0.58 $\pm$ 0.059	0.36 $\pm$ 0.032	0.59 $\pm$ 0.010	0.32 $\pm$ 0.026	0.71 $\pm$ 0.027	0.46 $\pm$ 0.097	0.78 $\pm$ 0.068	0.78 $\pm$ 0.026	0.70 $\pm$ 0.016	0.66 $\pm$ 0.032	0.44 $\pm$ 0.088	503 $\pm$ 22.6
	Mean	0.65	0.41	0.64	0.43	0.71	0.44	0.77	0.89	0.67	0.73	0.53	548
SEM		0.035	0.025	0.025	0.049	0.010	0.027	0.019	0.047	0.013	0.032	0.048	20.8
	CV	13.2	15.0	9.4	28.1	3.4	15.1	6.1	13.0	4.8	10.8	22.3	9.3
	Diff. ^a	0.26	0.52	0.35	0.51	0.26	0.30	0.15	0.09	0.15	0.13	0.15	–62.4

^a Diff. mean difference in FR and DMDap between equines and cattle; E1 and C1: *Lolium perenne* (1.00); E2 and C2: *L. perenne* (0.70) + heather (0.30); E3: *L. perenne* (0.70) + *U. gallii* (0.30); E4: *L. perenne* (0.40) + *U. gallii* (0.30) + heather (0.30); L and Q indicate linear or quadratic significance term for LCFA and Diet LCFA effects; and SEM: standard error of mean.

($P < 0.001$). The combination of LCFA and alkane data resulted in more accurate ($P < 0.05$) diet composition estimates in both animal species (96.2 and 97.7% for equines and cattle, respectively). In equines, the use of LCFA or alkanes alone resulted in similar estimates of diet composition (mean KSI indexes of 94.3 and 93.9%, respectively). By contrast, LCFA estimates in cattle (95.2%) were more accurate ($P < 0.05$) than those obtained using the alkane data (93.0%). As a result, an interaction ($P < 0.001$) between marker type and animal species was observed.

The accuracy of diet composition estimates was also affected ($P < 0.001$) by the diet composition. As expected, higher ($P < 0.05$) KSI indexes were observed in the less complex diet, i.e., composed only by *L. perenne* (i.e., E1 and C1) for both equines (96.2%) and cattle (96.4%). No interaction ($P = 0.287$) between diet composition and animal species was observed, whereas an interaction ($P < 0.001$) between marker data and diet composition was found.

For both animal species, the utilization of uncorrected marker faecal concentrations (FC0) and those corrected with mean recovery rate across all experimental diets (FC2) resulted in the worst ($P < 0.05$) estimates of diet composition when using LCFA data, alkane data and their combination. By contrast, high levels of accuracy were observed when using more accurate faecal corrections (i.e., FC1) with mean KSI indexes of 96.4 and 98.6% for equines and cattle, respectively. In general, the application of FC0 and FC2 corrections to LCFA, alkanes and LCFA + alkanes faecal concentrations resulted in an underestimation of *L. perenne* proportions in the diets, although in some occasions accurate estimates of diet composition were observed when applying these faecal corrections. As a result, an interaction between faecal correction and diet composition was found ($P < 0.001$).

4. Discussion

4.1. Marker profiles of the plant species

Results obtained in this study showed large differences in the LCFA and alkane profiles between plant species used as possible diet components, as previously reported for these epicuticular markers and plant species (Dove and Mayes, 2005; Ferreira et al., 2009a,b, 2010). Although *L. perenne* and *U. gallii* had similarities in the alkane profiles, they were clearly discriminated by the LCFA markers. Ali et al. (2005) and Ferreira et al. (2009a, 2010) also indicated that plant species with a similar pattern in one marker may differ in the pattern of a different marker. One important aspect that needs to be considered when combining different types of markers is if they are supplying similar discriminatory information or different and complementary data. In the present study, results suggested that LCFA markers provided additional discriminatory information to that given by the alkane markers, as also observed by Ferreira et al. (2009a).

Alkane and LCFA profiles of the plant species were quite different in terms of total marker concentrations and in the predominance of odd-chain or even-chain markers. In fact, LCFA markers had between 3.4 (heather), 10.2 (*L. perenne*) and 10.9 (*U. gallii*) times higher total concentrations than the alkanes. These results are consistent with those found in these plant species in previous reports by Ferreira et al. (2009a, 2010)

Table 3

Mean ($\pm$ standard deviation) alkane recovery in faeces of equines and cattle (3 animals per diet) according to carbon-chain length and apparent *in vivo* DM digestibility (DMDap, g/kg).

Species	Diet	Alkane carbon-chain length								DMDap
		C ₂₅	C ₂₆	C ₂₇	C ₂₈	C ₂₉	C ₃₀	C ₃₁	C ₃₃	
Equines	E1	0.94 $\pm$ 0.058	1.06 $\pm$ 0.176	0.87 $\pm$ 0.035	1.01 $\pm$ 0.073	0.88 $\pm$ 0.025	0.86 $\pm$ 0.158	0.88 $\pm$ 0.038	0.85 $\pm$ 0.023	575 $\pm$ 45.1
	E2	1.03 $\pm$ 0.105	1.19 $\pm$ 0.045	0.95 $\pm$ 0.081	1.09 $\pm$ 0.117	0.95 $\pm$ 0.080	0.92 $\pm$ 0.028	0.95 $\pm$ 0.072	0.92 $\pm$ 0.053	553 $\pm$ 39.8
	E3	1.12 $\pm$ 0.070	1.12 $\pm$ 0.062	0.99 $\pm$ 0.064	1.04 $\pm$ 0.052	0.95 $\pm$ 0.036	0.99 $\pm$ 0.032	0.95 $\pm$ 0.022	0.98 $\pm$ 0.051	420 $\pm$ 4.8
	E4	1.16 $\pm$ 0.018	1.09 $\pm$ 0.040	1.04 $\pm$ 0.038	1.03 $\pm$ 0.054	0.98 $\pm$ 0.017	1.02 $\pm$ 0.031	1.01 $\pm$ 0.025	1.05 $\pm$ 0.044	394 $\pm$ 2.2
	Mean	1.06	1.12	0.96	1.04	0.94	0.95	0.95	0.95	485
	SEM	0.031	0.028	0.024	0.021	0.016	0.028	0.018	0.025	25.9
	CV	10.2	8.6	8.7	7.0	5.8	10.1	6.5	9.0	18.5
Cattle	C1	0.73 $\pm$ 0.028	0.82 $\pm$ 0.025	0.79 $\pm$ 0.020	0.86 $\pm$ 0.010	0.91 $\pm$ 0.015	0.95 $\pm$ 0.013	0.97 $\pm$ 0.012	0.99 $\pm$ 0.016	592 $\pm$ 9.6
	C2	0.84 $\pm$ 0.019	1.00 $\pm$ 0.045	0.89 $\pm$ 0.014	0.90 $\pm$ 0.060	0.88 $\pm$ 0.024	0.98 $\pm$ 0.031	0.96 $\pm$ 0.029	1.11 $\pm$ 0.032	503 $\pm$ 22.6
	Mean	0.78	0.91	0.84	0.88	0.89	0.96	0.96	1.05	548
	SEM	0.026	0.042	0.022	0.018	0.009	0.010	0.009	0.029	20.8
	CV	8.2	11.2	6.5	4.9	2.5	2.6	2.2	6.8	9.3
	Diff. ^a	0.28	0.21	0.12	0.16	0.05	−0.01	−0.01	−0.10	−62.4

^a Diff. mean difference in FR and DMDap between equines and cattle; E1 and C1: *Lolium perenne* (1.00); E2 and C2: *L. perenne* (0.70) + heather (0.30); E3: *L. perenne* (0.70) + *U. gallii* (0.30); E4: *L. perenne* (0.40) + *U. gallii* (0.30) + heather (0.30); L and Q indicate linear or quadratic significance term for alkane and Diet alkane effects; SEM: standard error of mean.

and in native Chinese grass species (*Leymus chinensis*, *Leymus dasystachys* and *Elymus sibiricum*) by Lin et al. (2009). Also Ali et al. (2005) observed higher LCFA total concentrations in 25 rangeland species from Sudan, ranging between a minimum of 2.8 (*Ocimum basilicum*) to a maximum of 38.5 (*Balanites aegyptiaca*) times higher LCFA total concentrations. Differences between plant species in total marker concentrations were also observed. Heather had the highest total concentrations for both LCFA and alkanes, whereas *U. gallii* was characterized by lower total marker concentrations. It should be noted that, in general, individual LCFA concentrations were higher than 100 mg/kg of DM, while only the alkane C₃₁ exceeded this value in all plant species. Very low concentrations are considered an important limitation for any chemical compound to be used as a diet composition marker as their less accurate analytical measurements could interfere with diet composition estimation. Besides the herbaceous species and *U. gallii* used in this study, *Phalaris aquatica* (Charmley and Dove, 2007) and the conifers *Picea* and *Pinus* (Dove and Mayes, 2006) are plant species known to be characterized by very low alkane concentrations.

Results also showed that the even-chain LCFA prevailed over the odd-chain ones, representing over 83% of the total

LCFA content. These results contrast with those obtained for the alkane markers, where the odd-chain accounted for more than 89% of the total alkane concentrations. Although data on odd-chain LCFA are not presented, Dove and Mayes (2006) suggested the dominance of even-chain fraction not only in LCFA but also in LCOH markers.

4.2. Marker faecal recovery

Although some LCFA and alkane markers had a faecal recovery of 1.0, in general the results showed an incomplete faecal recovery of both markers in the faeces of equines and cattle. Faecal recovery values above 1.0 are as “biologically” unexplainable since to date there is no evidence of synthesis of alkane and fatty acids within this range of carbon-chain length in the digestive tract of both ruminant and non-ruminant species. In the case of alkanes, these results could be due to a higher degree of potential analytical error associated with their very low concentrations in the diet components and faeces (Dove and Mayes, 2005), especially for the even-chain and shorter alkanes, which could have led to more inaccurate estimates of their faecal recovery.

Table 4

Effect of diet composition, animal species, LCFA carbon-chain length and their interaction on LCFA faecal recovery.

Effects	LCFA data			Effects	Equines			Cattle		
	All	Odd-	Even-		All	Odd-	Even-	All	Odd-	Even-
D	<0.001	<0.001	<0.001	D	<0.001	0.006	<0.001	<0.001	<0.001	<0.001
AS	<0.001	<0.001	<0.001							
CCL	<0.001	<0.001	<0.001	CCL	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
L	<0.001	<0.001	<0.001	L	<0.001	0.413	<0.001	<0.001	<0.001	0.025
Q	<0.001	<0.001	<0.001	Q	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
D*AS	<0.001	<0.001	<0.001							
D*CCL	<0.001	<0.001	<0.001	D*CCL	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
L	0.029	<0.001	<0.001	L	<0.001	0.739	<0.001	0.748	0.661	0.355
Q	<0.001	<0.001	<0.001	Q	<0.001	0.078	<0.001	<0.001	0.067	<0.001
AS*CCL	<0.001	<0.001	<0.001							
L	<0.001	<0.001	<0.001							
Q	0.171	0.055	0.224							

D diet; AS animal species; CCL carbon-chain length; L linear; and Q quadratic.

Table 5

Effect of diet composition, animal species, alkane chain length and their interaction on alkane faecal recovery.

Effects	Alkane data			Effects	Equines			Cattle		
	All	Odd-	Even-		All	Odd-	Even-	All	Odd-	Even-
D	<0.001	<0.001	<0.001	D	<0.001	<0.001	0.189	<0.001	<0.001	<0.001
AS	<0.001	0.156	<0.001							
CCL	<0.001	<0.001	0.001	CCL	<0.001	<0.001	<0.001	<0.001	<0.001	0.005
L	0.125	0.067	<0.001	L	<0.001	<0.001	<0.001	<0.001	<0.001	0.025
Q	0.002	<0.001	0.576	Q	0.010	<0.001	0.726	0.057	0.024	0.007
D*AS	0.552	0.449	0.889							
D*CCL	<0.001	<0.001	0.601	D*CCL	0.358	0.668	0.499	<0.001	<0.001	0.005
L	<0.001	<0.001	0.293	L	0.605	0.683	0.253	0.003	0.164	0.003
Q	0.006	0.001	0.863	Q	0.168	0.086	0.764	<0.001	<0.001	0.088
AS*CCL	<0.001	<0.001	<0.001							
L	<0.001	<0.001	<0.001							
Q	0.698	0.955	0.057							

D diet; AS animal species; CCL carbon-chain length; L linear; and Q quadratic.

Our data suggest that LCFA and alkanes behave differently in the digestive tract of equines and cattle. Although to the authors' knowledge there are no published faecal recovery data of LCFA in equines, results obtained for alkanes in non-ruminant species (Sehested et al., 1999; Ordakowski et al., 2001; Monks et al., 2005) showed absence of any systematic relationship between chain length and the faecal recovery. By

contrast, Smith et al. (2007) and Ferreira et al. (2009b) observed an increase of alkane faecal recovery with alkane carbon-chain length in equids.

The comparison of marker faecal recovery between animal species indicated that, in general it was higher in equines, except for the longer alkanes (C₃₀ to C₃₃) as a result of a positive association between carbon-chain length and the faecal recovery of both markers in cattle. A similar trend was observed for other ruminant species in LCFA (Ali et al., 2004; Ferreira et al., 2009a, 2010), alkanes (Ferreira et al., 2005; Charmley and Dove, 2007; Elwert et al., 2008) and LCHO (Dove and Charmley, 2008). Differences between animal species in the faecal recovery pattern of both markers could have an impact on subsequent diet composition estimates based on uncorrected marker faecal concentrations. In fact, it is expected that cattle diet composition estimates can be more prone to bias towards the plant species with predominance of longer markers with higher faecal recoveries.

Results also showed an effect of diet composition on the faecal recovery of both markers in both animal species. Although Ferreira et al. (2007b) referred that this effect will depend on the particular species comprising the diets, these results are similar to those obtained for these markers in goats (Ferreira et al., 2005, 2009a) and sheep (Ferreira et al., 2010) fed on the same vegetation species used in the present trial. Also, Charmley and Dove (2007) observed a diet composition effect on the alkane faecal recovery of sheep fed with diets comprising *Trifolium subterraneum*, *P. aquatica*, *Lolium rigidum* and *Triticum aestivum* straw. By contrast, a lack of diet composition effect on the alkane faecal recovery was observed in other studies conducted in cattle (Hendricksen et al., 2003; Brosh et al., 2003), goats (Brosh et al., 2003) and sheep (Elwert et al., 2008).

In equines, the FR of both markers was negatively related with diet digestibility as a decrease of the disappearance of markers from the digestive tract with a decrease of diet digestibility was observed (Fig. 4). The lowest diet digestibility values were observed in diets composed by the woody species (i.e., heather and *U. gallii*), characterized by high levels of lignin (mean content of 341 and 217 g/kg DM). As these phenolic compounds are responsible for the linkages of cuticle and the cell wall components (Himmelsbach, 1993), it is expectable that cuticular waxes will be less accessible to the degradation in vegetation species with higher lignin content. These findings are in

Fig. 1. Relationship between the carbon-chain length of long-chain fatty acids (LCFA) and alkanes with their faecal recovery observed in the equine groups (E1 to E4).

Fig. 2. Comparisons of known and estimated proportions of dietary components obtained using different marker data (LCFA, alkanes or LCFA + alkanes) and faecal recovery corrections (FC0, FC1 and FC2) for the equine groups (E1 to E4).

agreement with those obtained elsewhere for the alkane markers (Ferreira et al., 2005; Charmley and Dove, 2007; Elwert et al., 2008). In opposition, results in cattle were rather contradictory. Although a higher but not significant alkane faecal recovery was observed in the diet with lower digestibility (i.e., *L. perenne* and heather), in the LCFA a positive association between diet digestibility and LCFA faecal recovery was detected. It should be noted that the range of diet digestibility in cattle was low (503 to

592 g/kg DM) and, for that reason, a consistent conclusion cannot be drawn.

4.3. Diet composition estimates

Results obtained in this study for both animal species showed higher levels of accuracy of diet composition estimates when LCFA and alkanes were combined, suggesting

Fig. 3. Comparisons of known and estimated proportions of dietary components obtained using different marker data (LCFA, alkanes or LCFA + alkanes) and faecal recovery corrections (FC0, FC1 and FC2) for the cattle groups (C1 to C2).

Fig. 4. Relationship between diet digestibility and faecal recovery of the LCFA C₂₂, C₂₃, C₂₇, C₂₈, C₃₀, C₃₂ and C₃₄ observed in the equine diets.

that an increase in the discriminatory power between plant species was achieved with this combination. These results are consistent with those obtained by Kelman et al. (2003) and

Dove and Charmley (2008) when combining alkanes with LCOH, and Ferreira et al. (2009a, 2010) when alkane and LCFA data were used together to estimate diet composition of goats

and sheep, respectively, fed on the same vegetation components used in the present trial.

The combination of markers is usually suggested in situations where the number of plant species available for animal consumption exceeds the number of alkanes or LCFA available for the calculations (Ferreira et al., 2009b) or when difficulties in distinguishing plant species occur due to a similar marker profiles. For example, Ferreira et al. (2009b) observed similarities in the alkane pattern of *Agrostis-Poa* leaf fraction, *L. perenne*, *T. repens* and “other species” that made their discrimination difficult. Also Oliván et al. (2007) found difficulties in differentiating herbaceous species (*L. perenne*, *P. longifolium* and *A. capillaris*) and gorse when alkane markers were used. In these situations, Ferreira et al. (2009a) suggested that the combination of several faecal markers (alkanes, LCFA, LCOH or alkenes) can provide a more specific fingerprint to each plant species, increasing the discriminatory power between them. However, Dove and Charmley (2008) indicated that the combination of markers does not always result in more accurate estimates of diet composition. In the present study, the combination of markers resulted in the most accurate estimates of diet composition for diets E2 (KSI of 97.1%), E4 (KSI of 96.6%) and C2 (98.7%), while LCFA estimates of diet composition were more precise for the animal groups fed only with *L. perenne* (i.e., E1 and C1 with mean KSI values of 97.1 and 98.1%, respectively). By contrast, although PCA results showed resemblances in the alkane profiles of *L. perenne* and *U. gallii*, and their clear discrimination when using LCFA markers, alkane diet composition estimates for E3 group (mean KSI value of 96.6%) were more accurate than those obtained by the LCFA (88.6%) or LCFA + alkanes (94.4%). Further investigation showed a lower correlation between the LCFA profiles of *L. perenne* and *U. gallii* (0.414) than that obtained between their alkane profiles (0.837). These results suggest that PCA analysis on markers profiles should be analysed with caution as a clear discrimination between plant species with this statistical procedure does not mean that accurate estimates of diet composition will be obtained.

The present study can illustrate a practical situation where LCFA or alkanes are selected for a diet composition estimation, and not their combination, as a very low number of possible diet components was used. Results showed that the use of both markers in separate resulted in accurate estimates of diet composition, although higher KSI values were observed with LCFA. These results are in opposition with the higher ability of alkanes than LCFA to estimate diet composition in sheep (Ali et al., 2005; Ferreira et al., 2010) and goats (Ferreira et al., 2009a). In our opinion, the very low alkane concentrations presented in all vegetation components could explain these results as they are more prone to measurement errors (Brosh et al., 2003; Dove and Mayes, 2006), making the discrimination between plant species more difficult (Charmley and Dove, 2007). As *L. perenne* and *U. gallii* used in this study, other plant species possess very low alkane concentrations (Charmley and Dove, 2007; Ferreira et al., 2009b), especially as the grazing season progressed due to a maturity stage effect on alkane concentrations (Dove et al., 1996), possibly related to a decrease of the leaf fraction in the whole plant, characterized by higher alkane concentrations than the stem fraction (Smith et al., 2001). According to Ferreira et al. (2010) a different ability of markers to distinguish plant species of different diets could explain the lack of superiority of one type of marker to another across the diets used in the present trial.

As expected, for all markers (i.e., LCFA, alkanes and LCFA + alkanes) the most accurate estimates of diet composition were obtained when using treatment mean recovery corrections (i.e., FC1), with mean KSI indexes above 96% for both animal species, indicating that a suitable correction for incomplete faecal recovery should be used before the application of these markers for diet composition estimation. Higher accuracy can be obtained if individual recovery data are used as they only reflect differences between marker profiles of the diet offered and the diet actually consumed by each animal, and a possible analytical error (Dove and Charmley, 2008). Similar levels of accuracy were observed in studies performed with alkanes (Charmley and Dove, 2007; Ferreira et al., 2005) and LCFA (Ali et al., 2005; Ferreira et al., 2009a, 2010) when this type of faecal correction was used.

Results obtained in this study showed similar accuracy of diet composition estimates obtained using uncorrected marker concentrations (FC0) or those corrected with mean recovery rate across all experimental diets (FC2), differing from those obtained with FC1. Discrepancies between known diet composition proportions and those estimated using FC2 were the result of a high and significant between diet variation (Ferreira et al., 2009a). In a practical perspective, FC2 is generally used to correct marker faecal concentrations as more accurate estimates of faecal recovery (i.e., individual data and FC1) are difficult to obtain in grazing studies. Data showed that if overall recovery data is used in field studies there will be a loss of accuracy in diet composition estimates. In our opinion, the high dependence of these markers on the use of suitable estimates of faecal recovery can be overcome by an increase of the number of markers (Ferreira et al., 2009a), providing a more specific fingerprint for each diet component, as also indicated by Ali et al. (2005) and Lin et al. (2009). To illustrate our suggestion, in this study the accuracy of diet composition estimates obtained with the less suitable faecal corrections (i.e., FC0 and FC2) was higher when LCFA and alkanes were combined than when markers were considered alone, for both animal species. Moreover, the proportion of heather in the diets (E2, E4 and C2) was, in general, estimated with accuracy as a result of a very distinct LCFA and alkane profiles.

Surprisingly, although the association between LCFA and alkane faecal recoveries and carbon-chain length differed between equines (negative) and cattle (positive), diet composition estimates obtained when applying FC0 showed similar trends for both animal species. In general, the proportion of *L. perenne* in the diets was overestimated by the LCFA both in equines and cattle, when using uncorrected faecal concentrations. By contrast, the use of FC0 in alkanes led to an underestimation of *L. perenne* in both animal species. Although the impact of faecal correction will be a balance between the effect of concentration and the bias toward the markers with higher concentrations, LCFA estimates can be explained by specific features that *L. perenne* had: 1) highest concentration on shorter LCFA (C_{22} – C_{27} , 0.648) that had higher faecal recoveries in equines, and 2) highest concentration on even-chain LCFA (0.881) that had higher faecal recoveries in cattle. Thus, it seemed that LCFA estimates tended for the plant species with predominance on the LCFA that had higher faecal recovery, as previously observed by Ferreira et al. (2009a, 2010). Similarly, FC0 alkane estimates in equines tended for the plant species with higher concentration on shorter alkanes (*U. gallii*, 0.177),

whereas in cattle they tended for the plant species with higher concentration on the longer ones (heather, 0.952).

Ferreira et al. (2009a) and Dove and Mayes (2005) indicated that faecal recovery correction would have little impact on the accuracy of diet composition estimates when marker recoveries are unaffected by the carbon-chain length, usually observed in non-ruminant herbivores. Results obtained in E4 group when using LCFA markers illustrate this feature, as recovery was not affected by LCFA chain length and accurate estimates of diet composition were obtained.

5. Conclusions

Results obtained in this study show a different behaviour of markers in the digestive tract of both animal species. The faecal recovery of both markers was negatively and positively associated with carbon-chain length in equines and cattle, respectively. Results also show that long LCFA can be used as faecal markers to estimate diet composition of equines and cattle fed with herbaceous and woody species if a previous and suitable correction of marker faecal concentrations to incomplete faecal recovery is applied. In our opinion LCFA can be particularly useful in situations where alkane concentrations are low, in particular for much of the plant species found in heathland vegetation communities. Moreover, the use of a more specific fingerprint, provided by marker combination, can result in an improvement of the accuracy of diet composition estimates.

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