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# Evaluation of very long-chain fatty acids and *n*-alkane epicuticular compounds as markers for estimating diet composition of sheep fed heathland vegetation species

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ABSTRACT

Application of very long-chain fatty acids (VLCFA) as an alternative or a complement to the alkane markers to estimate diet composition of sheep offered six different diets was evaluated. Twenty-four adult crossbred sheep were housed in metabolic crates and fed with diets composed of different proportions of herbaceous (*Lolium perenne* and *Trifolium repens*) and woody species (*Erica* spp., *Calluna vulgaris* and *Ulex gallii*). Diet composition was estimated from VLCFA (i.e., $C_{22}$–$C_{34}$) and alkane (i.e., $C_{25}$–$C_{33}$) concentrations in diet and faeces, by least-squares procedures, considering the plant species effectively offered to the animals (C1) or these plant species plus feeds that were not part of the diets but were available in the field at each experimental period (C2). Prior to this, faecal VLCFA and alkane concentrations were uncorrected for incomplete faecal recovery (FC0), corrected using recovery data for each individual ewe (FC1), corrected using mean recovery rate of the dietary treatment that the animal belonged to (FC2), or corrected using mean recovery rate across all experimental diets (FC3). For all diet components, total VLCFA concentrations were higher ($P<0.01$) than those observed for alkanes, with a predomination of even-chain ($P<0.001$) over odd-chain. Faecal recovery was incomplete and tended to increase with carbon-chain length in a curvilinear ($P<0.001$) fashion for the even-chain VLCFA, and a linear ($P<0.001$) one for odd-chain VLCFA and alkanes. Diet composition had an effect ($P<0.001$) on faecal recovery of both markers. Estimates of diet composition obtained using alkanes alone were more accurate ($P<0.05$) than those obtained with VLCFA alone while their combination resulted in an improvement ($P<0.05$) of the accuracy. Estimates obtained when considering plant species offered effectively to the animals were more accurate ($P<0.001$) than those obtained when considering more plant species as possible diet components. In general, use of faecal marker concentrations corrected with FC2 and FC3 provided equally accurate estimates of diet composition, indicating that an overall recovery data can be applied to these markers in grazing studies without losing accuracy in the estimation. Although the use of alkanes resulted in more accurate estimates of diet composition, results obtained in this study showed that VLCFA can be useful markers for studying diet selection of sheep

*Abbreviations:* FR, faecal recovery; DM, dry matter; DMDap, apparent DM digestibility; KSI, Kulcyznski similarity index; LW, live weight; PCA, Principal Components Analysis.

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grazing on shrubland vegetation communities. However, a suitable adjustment of their faecal concentrations prior to their application is recommended. It is also concluded that use of VLCFA in combination with alkanes could result in more accurate estimates of diet composition.



## 1. Introduction

Large areas of the northwest of the Iberian Peninsula are covered by shrubland vegetation communities, dominated by woody species (i.e., *Erica* spp., *Calluna vulgaris* and *Ulex gallii*) of very low nutritive value for livestock (Celaya et al., 2008; Mandaluniz et al., 2009). The abandonment of agricultural and livestock management initiated in the 1950s, led to an increase of these heathland areas, reaching in some Portuguese and Spanish regions more than 40–50% of total area (Álvarez et al., 2004; DGRF, 2007). This woody phytomass of high combustibility and flammability is the main fuel of wild fires observed in these areas, causing serious environmental problems as well as direct and indirect economic losses associated with cost of prevention and extinction of fires. Appropriate use of these vegetation communities by domestic herbivore species could provide both environmental and economical benefits by controlling shrub encroachment and thus reducing fire risks (Jáuregui et al., 2009) and by promoting typical animal products. The incorporation of improved pasture areas to these heathlands is recommended to meet the nutritional requirements of productive livestock. Based on previous results (Osoro et al., 1999; Celaya et al., 2008), it seems that small ruminants are more appropriate for maintaining sustainable animal production systems on these heathland–grassland associations. Particularly, sheep have been regarded as the most productive in this situation and have demonstrated the ability to include green shoots of heather and gorse in their diets, when the herbage availability is very low and when gorse is at early stages of development with a high crude protein and low fibre content (Celaya et al., 2007, 2008).

Knowledge on the impact of livestock production systems in these plant communities (i.e., grazing behaviour-diet selection) is essential to improve its management, aiming to enhance, simultaneously, livestock production and biodiversity of the ecosystem. In our laboratory, estimation of diet composition of domestic herbivores has been performed using the alkane markers (Ferreira et al., 2005, 2007a,b). Results showed that the proportion of heath species (i.e., *Erica* spp., *C. vulgaris*) in the animals' diets can be accurately estimated even if uncorrected alkane faecal concentrations are used, as a result of their distinct alkane profile. However, it seems that discrimination between herbaceous species and/or herbaceous species and gorse (*U. gallii*) is less clear due to similarities in their alkane profile, leading to diet composition estimation more prone to bias, especially when less accurate faecal recovery data is applied. An increase in the discriminatory power between these plant species can be accomplished by combining the alkanes with other epicuticular wax components such as very long-chain fatty acids (VLCFA) or long-chain fatty alcohols (LCOH) (Bugalho et al., 2004; Dove and Mayes, 2006).

The objective of this work was to evaluate the potential of using either VLCFA or VLCFA + alkanes as markers for estimating diet composition of sheep fed on diets consisting of grass–clover feeds combined with heather–gorse communities, and to compare these estimates with those obtained using only the alkane markers (Ferreira et al., 2007b). The effect of using different sets of faecal recovery data on both markers was evaluated. Additionally, the effect of including other plant components besides those effectively offered to the animals on the accuracy of the estimations was assessed.

## 2. Materials and methods

### 2.1. Experimental site and design

This experiment was carried out at the Carbayal Research Station, situated at 900–1000 m above sea level, at San Isidro's Mountain, Illano, Asturias, Spain (longitude 6°53′, latitude 43°21′), where the vegetation consists of natural shrubland of heather–gorse communities (*Calluno-Ulicetea*) interspersed with areas of improved pastures of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). The natural vegetation is dominated by short heather species (*Erica umbellata*, *E. cinerea* and *Calluna vulgaris*), tall heather (*E. australis* and *E. arborea*), and gorse (*U. gallii*), a thorny and woody legume.

Six experimental diets composed of different proportions of herbaceous species (*L. perenne* and *T. repens*) and woody species (*U. gallii* and heather species) were offered to six different groups of four non-lactating ewes, housed individually in metabolic crates, in three distinct periods of 11 days carried out in May, June and July of 2003, to make use of current season's green shoots of the main plant species, which are selected by sheep on these vegetation conditions (Celaya et al., 2008).

Each experimental period comprised a 7-day period for adaptation of the animals to the diets and experimental conditions, followed by a 4-day period of collection of samples of faeces and diet components. Freshly cut vegetation samples (*L. perenne*, *T. repens* and green shoots of the woody species) were harvested from random sites within the experimental field and were offered twice a day (9:00 and 18:00 h) in equal proportions to the animals. No refusals were allowed as the botanical separation of the diet components in the refusals would be very difficult to accomplish. One sample of the total faecal output was collected every 2 days and a daily representative sample of each diet component was collected before the morning meal.

### 2.2. Animals and diets

In May, 4 adult non-lactating crossbred (Gallega*Latxa) ewes (S1: 26 ± 2.3 kg live weight, LW) received a daily total amount of 1.0 kg dry matter (DM)/100 kg LW of a diet composed only of *Lolium perenne*.

In June, 16 adult non-lactating crossbred (Gallega*Latxa) ewes were divided in 4 groups of 4 animals each: S2 (24 ± 1.7 kg LW) received a diet composed of *L. perenne* (0.70) and *U. gallii* (0.30); S3 (25 ± 0.3 kg LW) diet included *L. perenne* (0.40), heather (0.30) and *U. gallii* (0.30); S4 (27 ± 0.8 kg LW) was fed on *L. perenne* (0.71) and heather (0.29); S5 (29 ± 1.4 kg LW) received a diet composed of *L. perenne* (0.41), *T. repens* (0.28) and heather (0.30). Sheep received a daily total amount of 0.8 kg DM/100 kg LW. As the *L. perenne* used in this experimental period was in the reproductive stage, the spike and leaf/stem fractions were separated and only the leaf/stem fraction was offered to the animals. The botanical composition of the heather component represented the field proportion of different plant species of the *Ericaceae* family, namely *E. umbellata*, *E. cinerea* and *C. vulgaris*. Among the woody species, *U. gallii* was considered as an individual vegetation component since it is a legume and the nutritive value and the *n*-alkane profile are different from the *Ericaceae* (Celaya et al., 2008).

In July, 4 adult non-lactating crossbred (Gallega*Latxa) ewes (S6: 30 ± 0.5 kg LW) received 0.8 kg DM/100 kg LW of a diet composed of herbaceous species *L. perenne* (0.70) and *T. repens* (0.30). Similarly to the second experimental period only the leaf/stem fraction of *L. perenne* was offered to the animals.

### 2.3. Chemical analysis

Samples of the diet components and faeces were immediately dried on the day of collection, using a forced-air oven at 60 °C for 72 h, for DM determination. Samples for alkane analyses were immediately frozen at −20 °C and then freeze-dried and milled through a 1-mm screen. After alkane analysis (Ferreira et al., 2007b), all samples were vacuum-packed in plastic containers. Before VLCFA analysis, samples of plant species used in each experimental period and faeces of each animal of each experimental period were composited, resulting in 1 sample per diet component and per animal.

#### 2.3.1. Very long-chain fatty acids analysis

Very 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 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 VLCFA extract, 2 mL of heptane were added and 0.5 mL of the extract were 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 VLCFA 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 VLCFA to their fatty acid methyl esters (FAME), 1 mL toluene and 2 mL of methanol, previously acidified with sulphuric acid, were added to the dried elute and 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 for chromatographic analysis.

Analysis of the VLCFA ($C_{22}$–$C_{34}$) was carried out by gas chromatography–mass spectrometry (GC–MS), using an Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) GC equipped with a 5973N mass spectrometer and an Agilent 7683 automatic liquid sampler injector. The target analytes were separated using a DB-1 capillary column, 30 m × 0.25 mm with 0.25 μm film thickness (Agilent Technologies, USA). Injection volume was 1 μL (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 VLCFA concentrations were quantified relative to known amounts of the internal standard hentriacontanoic acid ($C_{31}$) added at the beginning of the extraction procedure.

### 2.4. Calculations

Faecal recovery (FR) of each marker (i.e., VLCFA and alkanes) was calculated for each animal as the proportion of marker consumed in the diet which was recovered in the faeces.

The individual diet composition estimates were obtained using an optimization procedure which minimizes the sum of squared discrepancies between the actual VLCFA, alkane and VLCFA + alkane proportions in faeces (adjusted or not for incomplete faecal recoveries) and estimated proportions (different combinations of diet components), according to Salt et al. (1994), based upon the individual marker concentrations expressed as proportions of the total. These calculations were performed considering only the plant species effectively offered to the animals (C1) or these plant species plus feeds that were not part of the diets but that were available in the field at each experimental period (heather and *U. gallii* in May, and

*L. perenne* spike, heather and *U. gallii* in July) (C2). Estimates were based on VLCFA alone ($C_{22}$–$C_{34}$), alkanes alone ($C_{25}$–$C_{33}$) or a combination of LCFA with alkanes.

For these calculation methodologies, different VLCFA and alkane faecal recovery corrections were applied being: (1) VLCFA and alkane faecal concentrations without any correction for incomplete faecal recovery (FC0); (2) VLCFA and alkane faecal concentrations corrected with individual data (FC1); (3) VLCFA and alkane faecal concentrations corrected using mean recovery rate of the dietary treatment that the animal belonged to (FC2); and (4) VLCFA and alkane faecal concentrations corrected using mean recovery rate across all experimental diets (FC3).

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

$$\mathrm{DMD_{ap}\ (g/kgDM)} = \frac{I - F}{I} \times 1000 \quad (1)$$

where $I$ is the total DM intake and $F$ is the total DM faecal output.

### 2.5. Statistical analysis

Statistical analyses were performed using GenStat (2008). Differences between plant species in VLCFA, alkane and VLCFA + alkane profiles were explored using Principal Components Analysis (PCA). To assess if the discriminatory information provided by the VLCFA 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). The OPR rotates the axes of each PCA output in an attempt to minimize the residual sum of squares between the PCA scores based on VLCFA and those based on alkanes. Large values for unexplained residual variance after OPR imply that the two classes of marker are providing different types of discriminatory information.

Effects of diet composition and carbon-chain length on recovery of each VLCFA and alkane in faeces 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.

In order to assess the accuracy of diet composition estimates as a whole, overlap in diet composition between known values and those estimated using different sets of FR correction was calculated by the Kulczynski similarity index (Krebs, 1989) as:

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

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

## 3. Results

### 3.1. LCFA content of diet components

The VLCFA and alkane content of diet components used in each experimental period are presented in Table 1. In addition to the plant species offered to the animals, VLCFA and alkane contents of feeds available in the field at each experimental period (heather and *U. gallii* in May, and *L. perenne* spike, heather and *U. gallii* in July) that were not part of the diets, but that were included as possible diet components in the calculations, are also presented. The aim of this inclusion was to test if both markers (i.e., VLCFA and alkanes) and their combination would be able to estimate correctly the diet consumed by sheep even though some vegetation components (plant parts and/or plant species) are absent from the diet but are considered as possible diet components in the calculations. The concentrations of alkanes $C_{22}$- and $C_{34}$- and $C_{31}$-VLCFA are not shown as they were used as internal standards in GC–MS analyses and $C_{33}$-VLCFA was absent in all vegetation species.

With the exception of *U. gallii* in June, total VLCFA concentrations exceeded 1000 mg/kg DM in all diet components and were higher ($P$<0.01) than those observed for alkanes. Highest total VLCFA concentration was observed in *T. repens* in July (3977 mg/kg DM) while the other herbaceous species (*L. perenne*) had a mean total VLCFA concentration of 1820 mg/kg DM. Total VLCFA concentration in heather exceeded 3000 mg/kg DM across all periods while the other woody species (*U. gallii*) had the lowest mean total VLCFA concentration (1262 mg/kg DM).

Even-chain VLCFA predominated ($P$<0.001) over odd-chain ones, representing between 0.80 (*U. gallii*) and 0.93 (heather) of total VLCFA. *U. gallii* was characterized by high amounts of VLCFA of low carbon-chain length, with $C_{22}$ representing a mean of 0.30 of total VLCFA content. Heather and *L. perenne* VLCFA profiles were only distinguishable in the longer fatty acids ($C_{30}$–$C_{34}$) where heather had higher concentrations (i.e., 0.30 vs. 0.19 of total VLCFA content). *T. repens* was characterized by very high odd-chain VLCFA concentrations.

The extent to which diet components (marker profiles) could be discriminated was evaluated using principal component analysis (PCA, data not shown). In May, the first two principal components (PC) explained 1.0 of the variance among plant

**Table 1**
Mean very long-chain fatty acids (VLCFA) and alkane content (mg/kg DM) of the vegetation components used in the diet composition calculations at each experimental period.

| | Period 1 (May) | | | Period 2 (June) | | | | Period 3 (July) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *L. perenne* | Heather | *U. gallii* | *L. perenne*[a] | *T. repens* | Heather | *U. gallii* | *L. perenne*[a] | *L. perenne*[b] | *T. repens* | Heather | *U. gallii* | SD[c] |
| *VLCFA concentrations* | | | | | | | | | | | | | |
| $C_{22}$ | 514 | 594 | 447 | 322 | 438 | 583 | 319 | 252 | 284 | 613 | 700 | 347 | 30.1 |
| $C_{23}$ | 55 | 42 | 144 | 59 | 162 | 42 | 98 | 80 | 71 | 289 | 55 | 95 | 8.5 |
| $C_{24}$ | 382 | 487 | 309 | 267 | 529 | 475 | 233 | 257 | 285 | 716 | 556 | 164 | 17.7 |
| $C_{25}$ | 57 | 59 | 75 | 41 | 107 | 60 | 51 | 50 | 52 | 182 | 68 | 54 | 4.6 |
| $C_{26}$ | 559 | 516 | 128 | 259 | 451 | 440 | 54 | 187 | 158 | 608 | 516 | 68 | 24.8 |
| $C_{27}$ | 55 | 56 | 24 | 28 | 71 | 49 | 10 | 28 | 23 | 130 | 64 | 20 | 3.7 |
| $C_{28}$ | 396 | 476 | 114 | 247 | 584 | 432 | 52 | 204 | 183 | 792 | 572 | 156 | 30.1 |
| $C_{29}$ | 55 | 85 | 43 | 30 | 73 | 90 | 24 | 34 | 23 | 140 | 85 | 98 | 4.9 |
| $C_{30}$ | 287 | 481 | 134 | 228 | 390 | 559 | 111 | 171 | 139 | 440 | 445 | 324 | 18.4 |
| $C_{32}$ | 128 | 365 | 26 | 87 | 80 | 330 | 27 | 74 | 41 | 65 | 449 | 35 | 10.3 |
| $C_{34}$ | 32 | 83 | 0 | 20 | 12 | 69 | 1 | 16 | 1 | 1 | 127 | 1 | 3.8 |
| Total | 2521 | 3243 | 1448 | 1588 | 2896 | 3127 | 978 | 1352 | 1259 | 3977 | 3634 | 1361 | 82.8 |
| *Alkane concentrations* | | | | | | | | | | | | | |
| $C_{25}$ | 20 | 26 | 5 | 21 | 11 | 37 | 3 | 11 | 85 | 16 | 11 | 2 | 0.4 |
| $C_{26}$ | 5 | 9 | 3 | 4 | 4 | 11 | 2 | 2 | 7 | 4 | 5 | 2 | 0.2 |
| $C_{27}$ | 40 | 80 | 39 | 46 | 27 | 116 | 19 | 22 | 112 | 38 | 49 | 10 | 0.7 |
| $C_{28}$ | 13 | 20 | 11 | 12 | 10 | 25 | 8 | 7 | 12 | 11 | 15 | 6 | 0.6 |
| $C_{29}$ | 178 | 359 | 111 | 190 | 93 | 701 | 67 | 124 | 219 | 170 | 278 | 43 | 2.5 |
| $C_{30}$ | 19 | 51 | 19 | 17 | 10 | 71 | 17 | 11 | 20 | 17 | 40 | 12 | 0.3 |
| $C_{31}$ | 274 | 1143 | 270 | 273 | 124 | 1223 | 309 | 235 | 338 | 207 | 900 | 157 | 5.4 |
| $C_{32}$ | 12 | 78 | 7 | 8 | 6 | 80 | 10 | 5 | 8 | 7 | 69 | 5 | 1.1 |
| $C_{33}$ | 115 | 596 | 10 | 68 | 50 | 518 | 10 | 39 | 22 | 22 | 599 | 5 | 3.8 |
| Total | 699 | 2378 | 479 | 637 | 334 | 2781 | 444 | 463 | 849 | 501 | 1985 | 245 | 12.7 |

[a] Leaf/stem.
[b] Spike.
[c] Mean standard deviation of replicates for each marker.

components when using either data on VLCFA, alkanes or VLCFA combined with alkanes, with a clear discrimination between plant components. In June, the variance in VLCFA and VLCFA + alkanes profiles between diet components explained by the first two PC was similar (0.97) and lower than that obtained using only alkane data (1.0). Heather and *T. repens* showed very distinct VLCFA and VLCFA + alkane profiles while some similarities were observed between *L. perenne* and *U. gallii*. In the third experimental period, 0.94, 0.97 and 0.81 of the variance between diet components was explained by the first two PC when data on VLCFA, alkanes and VLCFA combined with alkanes were used, respectively. Heather was always clearly discriminated from the other four plant components, using either VLCFA or alkane markers as a result of its distinct profile on these markers, while *L. perenne* leaf/stem fraction and *U. gallii* presented some resemblances in alkane and VLCFA profiles. These results suggested that the combination of alkane and VLCFA data led to an improvement of the discrimination between diet components in all 3 experiments. The OPR results confirmed that this was the case, by demonstrating that when PCA scores based on alkanes or on VLCFA were compared, 0.429 (May), 0.696 (June) and 0.594 (October) of the residual variance, remained after rotation, indicating that VLCFA provide additional discriminatory information to that provided by alkanes.

### 3.2. Marker recoveries in faeces

Very long-chain fatty acid and alkane FR data are given in Tables 2 and 3, and indicated that, in general, FR of these markers was incomplete and tended to increase with carbon-chain length. It should be noted that FR of VLCFA $C_{34}$ was unexpectedly low in S6 diet (0.50) when compared with other diets and VLCFA with similar carbon-chain length. Results indicated a different recovery pattern of even- and odd-chain VLCFA. In fact, separate analysis of odd- and even-chain VLCFA indicated that FR of odd-chain tended to increase in a linear fashion ($P<0.001$), while FR of even-chain was best described by a quadratic function ($P<0.001$). For both odd- and even-chain alkanes a linear ($P<0.001$) association between carbon-chain length and FR was observed.

Results also showed that the FR of both markers were highly variable among dietary groups, resulting in a diet composition effect ($P<0.001$) on FR of both markers. Mean coefficient of variation (CV) of FR tended to be higher in VLCFA than in alkanes (0.142 vs. 0.114) and in the odd-chain markers (0.179 and 0.105 for VLCFA and alkanes, respectively). For both markers, variation in FR between diets decreased with carbon-chain length from a maximum of 0.271 (LCFA $C_{23}$) and 0.173 (alkane $C_{25}$) to a minimum of 0.060 (LCFA $C_{30}$) and 0.073 (alkane $C_{31}$).

Results in this experiment showed an interaction ($P<0.05$) between diet composition and carbon-chain length on FR of both markers. For VLCFA, a quadratic term ($P<0.001$) was observed for the even-chain VLCFA. By contrast, for the odd-chain VLCFA and both types of alkanes only a linear term ($P<0.001$) of the interaction was observed.

**Table 2**
Very long-chain fatty acid (VLCFA) recovery in faeces of sheep fed on six experimental diets in different experimental periods and according to chain length.

| Period | Diet | VLCFA carbon-chain length | | | | | | | | | | | DMDap | Effects | VLCFA carbon-chain length | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $C_{22}$ | $C_{23}$ | $C_{24}$ | $C_{25}$ | $C_{26}$ | $C_{27}$ | $C_{28}$ | $C_{29}$ | $C_{30}$ | $C_{32}$ | $C_{34}$ | | | All- | Odd- | Even- |
| First | S1 | 0.62 | 0.69 | 0.77 | 0.66 | 1.04 | 0.69 | 0.81 | 0.64 | 0.79 | 0.81 | 0.89 | 752 | Diet (D) | <0.001 | <0.001 | <0.001 |
| Second | S2 | 0.54 | 0.42 | 0.59 | 0.45 | 0.90 | 0.86 | 0.80 | 0.85 | 0.83 | 0.92 | 0.93 | 696 | VLCFA | <0.001 | <0.001 | <0.001 |
| | S3 | 0.71 | 0.53 | 0.73 | 0.55 | 0.91 | 0.85 | 0.84 | 0.82 | 0.80 | 0.85 | 0.79 | 622 | L | <0.001 | <0.001 | <0.001 |
| | S4 | 0.72 | 0.68 | 0.79 | 0.66 | 0.93 | 0.93 | 0.90 | 0.86 | 0.86 | 0.88 | 0.95 | 587 | Q | <0.001 | 0.170 | <0.001 |
| | S5 | 0.60 | 0.55 | 0.66 | 0.61 | 0.92 | 0.84 | 0.77 | 0.80 | 0.73 | 0.75 | 0.82 | 684 | D*VLCFA | <0.001 | <0.001 | <0.001 |
| Third | S6 | 0.46 | 0.32 | 0.45 | 0.36 | 0.84 | 0.72 | 0.77 | 0.73 | 0.84 | 0.72 | 0.50 | 589 | L | <0.001 | <0.001 | <0.001 |
| | Mean | 0.61 | 0.53 | 0.67 | 0.55 | 0.92 | 0.81 | 0.82 | 0.78 | 0.81 | 0.82 | 0.81 | 655 | Q | <0.001 | 0.991 | <0.001 |
| | SEM | 0.022 | 0.031 | 0.028 | 0.027 | 0.021 | 0.022 | 0.017 | 0.022 | 0.018 | 0.021 | 0.035 | 13.5 | | | | |

DMDap: apparent dry matter digestibility; S1: *Lolium perenne* (1.00); S2: *L. perenne* (0.70) + *Ulex gallii* (0.30); S3: *L. perenne* (0.40) + heather (0.30) + *U. gallii* (0.30); S4: *L. perenne* (0.71) + heather (0.29); S5: *L. perenne* (0.41) + *Trifolium repens* (0.28) + heather (0.30); S6: *L. perenne* (0.70) + *T. repens* (0.30). L and Q indicate linear or quadratic significance term for VLCFA and Diet*VLCFA effects; SEM: standard error of mean.

**Table 3**
Alkane recovery in faeces of non-lactating sheep fed on six experimental diets in different experimental periods and according to chain length.

| Period | Diet | Alkane carbon-chain length | | | | | | | | | DMDap | Effects | Alkane carbon-chain length | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $C_{25}$ | $C_{26}$ | $C_{27}$ | $C_{28}$ | $C_{29}$ | $C_{30}$ | $C_{31}$ | $C_{32}$ | $C_{33}$ | | | All | Odd- | Even- |
| First | S1 | 0.52 | 0.50 | 0.65 | 0.72 | 0.78 | 0.78 | 0.81 | 0.80 | 0.93 | 752 | Diet (D) | <0.001 | <0.001 | <0.001 |
| Second | S2 | 0.39 | 0.64 | 0.56 | 0.69 | 0.74 | 0.80 | 0.87 | 0.90 | 1.09 | 696 | Alk | <0.001 | <0.001 | <0.001 |
| | S3 | 0.52 | 0.73 | 0.62 | 0.83 | 0.76 | 0.83 | 0.85 | 0.88 | 0.90 | 622 | L | <0.001 | <0.001 | <0.001 |
| | S4 | 0.56 | 0.76 | 0.68 | 0.88 | 0.81 | 0.86 | 0.91 | 0.95 | 0.98 | 587 | Q | 0.014 | 0.254 | 0.395 |
| | S5 | 0.44 | 0.62 | 0.54 | 0.71 | 0.65 | 0.72 | 0.76 | 0.80 | 0.83 | 684 | D*Alk | <0.001 | 0.023 | <0.001 |
| Third | S6 | 0.65 | 0.59 | 0.69 | 0.73 | 0.83 | 0.96 | 0.92 | 1.17 | 0.99 | 589 | L | <0.001 | <0.001 | <0.001 |
| | Mean | 0.51 | 0.64 | 0.62 | 0.76 | 0.76 | 0.82 | 0.85 | 0.92 | 0.95 | 655 | Q | 0.410 | 0.865 | 0.194 |
| | SEM | 0.022 | 0.022 | 0.018 | 0.022 | 0.018 | 0.020 | 0.017 | 0.029 | 0.021 | 13.5 | | | | |

DMDap: apparent dry matter digestibility; S1: *Lolium perenne* (1.00); S2: *L. perenne* (0.70) + *Ulex gallii* (0.30); S3: *L. perenne* (0.40) + heather (0.30) + *U. gallii* (0.30); S4: *L. perenne* (0.71) + heather (0.29); S5: *L. perenne* (0.41) + *Trifolium repens* (0.28) + heather (0.30); S6: *L. perenne* (0.70) + *T. repens* (0.30). L and Q indicate linear or quadratic significance term for Alkane and Diet*Alkane effects; SEM: standard error of mean.

### 3.3. Estimates of diet composition

Diet composition estimates of the experimental diets S1 to S6 obtained using VLCFA, alkanes or a combination of both markers, with faecal concentrations corrected (individual, dietary mean and an overall mean) or not for incomplete FR are illustrated in Figs. 1 and 2. It should be noted that KSI values for S1 when considering only the plant species effectively offered to the animals (C1) were all 1.0 as the diet was only composed of *L. perenne* (1.0). The accuracy of diet composition estimates, expressed as KSI indexes, was affected by diet composition ($P<0.001$), markers ($P<0.001$), calculation procedure ($P<0.001$) and recovery corrections ($P<0.001$) used in the estimation. As expected, higher ($P<0.05$) accuracies of diet composition estimates were obtained when considering only the plant species effectively offered to the animals compared to C2 procedure (0.896 vs. 0.834 KSI values, respectively), even when S1 diet (with 1.0 and 0.987 for C1 and C2, respectively) was excluded from the analysis.

In general, the most accurate estimates of diet composition were obtained using combined VLCFA and alkane markers, with mean KSI value of 0.894, differing ($P<0.05$) from that obtained with the alkanes alone (mean KSI index of 0.867). The use of VLCFA alone resulted in less accurate ($P<0.05$) estimates of diet composition (mean KSI index of 0.834).

Diet composition had an effect ($P<0.001$) on the KSI values, with S1 showing the highest value (0.993) and S5 the lowest one (0.783). The worst estimates of diet composition obtained using VLCFA alone were in S6 diet (mean KSI of 0.659) for C2 calculation procedure, where very low KSI values were observed for FC0 and FC3 (0.299) as a result of a high overestimation of *T. repens* and heather in both FC0 and FC3, and *U. gallii* in FC3.

As expected, high levels of accuracy of diet composition estimates were obtained using individual recovery data (FC1), with a mean KSI index of 0.999, differing ($P<0.05$) from that obtained using mean recovery rate of the dietary treatment (FC2), with a mean KSI value of 0.911. The utilization of a mean recovery rate across all experimental diets (FR3) resulted in less ($P<0.05$) accurate estimates of diet composition (mean KSI value of 0.862), especially when based on VLCFA data (mean KSI value of 0.796). When estimates were based on alkane and VLCFA + alkanes corrected with FC3, an improvement ($P<0.05$) in their accuracy was observed with mean KSI values of 0.90 and 0.89, respectively.

In general, the utilization of uncorrected faecal marker concentrations (FC0) resulted in the worst estimates of diet composition with similar levels of accuracy for VLCFA (0.671), alkanes (0.645) and their combination (0.747). It should be noted that the use of FC0 in the less complex diet (S1, with three possible diet components) resulted in highly accurate estimates for all markers (mean KSI value of 0.988) while S5 had the lowest mean KSI value (0.519). In the second experimental period, the use of uncorrected faecal concentrations of VLCFA and VLCFA + alkanes for C2 calculation procedure, led to a general overestimation of heather and *T. repens*, while the use of alkanes with FC0 tended to overestimate the proportions of the woody species (heather and *U. gallii*). When only considering the plant species effectively ingested by the animals (C1), VLCFA overestimated *L. perenne*, while alkanes and VLCFA + alkanes tended to overestimate the proportions of heather and *U. gallii*.

In S6 diet, *L. perenne* tended to be underestimated by the VLCFA (for C1 and C2) and VLCFA + alkanes (for C2). In contrast, for the same diet, the estimated proportions of *L. perenne* based on alkane faecal concentrations uncorrected for incomplete faecal recovery and for both calculation procedures (i.e., C1 and C2) showed a tendency to be overestimated in addition to *U. gallii* (C2).

## 4. Discussion

### 4.1. LCFA content of diet components

As previously observed by Ali et al. (2005) and Ferreira et al. (2009a), VLCFA markers were characterized by (1) having a much higher total concentration than the alkane markers, ranging between 1.4 and 8.3 times higher for heather and

*T. repens*, respectively, and (2) a prevalence of even-chain VLCFA over the odd-chain ones (i.e., over 0.80 of total VLCFA total concentrations), contrasting with the predominance of odd-chain in the alkane fraction. Similar characteristics were observed elsewhere for other epicuticular wax compounds like the LCOH (Bugalho et al., 2004; Ali et al., 2005; Dove and Charmley, 2008). These results suggest that VLCFA may be particularly valuable to discriminate between plant species whose alkane concentrations are very low as is the case of *Phalaris aquatica* (Charmley and Dove, 2007), several conifer species (i.e., *Pinus sylvestris*, *Picea sitchensis* and *Pseudotsuga menziesii*) (Ali et al., 2005), *U. gallii* (Ferreira et al., 2007a) and herbaceous species in general, especially in advanced stages of maturity where alkane concentrations are even lower as a result of a

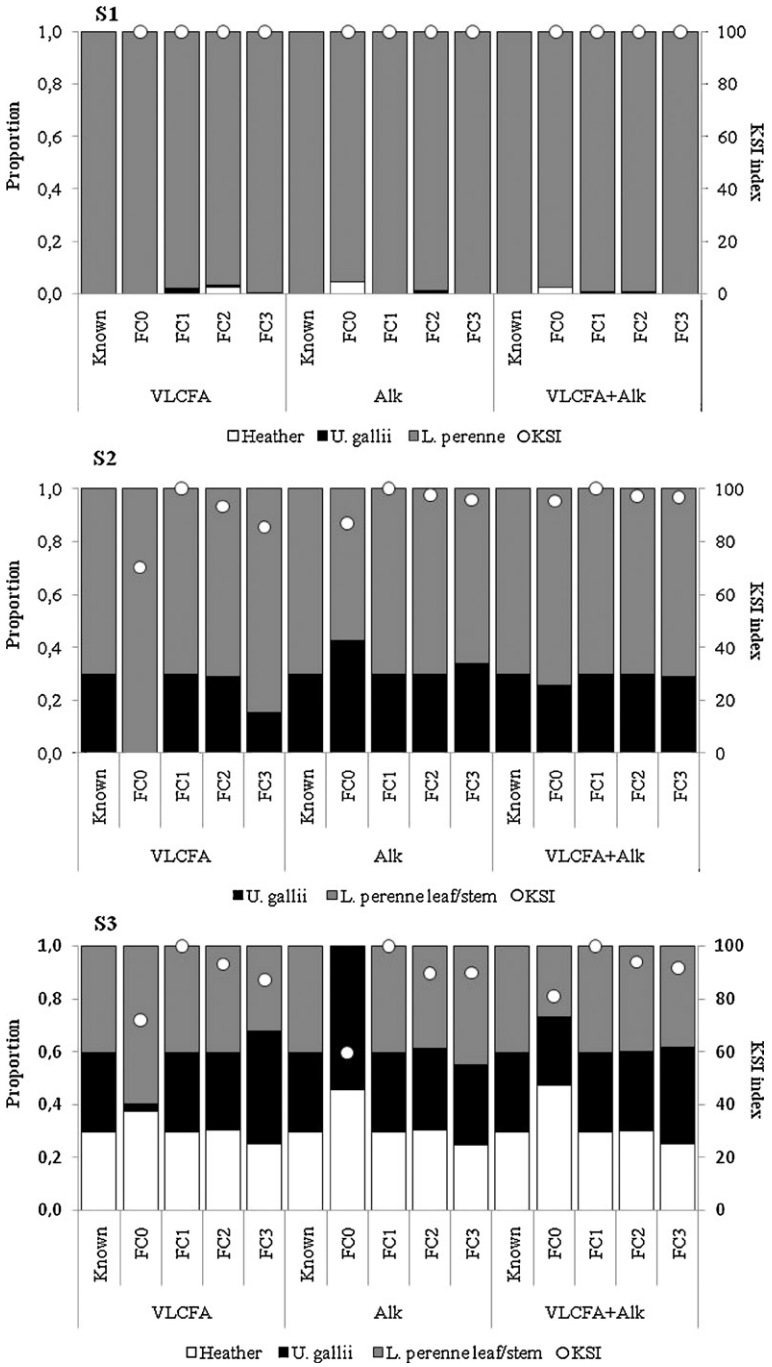


**Fig. 1.** Comparisons of known and estimated proportions of dietary components obtained using different markers' data (LCFA, alkanes or LCFA + alkanes) and faecal recovery corrections (FC0, FC1, FC2 and FC3) for each experimental diet (S1–S6) when considering only plant species effectively offered to animals (C1).

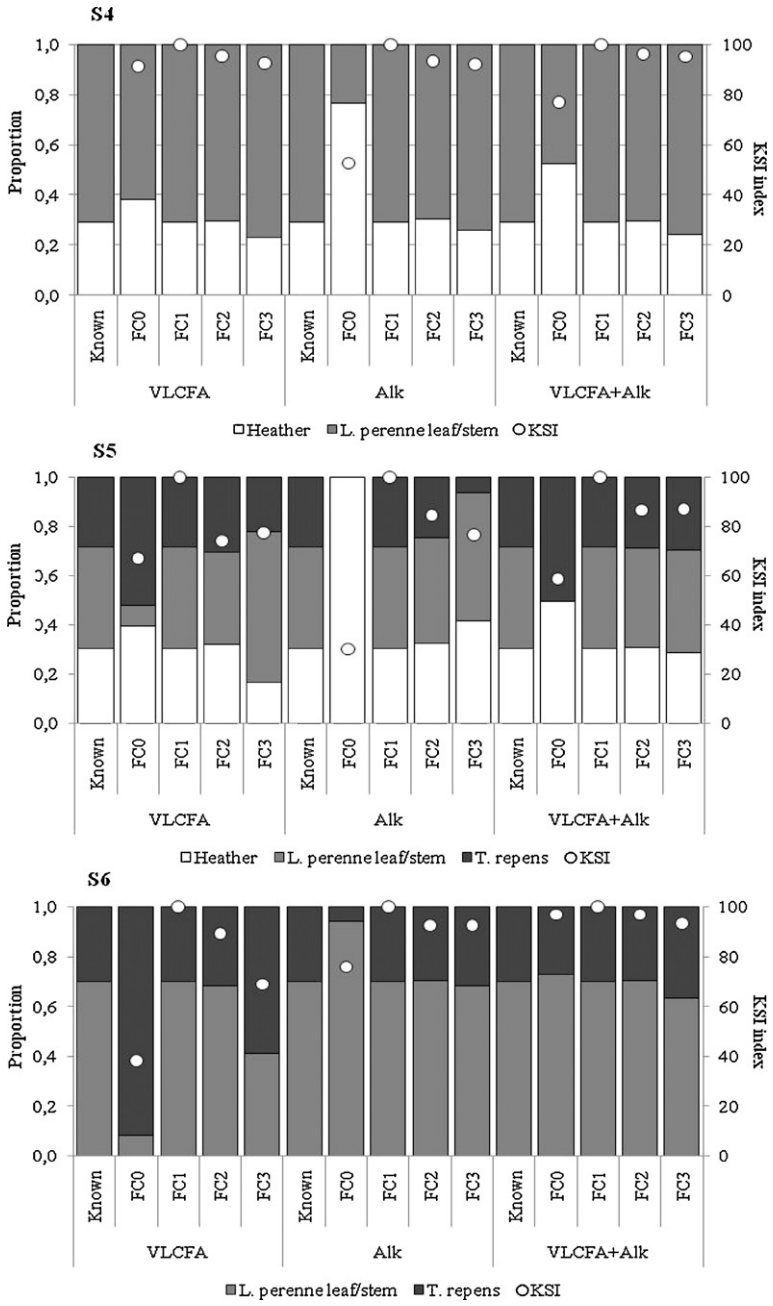


**Fig. 1.** (*Continued*).

higher stem fraction characterized by lower alkane concentrations than the leaf fraction (Dove et al., 1996; Ferreira et al., 2005).

Results also showed large differences between plant species in their VLCFA and alkane profiles. As previously reported (Ali et al., 2005; Ferreira et al., 2009a), plant species with similar profile in one type of marker may differ when using other markers. For example, in the present study *L. perenne* and *T. repens* used in June and July showed resemblances in their alkane profiles but presented very distinct VLCFA profiles. In contrast, similarities found in the alkane profile of *L. perenne* leaf/stem and *U. gallii* were also observed in their VLCFA profiles. Consequently, the VLCFA can be useful as supplementary diet composition markers to alkanes to distinguish *L. perenne* and *T. repens*. However, this would not be the case for discriminating *L. perenne* leaf/stem and *U. gallii*, and *L. perenne* leaf/stem from its spike fraction, as they showed similar VLCFA profiles. Dove and Mayes (2005) indicated that LCOH can also provide additional information to alkane markers to distinguish *L. perenne* and *T. subterraneum*, as the dicotyledon species shows higher concentration in $C_{30}$-alcohol.

### 4.2. Recovery of markers in faeces

Results obtained in this study showed that VLCFA and the alkane markers are similar in their behaviour in the digestive tract of sheep, as also observed by Ali et al. (2004). In fact, both marker types had an incomplete faecal recovery and showed a positive association between faecal recovery and marker carbon-chain length. The relationship was more pronounced for the alkanes as the faecal recovery increased from 0.51 to 0.95 from the minimum to the maximum alkane carbon-chain length when compared with the increase observed for the VLCFA (i.e., from 0.61 to 0.81). The association between VLCFA carbon-chain length and faecal recovery was better described by a curvilinear function ($P<0.001$), indicating that differences in the faecal recovery of these markers with adjacent carbon-chain length decreased with increasing carbon-chain length.

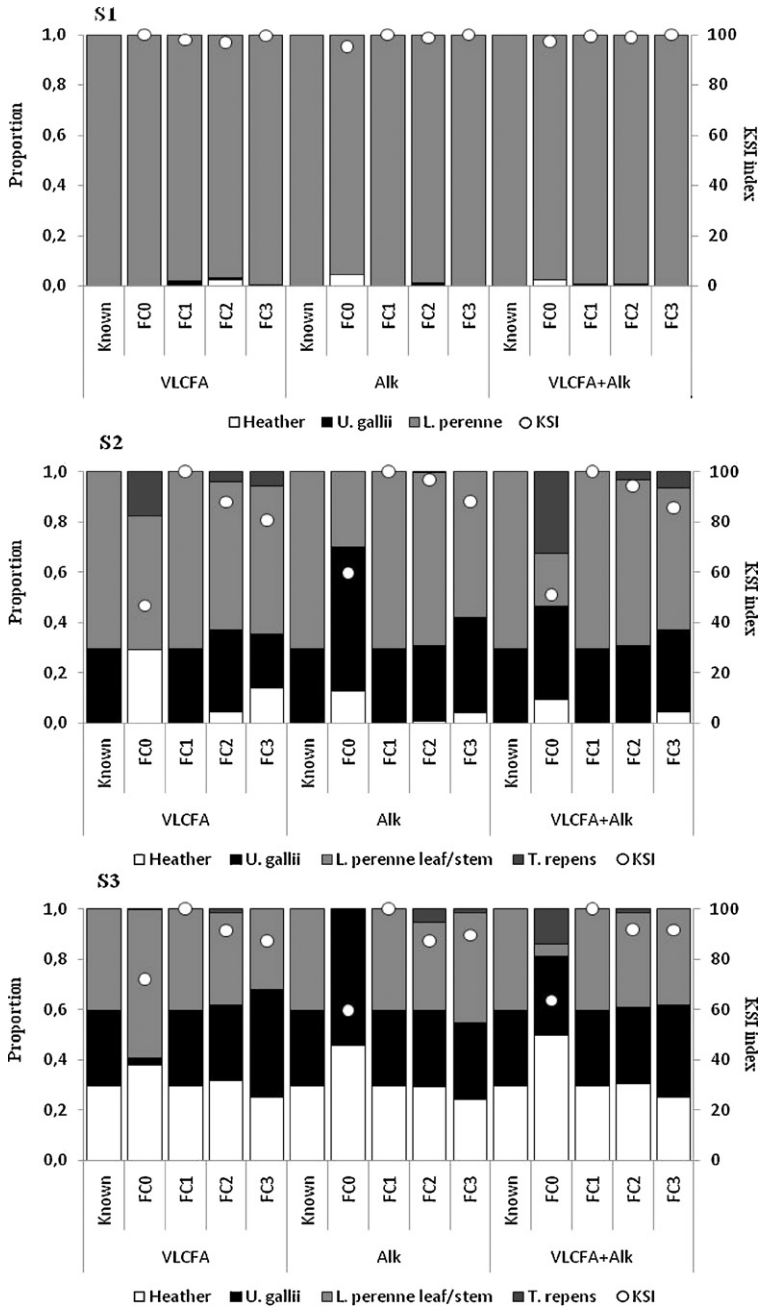


**Fig. 2.** Comparisons of known and estimated proportions of dietary components obtained using different markers' data (LCFA, alkanes or LCFA + alkanes) and faecal recovery corrections (FC0, FC1, FC2 and FC3) for each experimental diet (S1–S6) when considering plant species effectively offered to animals plus feeds that were not part of the diets but were available in the field at each experimental period (C2).

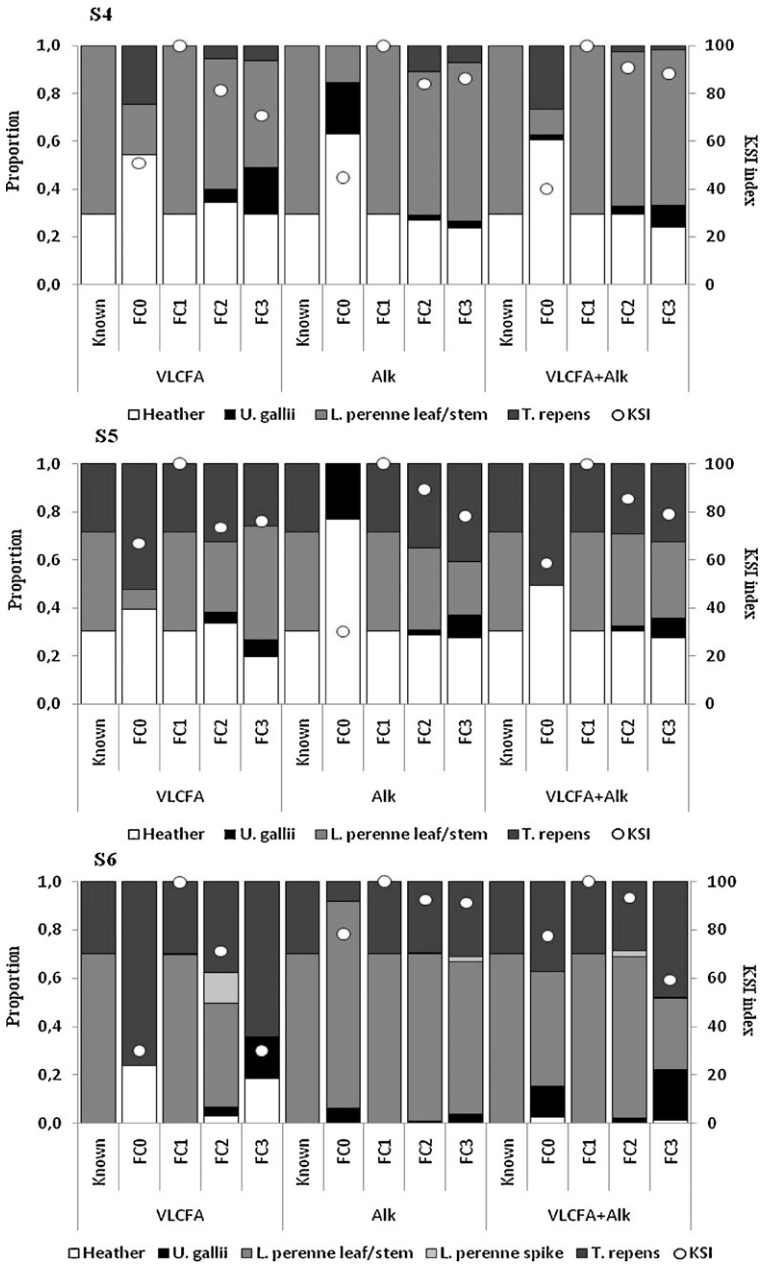


**Fig. 2.** (*Continued*).

In fact, a plateau in the faecal recovery values was observed between $C_{27}$- and $C_{34}$-VLCFA, with the values varying from a minimum of 0.78 to a maximum of 0.82. These are consistent with those obtained by Ferreira et al. (2009a) for the same markers in goats fed with diets composed with the same plant species used in this trial. A similar curvilinear pattern was also observed for the alkanes ($P$=0.014) when considering all data, which is in agreement with previous studies conducted with sheep (Elwert et al., 2008; Charmley and Dove, 2007) and other ruminant species (Piasentier et al., 2007; Ferreira et al., 2009a). However, the separate analysis of odd- and even-chain alkanes showed that the increase of their faecal recovery was linear rather than curvilinear, as also observed for the odd-chain VLCFA. Ferreira et al. (2009b) also observed a positive linear association between carbon-chain length and odd-chain alkane faecal recoveries in goats, sheep, cattle and equines fed with herbage or herbage plus heather.

As also observed by Ferreira et al. (2009a), the comparison of the marker faecal recovery data showed a higher FR of the VLCFA when considering the shorter carbon-chain markers (i.e., ≤29 carbon atoms), and a higher recovery of alkanes when considering the longer chain markers. These differences could have an important impact on subsequent diet composition estimates based on uncorrected faecal concentrations, which tend, in general, towards the diet components

with a predominance of longer chain markers. As differences in the FR between shorter and longer VLCFA were smaller than those observed in the alkane markers, VLCFA can be less prone to bias towards the plant components with longer VLCFA.

Results obtained in this study showed an effect ($P<0.001$) of diet composition on the FR of both type of markers. A similar effect was detected by Ferreira et al. (2009a) for both markers and by Charmley and Dove (2007) for the alkanes. Other studies suggest the absence of this effect in alkanes (Elwert et al., 2004) and in LCOH (Dove and Charmley, 2008). However, the analysis for each individual marker indicated clear differences between alkanes and VLCFA. Diet composition had an effect ($P<0.05$) on the faecal recovery of all individual alkanes, except for the $C_{33}$-alkane, and a lack of effect was observed for all individual VLCFA. In our opinion, more important than a numerical variation in the faecal recovery of a given marker between diets is the possible deviation in the relative recovery between markers and diets (Dove and Mayes, 2006). Any variability in relative recovery can compromise the application of a general set of FR correction for diet composition estimation in grazing studies. In this study, the interaction between the main effects (i.e., diet composition and carbon-chain length) indicated that the relative recoveries between markers differ between diets.

Although Ferreira et al. (2005) and Elwert et al. (2008) reported a negative association between alkane faecal recovery and diet digestibility (i.e., an increase in the alkane faecal recovery as diet digestibility decreases), in the present study we were not able to identify this association in both alkanes and VLCFA. Dove and Charmley (2008) also did not identify a relationship between diet digestibility and the faecal recovery of LCOH.

### 4.3. Accuracy of diet composition estimates

Results obtained in the present study showed that overall, the utilization of the combination of alkanes and VLCFA markers produced the most accurate estimates of diet composition. In comparison with alkanes, VLCFA estimates of diet composition were less precise although the diet components showed clear differences in their VLCFA profiles in all 3 periods. A lower ability of VLCFA than alkanes and LCOH markers to estimate diet composition of sheep was also observed by Ali et al. (2005), relating the results to a less robust analytical method for quantifying the VLCFA, as the extracts obtained showed several impurities. The same explanation cannot be drawn in the current study as mass spectral information allowed the confirmation of peak purity. It should be noted that the lower mean KSI value observed for the VLCFA was the result of a very low accuracy of diet composition estimates in S6 diet for FC0 in C1 (KSI value of 0.383) and C2 (KSI value of 0.299) and FC3 in C2 (KSI value of 0.299).

Moreover, results also showed that VLCFA performed better than alkanes in some diets (i.e., S3, S4 and S5 in C1, and S1, S3, S4 and S5 in C2) and vice-versa in other ones (i.e., S2 and S6 in C1, and S2, S4 and S6 in C2). A same lack of trend for a superiority of one class of marker to another was observed by Ali et al. (2005) when comparing alkanes, VLCFA and LCOH. In our opinion these results may be due to a different capability of markers (i.e., alkanes, VLCFA and LCOH) to differentiate diet components of different diets. This could also explain why, for some diets, the combination of markers did not result in better diet composition estimates for some diets, than when using single markers.

The objective of using a combination of markers was to increase the discriminatory power among plant species by using additional and/or complementary information of both markers that would result in a more specific marker fingerprint for each plant species (Bugalho et al., 2004; Ferreira et al., 2009a). Results obtained in the present study confirm the usefulness of combining markers in order to obtain more accurate estimates of diet composition. Kelman et al. (2003) were able to discriminate better grass, clover and *Lotus* spp. in the diet of sheep by combining alkanes and LCOH. However, results reported elsewhere (Ali et al., 2005; Dove and Charmley, 2008) indicate that a combination of markers is not always followed by improvements on the accuracy of diet composition estimates. As stated previously, this may occur when using markers that are not well correlated with the discrimination between diet components and in situations where the number of diet components is high (i.e., plant species that constitute the diet or that are considered possible diet components). It should also be stated that VLCFA + alkane combination was not able to maintain the accuracy on diet composition estimates obtained in C1 calculation procedure (mean KSI value of 0.935) when other plant species were considered as possible diet components (C2, mean KSI value of 0.853).

In the present study two different levels of data regarding the possible diet components were used (i.e., C1 and C2) aiming to evaluate its impact on the accuracy of diet composition estimates. Reduction in the number of possible diet components, based on preliminary information (Dove and Mayes, 2005) is usually suggested in grazing studies where the number of available plant species is high and the accuracy of diet composition estimates may be compromised (Oliván et al., 2007). Although the complexity of the diets used in the present trial was low, in terms of both effective and possible diet components, it is worth mentioning that a higher accuracy ($P<0.001$) of diet composition estimates were observed for C1 procedure (mean KSI value of 0.896 vs. 0.834 for C2).

Previous grazing behaviour studies conducted in shrubland vegetation communities that cover large land extensions of the northwest of the Iberian Peninsula (Oliván et al., 2007) showed difficulties in differentiation between herbaceous species and between herbaceous species and gorse (*U. gallii*) due to similarities in their alkane profiles, leading to diet composition estimate more prone to bias, especially when less accurate FR data was applied. Although PCA indicated clear differences between *L. perenne* and *T. repens*, improvement on the accuracy of diet composition estimates by using VLCFA was not clear. In fact, for S6 diet (*L. perenne* and *T. repens*) higher mean KSI values were observed for the alkane markers for both C1 (0.902 vs. 0.741) and C2 (0.925 vs. 0.582), while higher KSI values were observed for VLCFA for diet S5 (*L. perenne*, *T. repens* and heather) for C1 (0.796 vs. 0.729) and C2 (0.791 vs. 0.744). A similar lack of additional discrimination power between the

herbaceous species and *U. gallii* by using VLCFA was observed, although in this case preliminary PCA showed resemblances in the VLCFA profile between *L. perenne* and *U. gallii*.

As expected, results showed the need for a suitable correction of marker faecal concentration prior to diet composition estimation, as illustrated by the high KSI values observed for FC1 (0.999) and FC2 (0.911) for all markers, calculation procedures and diets. As suggested by Ferreira et al. (2009b), differences ($P<0.05$) between FC1 and FC2 were the result of deviations between the individual recovery sets and the group mean ones, due to individual variability within diets (i.e., animal variation). High levels of accuracy were also observed in previous studies conducted with alkanes (Charmley and Dove, 2007; Elwert et al., 2008; Ferreira et al., 2005, 2007a), VLCFA (Ali et al., 2005; Ferreira et al., 2009a) and LCOH (Ali et al., 2005; Dove and Charmley, 2008) when using this type of recovery corrections. However, these accurate recovery correction data (i.e., FC1 and FC2) are not usually used under grazing conditions as they are difficult to calculate. Alternatively diet composition calculations are performed using mean recovery data obtained in metabolic crate studies with animals fed with diets composed of plant species present in the field in proportions similar to those expected in the pasture (i.e., FC3 in the present study). The accuracy of the resultant estimates of diet composition will depend on differences between the overall recovery data (FC3) and the actual individual ones (FC1) as a result of a diet composition effect (Ferreira et al., 2009a). In the present study, diet composition estimates obtained using FC2 and FC3 with alkanes and their combination with VLCFA did not differ while for the VLCFA alone, diet composition estimates obtained using FC3 differed ($P<0.05$) from those obtained with the group mean faecal recovery (FC2). These results indicate that overall recovery data (FC3) can be applied to these markers (alkanes alone or their combination with VLCFA) in grazing studies without losing accuracy in diet composition estimates. It should be pointed out that a lower dependence on suitable recovery correction was expected when combining alkanes with VLCFA, due to a more specific marker fingerprint for each plant species (Ferreira et al., 2009a). However, KSI values obtained when applying FC3 to VLCFA + alkanes (0.890) were similar to alkanes alone (0.900), but higher ($P<0.05$) than those observed for VLCFA alone (0.796).

Results also showed that, in general, the lack of a suitable correction of marker faecal concentrations resulted in the poorer estimates of diet composition for all marker calculation procedures and diets, especially when using VLCFA (KSI of 0.671) and alkanes alone (KSI of 0.645). Higher ($P<0.05$) KSI values (0.747) were obtained when considering the combinations of markers. Diet composition estimates based on VLCFA and alkanes alone tended for the diet components with a predominance of longer chain markers, i.e., heather and *T. repens* for the VLCFA and *U. gallii* for alkanes. Similar trends were reported in previous studies with different epicuticular wax compound markers (Ali et al., 2005; Elwert et al., 2008; Dove and Charmley, 2008; Ferreira et al., 2009a).

## 5. Conclusions

Although the results obtained in the present study showed that VLCFA produced the poorest diet composition estimates, they fulfil all 3 basic features that any compound must have to be useful as a diet composition marker: (1) need to be accurately and precisely quantified, (2) its faecal recovery must be consistent, and (3) plant species should exhibit large between-species and small within-species variation in the markers' profiles. Thus, these markers can be particularly useful in situations where alkane concentrations are low, to distinguish plant species with similar alkane profiles, and in situations where the number of diet components to be differentiated is high, provided that suitable faecal recovery correction is applied prior to diet composition estimation. Moreover, our results also confirm that the combination of VLCFA with alkanes will improve the accuracy of diet composition estimates.

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