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Utilization of Biomarkers to Study the Grazing Behavior of Herbivore Species

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Additional information is available at the end of the chapter

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Abstract

Knowledge on diet selection of different herbivore species under each specific vegetation community is essential to develop and apply appropriate management decisions for each grazing system in order to, simultaneously, have a more efficient and sustainable utilization of pasture resources and the best animal performance level. In this chapter, traditional and more recent methodologies that can be used for studying diet selection of both domestic and wild herbivores are briefly presented, identifying the main advantages and limitations of their use. Particular emphasis is given to the utilization of epicuticular compounds, namely alkanes, long-chain fatty acids and long-chain alcohols, as faecal markers. The validation of their use is presented taking into account studies performed with different animal species under controlled conditions. The main advantages and shortcomings for their application to field studies with grazing animals are highlighted. Data indicate that the combination of these epicuticular compounds seems promising to overcome the enumerated constraints, allowing its application to more complex vegetation communities.

Keywords: diet selection, faecal markers, ruminant species

1. Introduction

The success of the strategies for the management of herbivores grazing on different plant communities, driven by production or environmental goals, requires the understanding of the processes involved in plant-herbivore interactions and their consequences for both plants and herbivores [1]. The plant-herbivore interaction is mutual and dynamic. The structure, composition, productivity, nutritive value and distribution of the different plant

communities determine the intake and nutritional status of the animals [2, 3]. In turn, the herbivores, through grazing, trampling, defecation, urination, etc., affect the dynamics of the vegetation community [4, 5]. These interrelationships are specific for each herbivore species and each vegetation type and are still poorly understood, leading to the use of less appropriate management strategies for agricultural and other land use objectives [4, 6]. The type (cutting, grazing or a mixed system) and intensity level of management will have a determinant role on the evolution of the habitat and on the biodiversity, being extremely important on the maintenance of species balance, maturity and nutritive value in plant communities, relating the timing and severity of defoliation in relation to patterns of plant growth and maturity, and proposed objectives (animal performance, biodiversity, sustainability, etc.).

The understanding of the grazing behaviour, especially diet selection, of different animal species under diverse conditions is essential to develop an appropriate grazing strategy for each specific situation in order to have a more efficient and sustainable utilization of the existing vegetation (**Figure 1**). The different dietary choices between plant species and plant parts in a specific vegetation community offered to the grazing animals are the main mechanism through which herbivores could increase sward heterogeneity [3, 7]. The diet selected by animals is constrained by temporal and spatial changes in the sward structure, plant defence mechanisms, food availability, plant phenology and animal factors [6, 7], and it differs between animal species [6, 8, 9] and also between breeds of cattle [10], sheep [11] and goats [12].

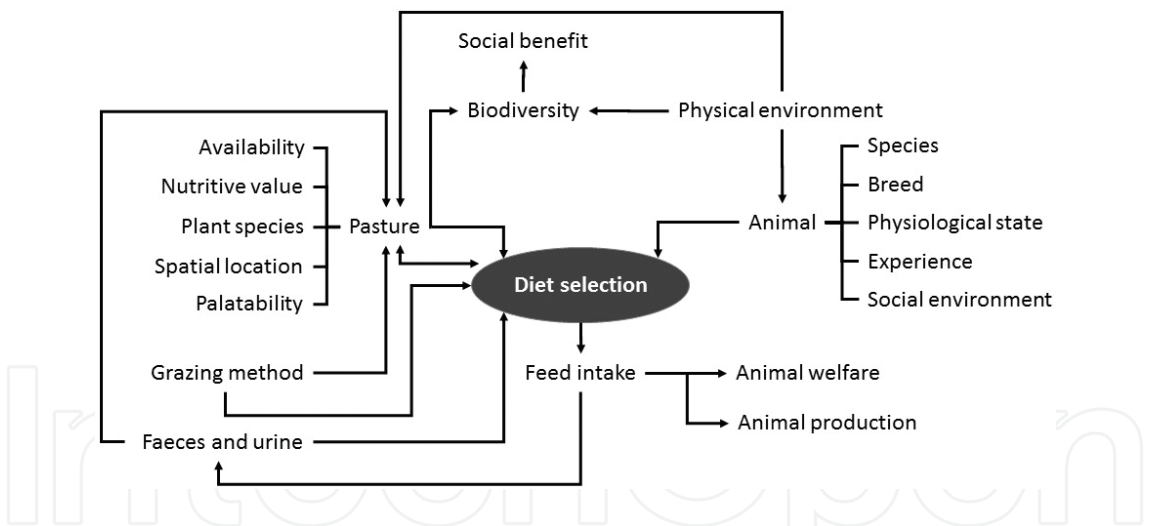


Figure 1. Schematic diagram of factors affecting diet selection of herbivores and its effects on animal production and biodiversity in a grazing system.

Generally, ruminant species are classified into three feeding types according to morphological and physiological adaptations of the digestive system [6, 13–15]: concentrate selectors (browsers), intermediate feeders and grass-roughage eaters (grazers). Based on this classification, it has been assumed that ruminant grazers, with greater body weight, achieve a higher extraction of nutrients from the diet consumed than browsers with low body weight [16]. According to Pérez-Barbería et al. [15] and Udén and van Soest [17], this is due to a higher extent of digestion of fibre by means of higher food retention in the rumen, larger stomach capacity, higher degree of stomach compartmentalization and smaller openings between the

rumen and omasum. In contrast, small ruminants would compensate this lower digestion capacity by selecting high-quality plant parts such as fruits, pods, young shoots and leaves.

Previous studies [9, 18], carried out in heathland vegetation communities with adjacent areas of improved pasture (*Lolium perenne* and *Trifolium repens*) (**Figure 2**) across the grazing season (May–December), indicate an almost total preference for herbaceous species by cattle, contrasting with the higher preference for the woody species (*Erica* spp., *Calluna vulgaris* and *Ulex gallii*) revealed by goats. By contrast, horses and sheep showed an intermediate behaviour, increasing the selection of the woody species through the grazing season as a result of the decrease in the availability of the preferred herbaceous species [18]. These differences in the grazing behaviour were reflected in animal performance [19]. This distinct behaviour and variable responses of different animal species allow alternative strategies to develop viable systems aiming to achieve production and biodiversity outcomes. Therefore, the evaluation of diet composition of grazing animals is important for the achievement of sustainable management and production systems for each vegetation community.



Figure 2. Different herbivore species grazing heathland vegetation communities with adjacent areas of improved pasture of the north of Spain.

In this review, we aim to describe several methodologies that are available to assess plant-animal interactions, with particular relevance to the utilization of epicuticular compounds. The main advantages and limitations of each method are also explored, comparing the accuracy of diet composition estimates.

2. Techniques used to estimate diet composition in herbivores

Traditional techniques used to estimate diet composition of grazing animals are based either on measurements on the plant biomass (the utilization techniques) or on animal-based measurements [20], namely the direct observation of the grazing animal and the microhistological examination

of plant fragments in different samples. However, all these techniques have important limitations associated with the measurement processes themselves, as the normal foraging behaviour may be compromised, and with the accuracy of the estimations [21].

Direct observation of the number of bites and the feeding times spent by the grazing animals on different plant communities is frequently used. The simplicity and minor equipment requirements are pointed out as advantages of this approach. However, as stated by Holechek et al. [20], it is extremely difficult to identify the plant species being consumed, especially when there is no spatial separation between plant species, and to convert the grazing times or number of bites to an accurate estimate of the amount of the plant consumed [22], besides being a time-consuming approach that is very difficult to accomplish during nocturnal periods.

The microhistological procedures rely on the visual identification of epidermal cuticular fragments in samples of oesophageal extrusa, in a gut compartment or in faeces [20, 21]. Diet composition is expressed in terms of the proportion of identifiable fragments coming from each plant species. Although microhistological approaches can be valuable to confirm the presence or the absence of a particular plant species or plant part in the diet [23], they are tedious to perform, require a lot of training of the researchers and involve sacrifice (stomach analysis) and fistulation (oesophageal extrusa) of the animals, unless faecal samples are used. Moreover, in the case of using faecal samples, possible differential digestion of the different plant species and the large proportion of unidentifiable fragments reduce the accuracy of diet composition estimates.

Another methodology that has been used for studying diet selection of herbivores is the near-infrared reflectance spectroscopy of faeces (F.NIRS) [24–27]. This methodology involves the association between faecal spectra with that of diets consumed, i.e. measurements of the reflectance of light between 700 and 2500 nm (for more details, see Dixon and Coates [28]). This spectrum gives a specific signature depending on the presence, character and number of important chemical bonds, such as OH, NH and CH [28]. According to Swain and Friend [29], one of the major limitations pointed out to NIRS applications (i.e. estimation of feed intake, digestibility and diet composition) is the need to have accurate calibration equations based on known and estimated nutritional parameters that will obviously vary for each specific situation (vegetation community). Nevertheless, these authors recognized the usefulness of this technique in identifying the presence of a specific feed item.

Results obtained by Ferreira et al. [18] suggest large variation in the spatial choice (i.e. plant communities where to graze) between animal species within a day and throughout the grazing season. The nutritive value, availability and the spatial distribution of the feed resources, and the distance to water and slope are major factors influencing grazing distribution patterns [30]. Early studies used visual field observations to assess these temporal and spatial modifications of rangeland use by both domestic and wild herbivores [30]. The utilization of recent available telemetry techniques can help grazing scientists to assess landscape vegetation preferences of herbivores [29], increasing the number of observed animals and reducing significantly the labour and allowing the collection of high-quality and unbiased data over a 24-h period. Identification of the preferred grazing sites can be accomplished by using telemetry devices, as global positioning systems (GPS). These devices are able to fine-scale spatio-temporal location data [31] with a spatial accuracy of <5 m [32] depending on the telemetry

devices. This information together with data on the spatial arrangement of the plant communities can be used to assess the animals' patch selection. According to Swain and Friend [29], the spatial arrangement of vegetation (number and size of the patches) will determine the level of local accuracy needed, i.e. small patches in larger number will need a higher accuracy of location data. In a recent study, Thompson et al. [33] used GPS collars to spatially register cattle location, and based on this, data were able to assess their activities (grazing, travelling or resting) on distinct plant communities of a rangeland, using an algorithm developed to classify cattle activity. Hebblewhite and Haydon [31] referred that the high cost of GPS collars that depends on its features (i.e. battery size, longevity, programmability, remote data access) has led researchers to opt for using fewer GPS units, limiting statistical inference. According to these authors, collar failures that could range from 5 to 50% of the units reduce even further sample size. In addition to these shortcomings, this method does not allow to quantify or estimate diet composition.

Analysis of stable carbon isotopes in animal faeces has also been used to discriminate C_3 and C_4 plants on the diet selected by domestic [31–37] and wild herbivores [38]. This methodology is based on differences between plants with different photosynthesis pathways in fractionating of ^{13}C , with C_3 plants discriminating more against the heavier isotope ^{13}C in favour of ^{12}C than C_4 plants. This results in different $^{13}C:^{12}C$ ratios that are expressed as $\delta^{13}C$ relative to the $^{13}C:^{12}C$ ratio of the international Vienna Pee Dee Belemnite standard. Using these markers De Smet et al. [39] were able to estimate accurately the proportion of C_4 plant material in the diet analysing stable carbon isotope ratios ($\delta^{13}C$ value) in different tissues (blood, plasma, liver, kidney fat, hair, muscle and ruminal contents) taken from beef animals at slaughter. Nevertheless, Dove and Mayes [21] pointed out some limitations to this technique: (1) limited to situations where C_4 plants are present, for example, tropical grazing systems; (2) when using faecal samples, differential recovery of feeds in faeces may lead to underestimation of those of higher digestibility; and (3) possible effect of faecal endogenous carbon on the faecal carbon isotope ratio.

Alternatively, plant-wax components, especially alkanes and other wax components, such as long-chain alcohols and long-chain fatty acids, have been suggested as possible markers to estimate diet composition. The main advantages of using these markers is the fact that for their quantification the same analytical procedure is used on samples of the diet components and animal faeces, reducing labour and analytical error. Moreover, it provides the necessary information for the estimation of diet composition, digestibility and intake for each individual, therefore accommodating possible differences between individuals [21].

3. Epicuticular compounds

The aerial surfaces of most higher plants are covered by a layer of (epicuticular) wax that is a complex mixture of hydrophobic compounds such as long-chain fatty acids, aldehydes, alcohols, triterpenes, sterols, ketones, esters, flavonoids and alkanes [40]. According to Dove and Mayes [41], the chemical composition of this layer varies within plant species and plant

parts, with leaves and floral parts tending to present higher wax concentrations than stems [21]. This layer has multiple functions, being the first line of protection between plants and the environment, acting as hydrophobic barriers, limiting nonstomatal water loss, and may constitute a defence mechanism against bacterial and fungal pathogens and other stress agents [40]. According to Eigenbrode and Espelie [42], it also plays an important role in the plant-insect interactions, repelling or attracting them.

Although the first studies on the possible use of epicuticular compounds as faecal markers were carried out with long-chain fatty acids (LCFAs) by Body and Hansen [43] and Grace and Body [44], alkanes are the ones most widely studied and applied in field studies due to their relative inertness and simplicity of analysis [21]. Alkanes present in the epicuticular mixture differ in carbon-chain length, varying from 21 to 37 carbon atoms [45]; those with odd number of carbon atoms represent more than 90% of the total content. Generally, the most abundant are the n-nonacosane (C_{29}), n-untriacontane (C_{31}) and n-tritriacontane (C_{33}) [22, 45]. The alkanes with less than 25 and more than 35 carbon atoms are present in very low concentrations. The alkane content varies between plant species (**Table 1**), plant parts and even cultivars of the same species [46, 47], plant stages of maturity and climatic conditions. In general, most of the herbaceous species, especially tropical forage species [48, 49], but also some shrub species (e.g. *U. gallii*, [50]), are known to possess very low alkane concentrations.

As can be observed in **Table 1**, differences in the alkane profiles between plant species occur in terms of absolute concentrations and relative proportions of the individual alkanes in the total content. Dove et al. [47] studied the effect of the plant species, age and part of the plant on the alkane profiles of different pasture species (*Phalaris aquatica*, *L. perenne*, *T. repens*, *Trifolium subterraneum* subsp. *subterraneum*, *T. subterraneum* subsp. *yanninicum* and *Medicago sativa*) and observed that species explained 85% and date of harvest only 6% of total variation. Differences in the alkane content between plant parts in the same pasture species were observed by Dove et al. [47]. Higher concentrations were found in the leaf than in the stem fraction. Also, much higher concentrations presented by the inflorescence of the perennial ryegrass (*L. perenne*) and by the flower of the white clover (*T. repens*) should be pointed out [47]. Less evident is the effect of age/stage of development in the alkane content of plant species. As stated above, the influence of the harvest date on the alkane content of the plant species studied by Dove et al. [47] accounted for only 5.7% of total variance. The results obtained by Oliveira et al. [57] indicate a decrease with age in the concentrations of C_{33} and C_{35} of hays of *Pennisetum purpureum* (C_{33} $r = -0.97$; C_{35} $r = -0.99$). Similar results were obtained by Laredo et al. [48] in leaves of *Pennisetum glaucum* (C_{33} $r = -0.81$; C_{35} $r = -0.85$) and *Sorghum* sp. (C_{33} $r = -0.96$; C_{35} $r = -0.95$) as age increased. However, opposite results were obtained by Smith et al. [58] when evaluating the effect of season of harvest on alkane concentrations of 40 common rangeland grasses found in Southern Africa. These authors did not observe a significant change in alkane concentrations either in leaf or stem components of the plant species between dry and wet seasons, suggesting that differences in alkane concentrations in whole plant samples could result from differences in the proportions of plant parts that present different alkane patterns.

Species	n-Alkanes (mg/kg DM)									References
	C ₂₅	C ₂₆	C ₂₇	C ₂₈	C ₂₉	C ₃₀	C ₃₁	C ₃₂	C ₃₃	
<i>Lolium perenne</i>	20.0	5.2	40.2	12.7	178.0	18.8	274.0	12.0	115.4	[50]
<i>Lolium multiflorum</i>	33.5	5.4	56.6	8.6	150.8	13.1	181.6	5.1	23.7	[51]
<i>Lolium rigidum</i>	17.2	7.6	51.0	17.7	254.0	22.9	411.0	–	7.6	[52]
<i>Festuca arundinacea</i>	23.6	3.2	42.3	7.6	129.3	12.1	215.7	6.8	58.7	[53]
<i>Holcus lanatus</i>	133.6	15.8	111.5	21.8	225.3	23.8	178.0	12.0	47.2	[51]
<i>Phalaris aquatica</i>	10.7	4.2	8.4	3.8	14.2	3.8	22.2	–	7.6	[52]
<i>Nardus stricta</i>	19.9	5.0	73.1	18.1	535.9	26.3	647.9	17.5	243.5	[54]
<i>Leymus chinensis</i>	3.0	2.0	11.0	4.0	26.0	4.0	57.0	2.0	15.0	[55]
<i>Leymus dasystachys</i>	10.0	4.0	28.0	4.0	47.0	4.0	46.0	2.0	12.0	[55]
<i>Elymus sibiricum</i>	8.0	2.0	16.0	4.0	114.0	7.0	185.0	4.0	25.0	[55]
<i>Trifolium repens</i>	16.4	3.8	38.2	11.3	170.0	16.6	206.9	7.3	22.2	[50]
<i>Trifolium striatum</i>	10.0	4.0	48.2	30.0	989.9	22.5	68.1	5.1	7.9	[53]
<i>Trifolium arvensis</i>	30.2	9.2	122.7	33.9	915.2	40.7	314.2	20.9	32.5	[51]
<i>Trifolium subterraneum</i>	4.9	4.7	52.3	18.1	361.0	10.9	80.8	–	6.0	[52]
<i>Vicia sativa</i>	15.8	3.7	67.0	12.3	204.2	16.3	502.8	15.1	29.7	[51]
<i>Ornithopus compressus</i>	17.4	5.3	40.3	9.7	570.1	8.4	60.8	1.1	10.7	[51]
<i>Ulex gallii</i>	4.7	3.1	38.5	11.1	111.2	18.5	269.5	7.3	9.6	[50]
<i>Calluna vulgaris</i>	15.8	9.9	75.4	26.6	289.9	35.1	939.8	58.8	685.5	[54]
<i>Erica cinerea</i>	18.0	7.1	45.1	12.9	215.6	38.7	1196.7	59.4	493.4	[54]
<i>Erica umbellata</i>	17.6	7.7	51.2	12.9	239.7	30.9	580.6	35.9	235.7	[54]
<i>Erica arborea</i>	14.5	4.6	67.8	21.4	408.7	93.7	1625.8	133.9	662.2	[50]
<i>Erica tetralix</i>	7.0	5.0	50.0	18.0	926.0	45.0	1838.0	46.0	687.0	[56]
<i>Vaccinium myrtillus</i>	13.0	10.0	45.0	42.0	151.0	33.0	201.0	11.0	46.0	[56]

Table 1. Alkane concentrations (mg/kg DM) of several herbaceous and shrub plant species.

Other epicuticular compounds, namely long-chain fatty alcohols (LCFAs) [59–63] and LCOH [53, 64–66], have also been suggested as possible diet composition markers. Also, alkenes (unsaturated aliphatic hydrocarbons) were tested with success by Dove and Oliván [67] to estimate diet composition of sheep fed with different proportions of chaffed perennial ryegrass and unpelleted sunflower meal labelled with beeswax. These epicuticular compounds have the advantage over any other possible markers as the separation and quantification of these wax components can be an extension of the alkane procedure, not adding much more analytical work [59].

It should be noted that, as stated by Dove and Mayes [21], all studies have been based on total LCFA and LCOH concentrations (i.e. free plus esterified LCFA and LCOH), as a result of the cleavage of wax esters promoted by the saponification of samples with ethanolic KOH (1 M) in the extraction process. The LCFAs present in the epicuticular waxes are mainly mixtures of straight-chain saturated compounds [41] with an even number of carbons (**Table 2**). Within the LCFA that can be detected in animal faeces, those with carbon-chain lengths between C₂₂ and C₃₄ are suitable for diet composition estimation as they are exclusively associated with plant epicuticular waxes and present high recovery in animal faeces [41, 60]. Various studies have shown clear differences in the LCFA profiles between different plant species [41, 60–63, 68, 69], making them useful as diet composition markers. In general, individual and total LCFA concentrations of plant species are much higher than those found for the alkanes, especially for the herbaceous species [60, 62]. In fact, Ferreira et al. [60] and Lin et al. [69] observed that the majority of LCFAs with even-chain length in herbaceous species have concentrations above 100 mg/kg DM, whereas only a few alkanes exceeded this value. Also, Ali et al. [68] and Lin et al. [69] found total LCFA concentrations that were in average 10 times greater than the total alkane concentrations of 25 different rangeland species from Sudan and native Chinese grass species (*Leymus chinensis*, *Leymus dasystachys* and *Elymus sibiricum*), respectively. As also found for alkanes, differences between plant parts can also be observed in their LCFA profiles. Although there is limited information on possible differences between plant parts in their LCFA profiles, results obtained by Ferreira et al. [60] indicated a trend for the leaf/stem fraction of *L. perenne* to present higher concentrations on the longer (>25 carbon atoms) LCFA than the inflorescence fraction.

Species	Even-chain fatty acids (mg/kg DM)								References
	C ₂₀ -acid	C ₂₂ -acid	C ₂₄ -acid	C ₂₆ -acid	C ₂₈ -acid	C ₃₀ -acid	C ₃₂ -acid	C ₃₄ -acid	
<i>Lolium perenne</i>	–	514.3	381.9	559.2	396.3	287.1	128.2	32.2	[60]
<i>Leymus chinensis</i>	212.0	247.0	322.0	91.0	159.0	152.0	144.0	–	[69]
<i>Leymus dasystachys</i>	207.0	346.0	229.0	171.0	338.0	191.0	55.0	–	[69]
<i>Elymus sibiricum</i>	196.0	257.0	217.0	169.0	165.0	114.0	55.0	–	[69]
<i>Trifolium repens</i>	–	612.9	715.8	607.9	792.4	440.4	64.7	1.2	[60]
<i>Ulex gallii</i>	–	447.2	308.9	128.0	114.1	133.9	25.5	0.2	[60]
<i>Agrostis-Poa</i> ¹	–	324.2	249.5	522.6	195.9	156.7	97.6	42.3	[63]
<i>Poa</i> spp. ²	–	236.1	156.7	366.6	120.1	45.3	24.0	6.5	[63]
Heather ³	–	549.9	485.6	482.7	550.0	432.8	394.3	105.7	[63]
<i>Calluna vulgaris</i>	148.0	347.0	255.0	195.0	199.0	168.0	6.0	31.0	[68]
<i>Erica arborea</i>	–	645.2	292.7	215.0	434.1	782.9	528.7	138.5	[60]
<i>Vaccinium myrtillus</i>	211.0	179.0	140.0	128.0	325.0	1132.0	176.0	6.0	[68]

¹Leaf fractions.

²Flowerstem fractions.

³Composed of *Erica umbellata* (0.76), *Erica cinerea* (0.16) and *Calluna vulgaris* (0.08).

Table 2. Even-chain fatty acid concentrations (mg/kg DM) of several herbaceous and shrub plant species.

Similarly to the LCFA, free LCOHs found in epicuticular wax of plant species are straight-chain saturated compounds with an even number of carbons within the same range of carbon-chain length referred to the LCFA (C₂₀–C₃₄) (**Table 3**). They are mainly primary alcohols, although many conifers present high concentrations of the odd-chain secondary alcohol 10-nonacosanol (C₂₉) [41]. As observed for the other epicuticular markers, LCOH profiles vary among plant species [52, 64, 65, 68, 69]. Generally, grass species are characterized by very high concentrations in C₂₆ and C₂₈ alcohols [52, 53, 64, 68], whilst C₃₀ alcohol can be detected in large amounts in legumes [52, 64]. In general, total LCOH concentrations are within those of alkanes and LCFA, although Lin et al. [55, 69] reported a predominance of LCOH over the LCFA in *L. chinensis*, *L. dasystachys*, *E. sibiricum*, *Stipa baicalensis*, *Stipa grandis* and *Cleistogenes squarrosa*. As also found for the alkanes and LCFA, results suggest clear differences between vegetative and reproductive parts of herbaceous species. In fact, Ferreira et al. [64] indicated that the reproductive parts of *L. perenne* are characterized by having higher proportions of shorter LCOH than the vegetative tissues.

Species	Even-chain alcohols (mg/kg DM)							References
	1-C ₂₀ -ol	1-C ₂₂ -ol	1-C ₂₄ -ol	1-C ₂₆ -ol	1-C ₂₈ -ol	1-C ₃₀ -ol	1-C ₃₂ -ol	
<i>Lolium perenne</i>	47.3	61.5	162.5	2159.6	517.4	149.0	54.8	[64]
<i>Lolium rigidum</i>	–	12.3	60.0	1751.0	363.5	176.1	–	[52]
<i>Festuca arundinacea</i>	–	–	26.8	638.8	100.5	58.0	–	[53]
<i>Phalaris aquatica</i>	–	14.6	37.8	2813.0	134.9	65.3	–	[52]
<i>Leymus chinensis</i>	0	21.0	73.0	361.0	846.0	252.0	116.0	[69]
<i>Leymus dasystachys</i>	9.0	28.0	142.0	3815.0	4418.0	442.0	70.0	[69]
<i>Elymus sibiricum</i>	11.0	14.0	126.0	2374.0	185.0	50.0	0	[69]
<i>Trifolium repens</i>	26.5	35.6	45.7	415.3	167.7	1077.5	84.9	[64]
<i>Trifolium striatum</i>	–	–	37.0	214.4	443.5	1259.3	–	[58]
<i>Trifolium subterraneum</i>	–	23.7	240.5	503.9	369.9	2141.0	–	[53]
<i>Ulex gallii</i>	30.8	81.7	189.1	120.1	133.4	111.7	153.9	[64]
<i>Calluna vulgaris</i>	221.0	1190.0	474.0	203.0	450.0	829.0	–	[56]
<i>Erica arborea</i>	157.9	315.0	210.8	120.8	206.0	262.1	46.1	[64]
<i>Erica tetralix</i>	62.0	560.0	1124.0	1015.0	1496.0	4465.0	–	[56]
<i>Vaccinium myrtillus</i>	271.0	511.0	362.0	334.0	383.0	931.0	–	[56]

Table 3. Even-chain alcohol concentrations (mg/kg DM) of several herbaceous and shrub plant species.

4. Application of epicuticular compounds as biomarkers

The differences in the profiles of the epicuticular compounds mentioned above can be explored to estimate the proportions of different plant species and plant parts in different samples,

such as herbage mixtures [70]; extrusa from oesophageal-fistulated animals [71] or faeces of sheep [52, 61, 65, 69, 72–75], goats [50, 60, 64, 76], cattle [62, 63, 66, 76, 77] and horses [62, 66, 77]. The principle of the application of the technique is simple and relies on the comparison of marker concentrations in a mixture (extrusa, digesta or faeces) and in diet components, plant species and/or plant parts that contribute (or could contribute) to that mixture. The comparison of the marker profiles can be made using different calculation procedures. It should be pointed out that more important than choosing the calculation procedure used, it is necessary to ensure that the information used (marker profiles of the possible diet components and the resultant mixture—faeces) is as accurate as possible.

Dove [70] proposed the utilization of simultaneous equations to estimate the proportions of the possible diet components when using alkanes as diet composition markers. In order to obtain unique solutions, the number of markers used is equal to the number of diet components and to the number of equations created [22]. The result of the equations indicates the amounts of the different diet components necessary to produce 1 kg of faeces, making possible to estimate the digestibility of the estimated diet. According to Dove and Mayes [78], this calculation procedure can be used in simple dietary mixtures, being more difficult to compute in complex mixtures. The main limitation of this procedure is in situations where there are more markers than the possible diet components, being necessary to select the markers to be used in the calculations. This selection involves arbitrary choices of the markers and the loss of information provided by the markers which were not used in the calculations. Moreover, this procedure may occasionally produce meaningless biological results as negative proportions of the diet components considered in the calculations.

In order to surpass these limitations, least-squares optimization methods can be applied, for which several algorithms have been developed [71, 79–81]. These calculation methodologies allow us to accommodate concentrations of different marker types (alkanes, LCFA, LCOH). The solution achieved by these algorithms attempts to minimize the squared deviations between the observed (O) marker concentrations in faeces and the concentration profile (E) arising from the diet composition estimate [21]:

$$\sum_{i,j,k=1}^n [O - E]^2 = \sum_{i,j,k=1}^n [(F_i - (x A_i + y B_i + z C_i + \dots)) + (F_j - (x A_j + y B_j + z C_j + \dots)) + (F_k - (x A_k + y B_k + z C_k + \dots))]^2 \text{ minimal} \quad (1)$$

or

$$\sum_{i,j,k=1}^n [O - E]^2 = \sum_{i,j,k=1}^n \left[\left(\frac{F_i}{F_t} - \frac{x A_i + y B_i + z C_i + \dots}{x A_t + y B_t + z C_t + \dots} \right) + \left(\frac{F_j}{F_u} - \frac{x A_j + y B_j + z C_j + \dots}{x A_u + y B_u + z C_u + \dots} \right) + \left(\frac{F_k}{F_v} - \frac{x A_k + y B_k + z C_k + \dots}{x A_v + y B_v + z C_v + \dots} \right) \right]^2 \text{ minimal} \quad (2)$$

where x , y and z are the proportions of components A , B and C in the diet; F_i , A_i , B_i and C_i are the concentrations of alkane i in faeces and diet components A , B and C ; F_j , A_j , B_j and C_j are the concentrations of LCOH j in faeces and diet components A , B and C ; F_k , A_k , B_k and C_k are the concentrations of LCFA k in faeces and diet components A , B and C ; F_t , A_t , B_t and C_t are total alkane concentrations in faeces and diet components A , B and C ; F_u , A_u , B_u and C_u are

total LCOH concentrations in faeces and diet components *A*, *B* and *C*; *Fv*, *Av*, *Bv* and *Cv* are total LCFA concentrations in faeces and diet components *A*, *B* and *C*. It is possible to express the individual marker concentrations in the feeds and faeces as absolute concentrations (Eq. (1)) or as proportions of the total concentration (Eq. (2)). The advantage of using concentrations instead of proportions is that *x*, *y* and *z* estimates using Eq. (1) are the amounts which will result in 1 kg of faeces. Thus, this information can be used to obtain an estimate of diet digestibility as

$$\text{Dry matter digestibility} = \frac{(x + y + z + \dots) - 1}{(x + y + z + \dots)} \quad (3)$$

5. Major constraints to the application of biomarkers in herbivory studies

As stressed by Dove and Mayes [21], it is important to ensure that the information used in both sides of Eqs. (1) and (2) (marker patterns of diet components and animal faeces) is as accurate as possible. An important source of error, often forgotten by researchers when applying the epicuticular markers to estimate diet selection in grazing studies, is the representativeness of the hand-collected samples of the vegetation components, in terms of marker profiles. This task can be difficult to accomplish as there can be significant variations in the marker profiles between plant species and plant parts within a specific plant species, as mentioned earlier. Other aspects requiring special attention are the continuous modification of each vegetation component available in the pasture, the relationship between plant parts and its stage of maturity and, consequently, their marker patterns. For this reason, it is recommended to collect samples of the plant species corresponding to each measuring period. Another important constraint associated with feeds/plant species is their very low marker concentrations. For example, herbaceous species (*L. perenne*, *T. repens*, *Pseudarrhenatherum longifolium*, *Agrostis capillaris*, [82] and *P. aquatica* [83]) and some shrub species (*U. gallii* [82]) are characterized by having low alkane concentrations and, for that reason, are more prone to analytical errors. Ferreira et al. [60] suggested that in these situations other marker types (e.g. LCFA or LCOH) should be used.

An additional concern is the collection of representative samples of faeces in terms of marker profiles. As occurs with other types of markers, the variation within and between days in the faecal concentrations can limit the utilization of this technique. In general, this variation is observed for dosed even-chain alkanes that are used for intake estimation [49, 84–91] due to their tendency to be associated with the liquid phase of the digesta. For that reason, an adaptation period of 5 days for the synthetic alkanes to reach a steady-state excretion pattern in animal faeces is generally suggested [21]. Regarding the natural markers, in grazing studies it is likely the existence of variation in the diet selected by the animals and, consequently, in feed intake, digestibility and faecal output from day to day [78]. For this reason, these authors suggest a sampling period of 5–7 days to obtain a more representative sample of faeces.

An assumption inherent to the application of the epicuticular compounds as diet composition markers is that they are totally recovered in the faeces. The results obtained in metabolic crate studies clearly indicate an incomplete recovery of alkanes [21, 73, 76], LCFA [59–63] and LCOH [52, 59, 64–66, 69] in the faeces of ruminant species (**Figure 3**), suggesting a close association between the length of the carbon chain of markers and its faecal recovery. Generally, results

suggest a higher faecal recovery in the LCOH than in alkanes and LCFA [59, 64]. This is possibly related to the different location of these compounds in the wax layer (i.e. alkanes in greater concentrations in the epicuticular layer, whilst primary alcohols are found in greater concentrations

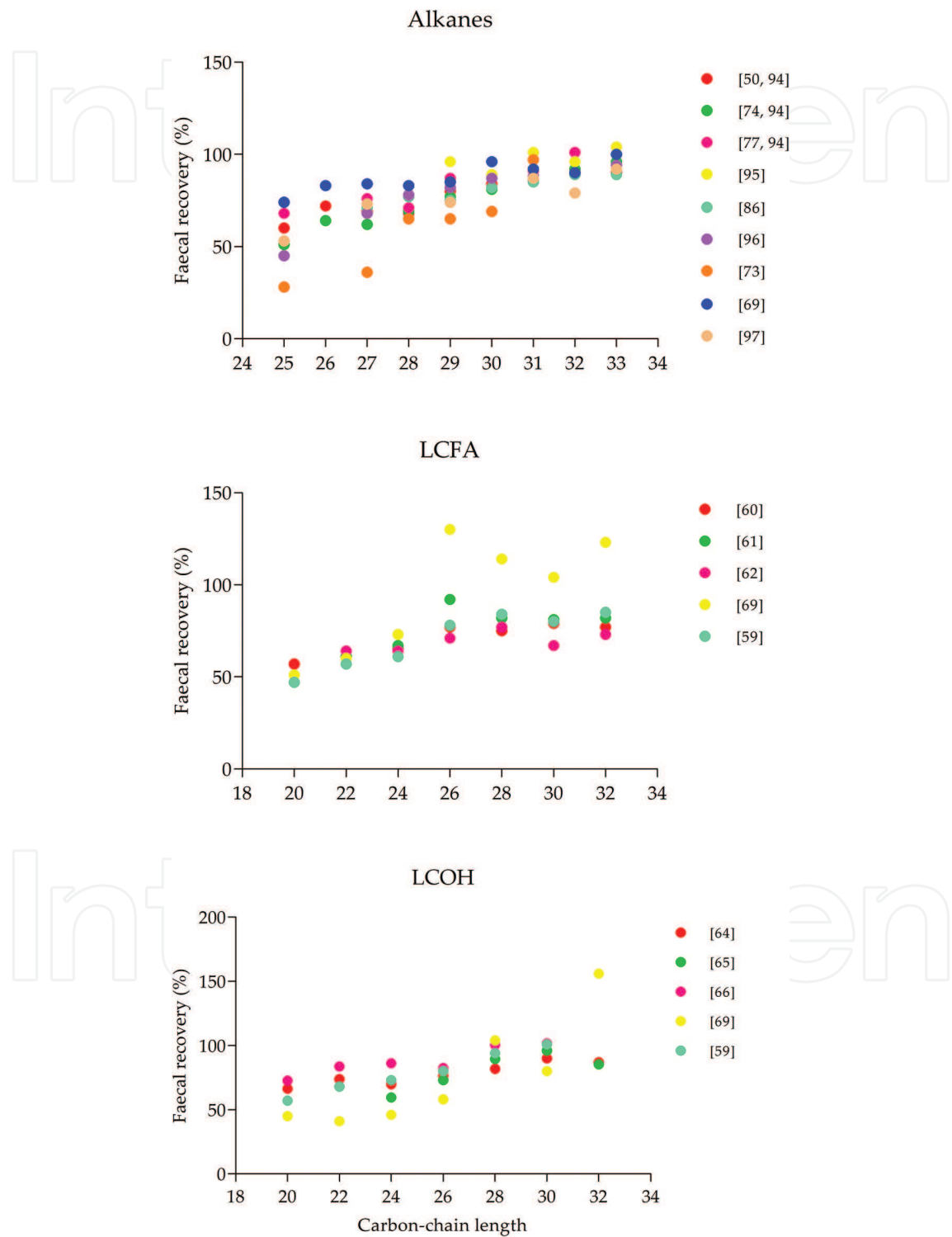


Figure 3. Effect of carbon-chain length on the faecal recovery (%) of alkanes [94], long-chain fatty acids (LCFA) and long-chain alcohols (LCOH) observed in ruminant species.

in the intracuticular wax [92] that could interfere with the efficiency of the extraction of these compounds and/or their absorption in the ruminants' digestive tract [65, 69, 93].

In several studies, the relationship between carbon-chain length and faecal recovery is better described by curvilinear functions for alkanes [60, 83, 93], LCFA [60, 61] and LCOH [65] as a result of the decrease in the difference of faecal recovery of markers with adjacent carbon-chain length with increasing carbon-chain length. By contrast, other studies have reported a linear association between carbon-chain length and faecal recovery in alkanes [75, 76, 96] and LCOH [64]. It seems that the association is dependent on the feeds/plant species comprising the diets and has an important effect on the accuracy of diet composition estimates on ruminant species. In fact, if uncorrected marker faecal concentrations are used in the calculations (Eq. (1) or (2)), estimates of diet composition will be biased towards those feeds/plant species with a predominance of longer carbon-chain length markers that have higher faecal recovery rates. For that reason, a suitable correction of marker faecal concentrations for incomplete faecal recovery, before applying them for diet composition estimation in grazing animals is generally suggested. Nevertheless, in situations where feeds/plant species do not have any chain-length bias, the effect of the recovery correction has little effect on the accuracy of diet composition estimates [60].

Data on marker faecal recoveries can be obtained in metabolic cage studies with animals fed on different mixtures of feeds/plant species that are available for each specific situation. It should be noted that in complex situations in terms of number of possible diet components, it will be difficult to decide which combination of plant species and/or plant parts will reflect the diet selected by each different animal species. For alkanes, Dove and Mayes [21] suggested another option that consists of dosing a range of synthetic even-chain alkanes and collecting the total faecal production by wearing faeces bags and calculating the faecal recoveries of natural odd-chain alkanes by interpolation. Nevertheless, it has been found that the synthetic dosed alkanes may have higher recoveries than those expected from interpolation of adjacent natural odd-chain alkanes [82, 87, 98].

For non-ruminant species such as horses [97, 99–102], pigs [103, 104], mountain hares [105] and pigeons [106], marker faecal recoveries seem to be unrelated to their carbon-chain length (**Figure 4**), indicating that these markers behave differently in the digestive tract of ruminants and non-ruminants, especially those with lower carbon-chain length. In fact, the comparison of faecal recovery data between ruminant and non-ruminant species for alkanes [77], LCFA [62] and LCOH [66] indicates a greater disappearance of the shorter markers in the gut of ruminants than in non-ruminants. The site and the mechanisms underlying marker losses in the animal gastrointestinal tract are still far from being completely elucidated. Earlier studies undertaken by Mayes et al. [107] suggested that the disappearance of the dosed alkanes C_{28} , C_{32} and C_{36} occurred mainly in the small intestine in sheep. More recently, Keli et al. [25] also suggested that alkane disappearance should mainly occur in the small intestine as they were not be able to find evidences of rumen microorganisms' capability to synthesize or metabolize alkanes in in vitro conditions. By contrast, Ohajuruka and Palmquist [108] found that the loss of dosed C_{32} alkane in dairy cows occurs mainly in the rumen.

The lack of a clear relationship between the carbon-chain length of the epicuticular compounds and their faecal recovery in non-ruminant species has an important effect on diet

composition estimates. In fact, Ferreira et al. [77] were not be able to observe an increase in the accuracy of diet composition estimates in horses when alkane faecal concentrations corrected for their incomplete recovery were used. This lower dependence of markers for a suitable faecal recovery correction was also found in LCOH and LCFA by López López et al. [66] and Ferreira et al. [62], respectively, although in their cases a linear association between carbon-chain length and faecal recovery was observed. These results indicate that, for this animal species, accurate estimates of diet composition can be obtained even when raw data of the faecal

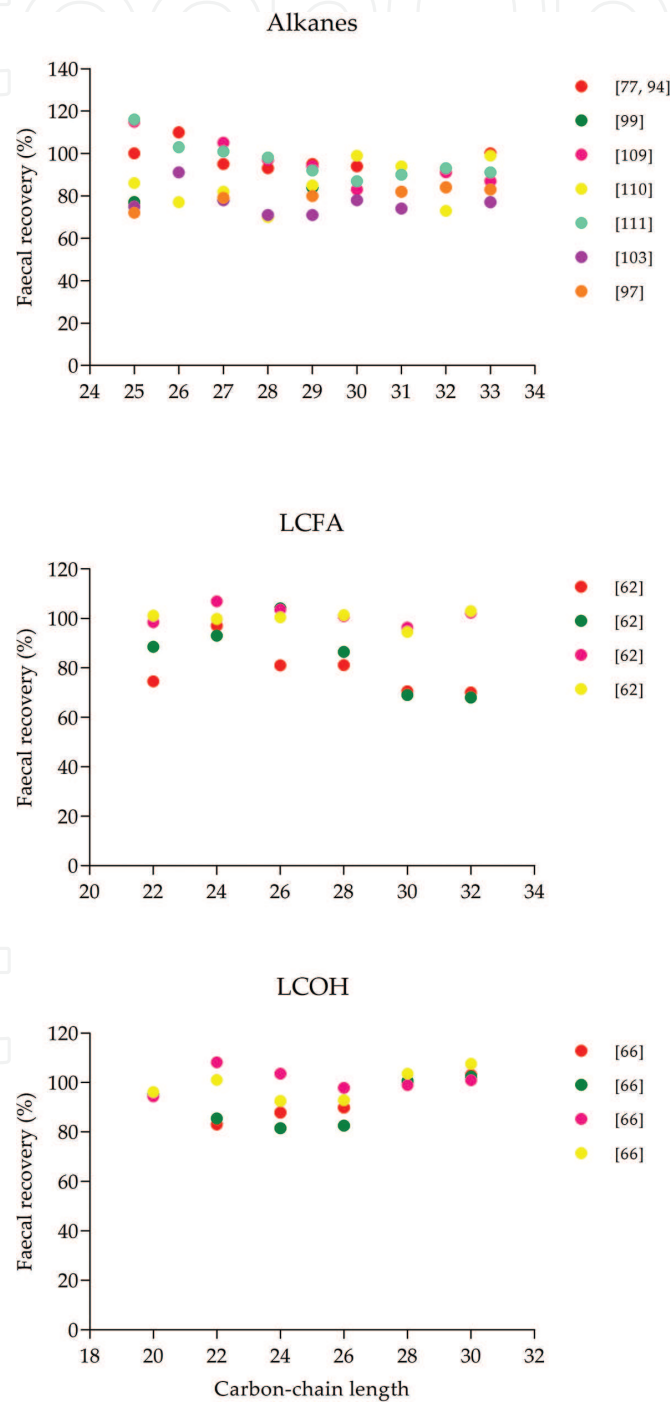


Figure 4. Effect of carbon-chain length on the faecal recovery (%) of alkanes [109–111], long-chain fatty acids (LCFA) and long-chain alcohols (LCOH) observed in non-ruminant species.

concentrations of these epicuticular compounds (i.e. without previous corrections of the faecal concentrations) are used.

Another important constraint that limits a wider applicability of epicuticular compounds as markers in grazing studies is that their faecal recovery may depend on the diet composition, compelling researchers to calculate faecal recoveries for each specific situation (i.e. diet composition), making it impossible to use recovery data available in literature. This effect was observed in several studies performed with alkanes [50, 83], LCOH [64, 65, 69] and LCFA [60, 61, 69], whilst others were not able to detect it [52, 73, 76, 96]. According to some authors [64, 69, 96], this inconsistency may be due to the particular plant species comprising the diets. Lin et al. [69, 75], using sheep fed distinct grass species (*E. sibiricum*, *L. chinensis* or *L. dasystachys*) obtained different faecal recoveries of LCOH, LCFA and alkanes. According to the same authors, these results could be explained by differences among plant species in their plant cuticular wax morphology, influencing the level of extraction and the absorption of these compounds in the digestive tract of animals. Diet digestibility may also explain these differences in the faecal recovery of the epicuticular compounds among different diets. In fact, a general tendency for higher faecal recoveries of alkanes [50, 74, 112] and LCOH [64] in diets with lower digestibility was observed. Lower accessibility to the cuticular waxes of those feeds as a result of a higher association of cuticle with cell wall components [113] may explain the lower availability of epicuticular compounds to be absorbed in the digestive tract of animals.

The first epicuticular compounds suggested as diet composition markers were the alkanes [45], and the limited number of components (e.g. plant species and/or plant parts) that can be discriminated in the diet that is restricted to the number of n-alkanes available was soon recognized. The number of alkanes available for diet composition calculations is generally limited to 9 (C_{25} – C_{33}), due to the higher potential analytical error associated with those of very low concentrations both in plants and in faeces, which may contribute to the discrepancy in sum of squares in the calculation method [78, 95]. Moreover, it is accepted that the increase of the number of diet components to be discriminated will likely result in less accurate diet composition estimates, as it increases the likelihood that an observed alkane pattern in faeces may result from different combinations of diet components [21]. To overcome these limitations, a possible approach to obtain reliable diet composition estimates is to increase the number of ‘discriminators’ by combining the use of alkanes with other plant-wax markers, such as alkenes [67], LCOH [52, 53, 64, 69] and LCFA [59–63]. According to Bugalho et al. [53], combination of markers should only be performed when additional discriminatory information is provided. Combination of different marker types may improve the accuracy of diet composition estimates as markers with greater concentrations, less prone to analytical error, can be selected in situations where the possible dietary feed items have similar alkane profiles. Moreover, it is likely that the use of a greater number of markers provides a more specific ‘fingerprint’ for a particular plant component [60]. This is also very important even when the number of plant species to be discriminated is low, but they present high similarities in alkane profiles, making it difficult to discriminate them.

In several studies, it was possible to observe an increase in the accuracy of diet composition estimates when combining two [52, 60, 114] or three marker types [64–66, 68, 69]. It should be pointed out that the combination of epicuticular compounds does not necessarily result in more accurate estimates of diet composition [21, 53]. For example, Vargas-Jurado et al. [115] did not observe an improve-

ment of the predictions of the composition of mixtures of tall fescue (*Festuca arundinacea*) and red clover (*Trifolium pratense*) when LCOH was combined with alkanes. Nevertheless, results obtained by Ferreira et al. [60, 64, 65] suggest that the combination of markers reduce their dependence on accurate faecal-correction data for an accurate diet composition estimation as more specific fingerprints of each plant species are achieved, increasing their ability to discriminate them.

Another approach that is suggested when the number of possible diet components is high is to decrease the number of possible diet components by pooling the available plant species into groups [81, 116]. These groups are formed by plant species with similar marker profiles, based on multivariate statistical analysis, that are then treated as dietary components in the calculations. One aspect that needs particular attention is the fact that the accuracy of diet composition estimates can be influenced by different availability or selectivity levels of some plant species within each group, especially if the marker profile of a particular plant species is distinct from the mean marker profile of the group in which that species is included [51]. As pointed out by Ferreira et al. [116], feeding selectivity effect will depend on the particular species that could be selected within the group and on the similarity in the marker profile of the plant species of the dietary group. Bugalho et al. [51] did not find any feeding selectivity effect within a group of 19 herbaceous species on diet composition estimates of red deer. Similar results were observed by Ferreira et al. [116] when applying different levels of feeding selectivity to a dietary group formed by heather species (*C. vulgaris*, *Erica cinerea*, *Erica umbellata* and *Erica australis*) with similar alkane profile. By contrast, when the same procedure was applied to a dietary group formed by three grass species (*L. perenne*, *P. longifolium* and *A. capillaris*), significant modifications of the proportions of each dietary group were observed. A decrease in feeding selectivity effect can be achieved by the formation of more uniform dietary groups in terms of marker profiles. However, Ferreira et al. [63] observed higher levels of accuracy of diet composition estimates when considering all plant species that animals had at their disposal compared to its grouping according to the similarity of alkane and LCFA profiles.

The exclusion of plant species based on preliminary information is another approach suggested by Dove and Mayes [21] to reduce the number of possible diet components. The observation of the animals' feeding behaviour, plant-derived data or information based on other methodologies that indicate the rejection of a particular plant species and/or vegetation community, can help the researcher to use more accurate data on the plant species that should be considered in the calculations.

The utilization/combination of different types of markers has also advantages in less complex plant communities (i.e. lower number of plant species to be discriminated), by giving the opportunity to the researcher to choose those with higher concentrations less prone to measurement errors in their analytical determination [21]. According to Charmley and Dove [83] and Ferreira et al. [60, 61], the utilization of markers with low concentrations can turn discrimination of plant species more difficult and may result in less accurate estimates of diet composition. In fact, Oliván et al. [82] attributed the difficulties in distinguishing three grass species (*L. perenne*, *P. longifolium* and *A. capillaris*) and gorse to their low alkane concentrations. Also, Charmley and Dove [83] had difficulties in obtaining accurate estimates of diet composition when *P. aquatica* (plant species characterized by very low alkane concentrations) was a component of diets fed to sheep. In our opinion, the exclusion of markers based on their low concentrations should be performed with caution as, in some situations, they may dis-

criminate better plant species than those with higher concentrations. Thus, Dove and Mayes [21] suggested that the balance between the capability of markers to discriminate plant species and the level of potential analytical error should be considered when choosing markers.

6. Conclusions

Taking into account all the data presented in this chapter, it is certain that the application of the epicuticular compounds as faecal markers can improve our knowledge on the grazing behaviour, particularly diet selection, of free-ranging herbivore species under different vegetation conditions. Although some shortcomings can be pointed out to these faecal markers, namely the variation of profiles within plant species and morphological parts, lack of inertness in the digestive tract of ruminant species and, for that reason, the need for a suitable recovery correction of their faecal concentrations, they have been used quite successfully. Its application allows to overcome major limitations recognized to the traditional techniques in terms of accuracy and extent of the results (i.e. identification of plant species and/or plant parts), animal welfare issues (i.e. avoid the need for fistulated animals; lower disturbance of animals compromising its normal grazing behaviour) and intensive labour. The combination of different marker types (alkanes, LCOH, LCFA) seems promising to overcome the enumerated constraints and to extend their application to more complex vegetation communities. Therefore, research on the identification of other chemical compounds should continue to be developed. Finally, data obtained from different available techniques (microhistological procedures, NIRS, Fourier-transform infrared (FTIR) spectroscopy and fluorescence spectroscopy, telemetry solutions) should be integrated in order to enhance the accuracy of diet composition being selected by herbivore species. This will further improve the precision of information (i.e. possible diet components) used when applying the epicuticular markers.

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