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Review

Techniques Used to Determine Botanical Composition, Intake, and Digestibility of Forages by Ruminants

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Abstract: The botanical and chemical composition of diets consumed by ruminants is different from the composition of plant species available in the rangeland or pastures on which they graze. Exploring alternative and improving existing methods of estimating botanical composition (diet selection) is imperative in advancing sustainable feeding practices in extensive production systems. The ability to predict the intake and digestibility of the diet consumed is important in designing grazing management for different feeding systems as well as supplementation strategies. This facilitates the efficient use of feed resources for optimal animal performance. This review assesses the merits, limitations, and potential advancements in techniques used to estimate botanical composition, forage intake, and digestibility in ruminants. Supplements containing sufficient quantity and identifiable *n-alkanes* can be used to determine the total forage intake in grazing ruminants without dosing the animals with synthetic even-numbered *n-alkanes*. When the botanical composition, intake, and digestibility of diet are estimated using internal markers, the results should be validated with those of faecal near-infrared reflectance spectroscopy (NIRS) or plant cuticular compounds to enhance the prediction accuracy. This should be done to determine the degree of error in the use of internal markers. Conclusively, the use of internal markers with automated solver routine software is a prudent approach to predicting botanical composition due to the analytical ease of the markers involved and the associated model assumptions.

Keywords: diet selection; grasslands; internal markers; NIRS; plant cuticular compounds; solver routine software

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1. Introduction

Natural grasslands and crop residues are the major feed resources for ruminants across Sub-Saharan Africa [1]. Although the nutritional value of feed resources is highest during the wet season, it varies widely across seasons, and often, the nutrients available are inadequate to sustain optimal animal performance [2]. Forages selected by grazing ruminants are usually different in composition and quality compared to the average available within the rangeland. Therefore, the knowledge of the nutritive value and intake of selected dietary components is important for managing different feeding systems and facilitates the choice and quantity of supplement feed required by the animals [3].

The quantity and quality of herbage selected and consumed in rangelands are influenced by several factors, which include but are not limited to the nutritional status and post-ingestive feedback of grazing herbivores, the presence of hair on and leaf size of the plant species, forage availability, as well as the botanical composition of the pasture and furthermore, differences in plant distribution, presence of chemicals as herbivory tolerance mechanism, and the number of plant species [4]. The magnitude of the influence caused

by the factors that affect plant selection choices and the extent of their consumption by animals under natural conditions remains unclear [5]. The feed preference and chemical composition of the diet selected, as well as digestibility, are interrelated in regulating intake in ruminants [5,6]. These serve as important tools in evaluating animal performance in the dynamic ecosystem under which animals graze.

The existing techniques used to determine botanical composition (diet selection), dry matter intake and digestibility in free-ranging animals have several limitations [7]. The traditional methods of predicting diet selection, dry matter intake, and digestibility in ruminants have presented results with a varying magnitude of error [3]. Therefore, there is a need to improve the existing methods and identify more accurate alternatives for predicting the botanical composition of the diet consumed, dry matter intake, and digestibility of selected diets by grazing animals. This review aims to assess the merits and limitations and provide suggestions for potential improvement to some of the techniques used to estimate botanical composition, forage dry matter intake, and digestibility in ruminants.

2. Methods of Estimating the Botanical Composition of Selected Diets

2.1. Utilisation Technique

Diet composition (the quantitative botanical composition of what a grazing animal selects) is usually different from the average composition of the herbage present in a rangeland. The utilisation technique is one of the primitive methods used in assessing the botanical composition and intake of diet consumed by grazing herbivores [8]. This approach shows the locations grazed and the extent to which the rangeland has been utilised. This is done by estimating the biomass composition of the rangeland before and after grazing. Some of the limitations associated with this approach include the inability to measure the frequency and time of utilisation of the various plant species consumed. Furthermore, biomass regrowth after defoliation and the invasion of the rangeland by other animals, rather than those of interest, are not quantified and could negatively influence the accuracy of measurements and reduce the credibility of the results from this technique [9].

When the range is solely grazed by the animals of interest, the technique can be used to monitor longer-term (seasonal) grazing responses of herbivores as compared to shorter-term (hours to days) grazing responses. The influence of the preferred plant species in the grazed or browsed area can be estimated based on production performance indexes such as body weight changes, milk, wool, or cashmere production at the end of the seasons. Therefore, this approach can be used for seasonal formulation and allocation of supplements to grazing animals, given that the requirements of animals and the nutrient content of the plant species consumed in the range are known (Tubiello, personal communication).

2.2. Direct Observation

Direct observation involves the visual assessment of plant species selected by the animals in comparison with the overall forage species in the grazed area at a given time. The botanical composition of diets consumed and the ingestive behaviour of ruminants can be evaluated using this method [10,11]. The principal advantage is that direct observation does not require the use of sophisticated equipment. However, the proper identification of plant species consumed remains a key drawback of this approach because animals graze continuously, and successive sample recognition becomes biased. Additionally, it is difficult to approach untamed animals close enough to observe what they are foraging accurately, and it can be hard even to locate them. In contrast, tamed animals can be used for close observation. However, only one or two animals can be observed by one observer at any given time, while diet selection is a complex concept involving constant interaction among animals [12], with the likelihood of reducing the accuracy of this technique.

The development and application of video recording tools in conjunction with global positioning system (GPS) collars and thermal imagining systems have enhanced the extent and accuracy of the direct observation method [13]. Managing stocking rate and animal distribution are crucial factors to consider when using video recording devices because

the devices might not cover all the grazing areas, resulting in missing animals grazing outside the coverage area, while some animals may be difficult to distinguish from others. Remote sensing devices have been effective in assessing animal distribution in natural habitats [14]. Furthermore, bite size and time spent on foraging and rumination have been found effective in predicting the intake of grazing animals [15]. Time spent on grazing could be used as an index of species preference and/or the importance of the plant species among the forage species available to the animals. According to the equation proposed by Sanon et al. [16], the grazing time (GTi) of i^{th} species in the range is defined as:

$$GTi = RGTi \times G \times T \quad (1)$$

where RGTi is the ratio of time spent on grazing i^{th} species to the total time spent on all species grazed in the range, G is the proportion of the grazed species to the total biomass in the range, and T is the total time spent by animals in the range (T).

Nevertheless, the complexities in distinguishing between hedonic and active grazing make it difficult to capture and predict the actual preference of each plant species consumed in the rangeland. The experience of the observer and the number of animals grazing in the range may also hugely impact the accuracy and precision of the direct observation method. The plant stage of development and the number of plant species in the grazed area could also affect the accuracy of this method because as plant species mature, they become easier to identify [17] as opposed to the early stages of growth. Furthermore, it is easier to identify the forage species consumed when there are only a few plant species in the grazed area.

A possible improvement to the direct observation technique is the use of tamed animals by experienced observers familiar with the plant species in the particular range [18]. Furthermore, calibrating the direct observation data against other techniques (e.g., micro histological procedures, discussed later in Section 2.3) based on results obtained for the specific grazed range could enhance the accuracy of the information obtained. The adaptability of animals to tropical rangelands can advantageously be estimated from behavioural data collected using the direct observation technique of diet selection monitoring. Urination frequency, respiration rate (estimated by flank movement), and drinking frequency could be used as an index of the adaptability of different grazing animals to the harsh conditions associated with most tropical rangelands [19]. Equally, several approaches to monitoring chewing behaviour responses, such as jaw movement, bite frequency, and bite size, may enhance the accuracy of the direct observation method in predicting forage and nutrient intake [20,21]. The use of video recording devices combined with sensor technologies such as the RumiWatch system [15] and other technologies that monitor jaw movement and bite characteristics can be used to estimate intake with higher accuracy and reliability without the need for close human observation of the grazing animals [22,23].

2.3. Microhistogical Procedures

This technique is based on the microscopic identification of plant fragments in faecal samples, stomach contents (or rumen evacuation), or oesophageal extrusa to determine diet composition and preference [24]. The stomach content method requires the slaughter of the animal, while the oesophageal extrusa and rumen evacuation methods are invasive techniques, requiring the fistulation of animals with a cannula in the oesophagus and stomach, to gain access to bolus and digesta, respectively. Generally, in microhistological analysis, the number of plant species found in the sample, measured volume, weight, and frequency of occurrence of each plant species, are used to evaluate the botanical composition of the diet consumed [25,26]. Samples collected from the rumen are contaminated with rumen contents making it difficult to analyse botanical diet composition [27].

Another important drawback of the stomach content method is the variation in the degradability of the plant species consumed. This results in a wide variation between the proportions of individual plant species in the diet consumed and their proportions in the stomach which is collected after slaughtering [28]. Furthermore, the analysis of

stomach contents does not provide information about where and when the animal ingested the forage.

Wilson et al. [29] proposed the trocar method as a modification to the stomach evacuation method as a means of preventing the slaughtering of animals. In the trocar method, animals are tranquillised, cut open, and samples are taken using a trocar; the wound is then sewn up. However, an overdose of the sedative, wound infections, and illnesses are some of the complications associated with this method, which often result in the eventual death of many animals subjected to this surgical procedure. This method is not recommended for use on endangered animal species, but animals killed by wild carnivores and animals culled or slaughtered for consumption could be used to estimate the botanical composition of intake using the stomach content method. The stomach content method can also be used as a reference for calibration and validation or to improve the accuracy of other techniques, such as direct observation and marker techniques.

2.3.1. Faecal Analysis Technique

The faecal analysis technique has received more attention than the oesophageal and stomach evacuation methods because of the many advantages it possesses, as detailed in previous reviews [30–32]. For example, the analysis of faecal samples does not interfere with the natural foraging behaviour of animals, is non-invasive and applicable to both domesticated and wild ruminants. However, the size of the faecal samples that can be analysed is limited by the volume of faeces excreted and how frequently the animals defecate. Furthermore, the proportion of diet components found in faeces is often not the same as in the consumed diet as a result of the differences in the passage rate and degradation rate of the different classes of plants consumed across and within animal species. Nevertheless, the faecal analysis technique is valuable in identifying the preference and botanical composition of forage species consumed by grazing animals [24].

2.3.2. DNA Sequencing of Faecal Samples

The DNA composition of faecal samples of ruminants could be used to estimate the botanical composition of an unknown diet consumed, such as those in a typical rangeland. This analysis is achieved using genetic sequence analysis which targets DNA segments (loci) that distinguish one plant from the other at the genus and specie levels, also called their DNA bar code [33]. The chloroplast DNA loci commonly used include the ribulose-bisphosphate carboxylase gene (rbcL), maturase K (matK), and the intron region of chloroplast tRNA gene (trnL), alone or in combination [34–36] to predict the taxonomic composition of feed consumed from faecal samples obtained. The technique involves total DNA extraction from faecal samples, sequencing of specific or different chloroplast DNA segments, and comparing the sequences with existing genomic databases [34].

While there has been much higher success with DNA bar code analysis of diets of carnivores, herbivore diets have specific limitations, which include the need to target specific DNA segments with maximal variability across plant species while also allowing for sufficient amplification from the DNA available. Furthermore, there is not enough reference genetic database for validating DNA sequences across the wide array of plant species that exist in natural rangelands [33,34]. In the study by Palumbo et al. [34], it was noted that the sequence abundance of the trnL gene in faeces also depends on the density of chloroplasts in the different species and on the digestibility of their plant tissues, how closely the faecal plant composition reflects the botanical composition of the diet ingested is still unclear. Therefore, while the DNA bar coding technique may be used to estimate the biodiversity of species consumed (diet composition), the quantitative estimation of each herbage consumed cannot be predicted. The cost of using this technique in practical contexts may also be a major drawback, especially in resource-limiting scenarios.

Nevertheless, this technique has been noted to be more accurate than other indirect methods of determining diet selection [35], although only limited studies have compared these methods across a wide range of ecological/animal feeding conditions in cattle [34].

Nevertheless, the DNA bar coding technique has been used as a proxy to predict diet composition in white-tailed deer, bison (Bos bison), goats (*Capra hircus*), western lowland gorillas (Gorilla gorilla), and colobus monkeys (colobus guereza) [33,37–39]. Therefore, more studies are recommended for a better understanding of the interaction between specie, feeding behaviour, and diet selection in natural and controlled environment scenarios using the DNA bar coding technique.

3. Analytical Procedures in Estimating Diet Intake in Grazing Animals

Biomarkers can be administered orally or incorporated into animal feed with the purpose of monitoring intake and/or digestibility. For a substance to be regarded as a marker, it should pass certain criteria. Generally, a suitable marker should be indigestible, inert and not bulky, neither affect nor be affected by the gastrointestinal tract (GIT) and its microbial population [40], as well as mix and remain uniformly distributed in the digesta [41]. The internal markers are natural components of feedstuffs, whereas external markers are synthetic and administered orally through dosing or inserted into the rumen via cannula, both of which are quantitatively recovered in faeces [42]. Diet components that qualify as markers must be metabolised to a low extent [43]. Some of the internal markers commonly used in animal nutrition studies include the modified acid detergent fibre (MADF), acid detergent lignin (ADL), indigestible neutral detergent fibre (INDF), indigestible acid detergent fibre (IADF), acid detergent insoluble nitrogen (ADIN), plant cuticular compounds (*n-alkanes*, alkenes, long-chain alcohols, sterols, aldehydes, long-chain fatty acids and flavonoids), and acid-insoluble ash (IAI), whereas examples of external markers include cobalt-ethylenediamine tetraacetic acid (Co-EDTA), titanium dioxide (TIO_2), chromium (III) oxide (Cr_2O_3) and ytterbium [44,45]. However, most of these markers do not fully fulfil all the requirements of an ideal marker [45]. External markers are often used to estimate faecal output and digesta passage rate, whereas internal markers are usually used to estimate dry matter digestibility in ruminants [46,47]. These measurements are subsequently used to estimate diet intake [45].

3.1. Plant Cuticular Wax Compounds

The surface of most browse and forage species contains a layer of epicuticular wax classically made up of aliphatic lipid compound mixtures such as *n-alkanes*, alkenes, long-chain alcohols, sterols, aldehydes, long-chain fatty acids, and flavonoids [48–50]. These can serve as biomarkers and can be unique to each forage and its parts. When oesophageal extrusa, stomach contents, or faces from animals consuming these plant materials are obtained and analysed, it can reliably be used to estimate the proportion of the diet composed of these plant or plant parts. Such can be translated to the botanical composition of the overall diet selected by such ruminants.

The abundance and composition of these wax compounds vary within plant parts and between plant species, with roots having the least while floral parts and leaves have the highest concentrations [51,52]. The variability in composition and distribution of these compounds in different parts of the plant (floral, leaves and stems) necessitates that these different plant fractions would need to be analysed separately to facilitate the predictions of selected dietary components through the use of the concentration of these plant epicuticular compounds. In the study by Fraser et al. [53], sheep selected different plant parts, showing a preference for leaves rather than stems and shoots, as would be expected under natural pasture conditions.

Some of the frequently used plant cuticular compounds are shown in Table 1. *N-alkanes* (C21-C35) are the prevalent class of these compounds and are frequently used as biomarkers in nutritional studies on ruminants and non-ruminants [54].

Table 1. Main properties of plant cuticular wax compounds used as biomarkers in animal nutrition studies.

Class	C-Length	Properties	Abundance	References
N-alkanes	C_{21}-C_{37}	Odd-numbered C-chains	Highly common	[55]
Branched *n-alkanes*	C_{28}-C_{32}	Iso- and ante-iso-branched chain	Rare	[45]
1st-OH	C_{20}-C_{34}	Saturated even-numbered C-chain	Common in high concentrations	[55]
2nd-OH	C_{29}	Odd-numbered C-chain, mainly C29	High concentrations in conifer leaves	[45]
LCFA	C_{20}-C_{34}	Even-numbered C-chains	Common in higher plants	[56]

C-length, carbon-chain length; 1st-OH, primary alcohols; 2nd-OH, secondary alcohols; LCFA, long-chain fatty acids.

About 10% of these markers have an even-numbered chain length, while the rest are odd-numbered [8]. The most prevalent *n-alkanes* are the ones with 29, 31, and 33 carbon atoms [51]. Alkenes are the other class of hydrocarbons with potential use as markers [45,49]. They are mostly odd-numbered mono-enes and are prevalent in the floral parts of plants, with chain lengths ranging from 23 to 33 carbon atoms [57].

Only a few studies have reported the estimation of composition, intake, and digestibility of diet selected by ruminants using alkenes. Therefore, further validation of this method is needed to establish its application as a biomarker for diet botanical composition and intake. Nevertheless, one of the major challenges associated with the use of alkenes is that these markers tend to be impure when running analyses using gas chromatography-mass spectroscopy (GC-MS), suggesting the need for additional effective analytical procedures. These procedures include the use of silica gel to purify the alkenes. This laborious analytical process limits the use of alkenes as a suitable alternative to the plant cuticular markers (*n-alkanes*) [58].

Long-chain alcohols [55,58] and long-chain fatty acids [56] have been assessed and validated as markers for predicting dietary parameters in ruminants. These are analysed using a similar procedure as *n-alkanes* but with additional analytical steps [59,60]. In the analysis of long-chain alcohols, additional analytical steps involve the crucial formation of trimethylsilyls (TMS) alcohol derivatives, making long-chain alcohols more suitable for GC-MS to yield better mass spectra that are distinct from other classes of aliphatic compounds [45]. Therefore, analytically, long-chain alcohols and fatty acids are suitable additional markers to *n-alkanes* compared to other alternative plant cuticular wax compounds (i.e., alkenes, sterols, triterpenes, and aldehydes).

According to Fraser et al. [53], using *n-alkanes* as the sole biomarker does not limit the number of dietary components that can be identified in the consumed diet. In contrast, Bugalho et al. [59] reported that the small number of *n-alkanes* available for analysis in comparison to the numerous dietary components available for an animal to choose from limits its application in predicting diet intake in natural grazing conditions. In agreement with Bugalho et al. [59], when the dietary choices varied from 4, 6, or 8 forage species, the accuracy of prediction (Kulczynski Similarity Index; KSI) when using *n-alkanes* as markers reduced from 85.7 to 77.7 and 76.2%, respectively [61]. The Kulczynski similarity index is used as a proxy for accuracy in predictions. This measure is calculated as follows:

$$\mathrm{KSI} = 100 \times \sum 2\,ci / \sum (ai + bi) \quad (2)$$

where KSI is the Kulczynski similarity index, *ci* is the lesser proportion of dietary component *i* between the observed and the predicted values, and *ai* + *bi* is the sum of the observed and the predicted values of dietary component *i*.

3.2. Evaluation of Multiple Internal Biomarkers

From samples collected via stomach evacuation and oesophageal extrusa or from faeces, it is possible to analyse several markers in predicting the botanical composition of feed consumed. When more than one type of biomarker is used, the accuracy of diet intake prediction seems to be higher. In the study by Lin et al. [61], long-chain alcohols (LCOHs) and long-chain fatty acids (LCFAs) were added to *n-alkanes* as biomarkers to estimate overall botanical composition, and the accuracy (R^2, coefficient of determination) increased to 81.9% (*n-alkanes* and LCOHs) and 82.0% (*n-alkanes* and LCFAs), as opposed to 75.4% with *n-alkanes* alone. These results indicate that combining these classes of markers will improve the accuracy in estimating the botanical composition of selected diets in ruminants grazing rangelands. In a similar study involving dairy cows consuming a ryegrass- and heather-based diet under a cafeteria feeding system, López et al. [54] observed that the accuracy of prediction (KSI) improved with the use of LCOHs and LCFAs (KSI = 98.90%) compared to the use of LCOHs alone (KSI = 97.2%; Table 2). However, LCOHs and *n-alkanes* alone had lower accuracy (KSI = 95.6%). It may be noted that the number of distinctive *n-alkanes* is limited and should be greater or equal to the number of plant species in the range before *n-alkane* analysis can be used in estimating botanical diet composition [44,51]. This renders *n-alkanes* less accurate and less suitable, compared to LCOHs and LCHFAs, in the estimation of the botanical composition of diets selected in typical rangeland with a complex diversity of plant species. Furthermore, in nutritional studies serving as proof of concept where animals are fed under a simulated environment by providing different feeds from ad-libitum to restriction feeding and at different stocking rates, the results of intake and preference under such conditions should be validated in a typical rangeland scenario.

Table 2. Comparison of the accuracy of techniques used to determine diet selection in ruminants.

Diet	Animals	Method [1]	Feeding System	Accuracy	Reference
Aristida multicaulis, Urochloa brizantha, Hyparrhenia rufa, Imperata cylindrica and Stylosanthes guyanensis	Cattle (237 ± 16 kg BW)	ALK	Indoor (mixed diet)	KSI = 70.75%	[47]
Themeda triandra, Zea mays and *Sorghum bicolor hay*	Sheep (46.5 ± 3.3 kg BW)	MADF, ADL and AIA	Indoor (cafeteria)	KSI = 90.90%	[5]
Ryegrass and heather	Cattle (499 ± 36 kg BW)	LCOH	Indoor (cafeteria)	KSI = 97.2%	[54]
Ryegrass and heather	Cattle (499 ± 36 kg BW)	LCOH and ALK	Indoor (cafeteria)	KSI = 96.50%	[54]
Ryegrass and heather	Cattle (499 ± 36 kg BW)	LCOH and LCFA	Indoor (cafeteria)	KSI = 98.90%	[54]
Lolium multiflorum and *Calluna vulgaris*	Cattle (499 ± 36 kg BW)	LCOH, ALK and LCFA	Indoor (cafeteria)	KSI = 92.00%	[54]
Medicago sativa and *Lolium rigidum*	Sheep (64.6 ± 2.48)	NIRS	Indoor (mixed diet)	R^2cv = 0.98	[62]
Poaceae, *Trifolium* and *Cynodon dactylon*	Sheep (52.5 ± 7.5)	NIRS	Indoor (mixed diet)	R^2 = 0.83	[63]
Lolium perenne and *Ulex gallii*	Goats				

[1] MADF, modified acid detergent fibre; ADL, acid detergent lignin; AIA, acid insoluble ash; KSI, Kulczynski similarity index (%); BWT, average body weight; LCOH, long-chain alcohols; ALK, alkane; LCFA, long-chain fatty acids; NIRS, Near-infrared reflectance spectroscopy; R^2cv, coefficient of determination in cross-validation; R^2, coefficient of determination.

Pepeta et al. [5] evaluated the use of combined use of ADL, AIA, and MADF in predicting the botanical composition of diets selected by sheep fed in a cafeteria feeding system at different stocking rates and reported a KSI = 90.90% when predicted values were compared to the observed intake values. The combined use of various classes of biomarkers (MADF, ADL, AIA, LCFAs, and LCOHs) has great potential to increase the accuracy of the marker procedure when applying the least square optimisation approach. Furthermore, the procedures required in analysing MADF, ADL, INDF, IADF, and ADIN are relatively cheaper and easier compared to the analysis of plant cuticular wax compounds (*n-alkanes*, alkenes, LCOHs, sterols, aldehydes, LCFAs, and flavonoids).

When the botanical composition of the diet consumed by grazing ruminants is estimated using biomarkers, the profiles of these markers in faeces arising from the se-

lected/consumed diet are compared with the profiles of dietary components on offer. Moreover, theoretically, only when the number of inert markers present in the diet is greater or equal to dietary components, then diet composition can be estimated using simultaneous equations [64]. When animals are grazing natural pastures characterised by complex vegetation, this becomes more difficult because of the complexities associated with solving the equations arising from a large number of diet components resulting in the prediction of "nonsensical" outputs [65,66]. In such situations, the least-square optimisation method becomes more useful than matrix equations.

Several studies carried out using software based on the principles of the least-square procedures yielded results with high accuracy [5,8,47,53,65,66]. One commonly used software is the Excel-based programme Solver. The Solver programme runs iterations to minimise the sum of the squared differences between the concentrations of markers in the diet, and the concentration of the markers in faeces (corrected for incomplete faecal recovery) arising from the consumed diet, as follows:

$$\sum_{i=1}^{n}[A-E]=\sum_{i=1}^{n}\left[\frac{Fi}{ft}-\frac{a\times D1i+b\times D2i+c\times D3i\ldots\ldots z\times Dni}{a\times D1t+b\times D2t+c\times D3t\ldots\ldots z\times Dnt}\right]^{2} \quad (3)$$

where a, b, c and z are the proportions (p) of diets D1, D2, D3, and Dn in the feed; Fi, D1i, D2i, D3i, and Dni are the concentration of the marker in faeces and diets; and Ft, D1t, D2t, D3t, and Dnt are the total concentration of markers in faeces and the feed components. A and E are the quantities of markers found in plant species or diet offered to animals as a choice and quantities found in faeces arising from selected and consumed plant species, respectively.

The constraints and assumptions used in the Excel Solver routine program include the following: all diets are assumed to have equal selectivity, and their proportions (p) should be constrained to be $0 < p \leq 1$, and their sum adds up to 1; the predicted marker concentrations in the selected diet should be calculated as the sum of the product of the proportions of dietary components and corresponding marker concentrations in the diet components ($a \times D1i + b \times D2i + c \times D3i + \ldots ..z \times Dni$). The optimiser runs iterations and stops when the objective cell (sum of the squared differences) has a minimum value at the point at which the concentrations of markers in the diets are the closest to the concentrations of markers in faeces.

3.3. Near-Infrared Reflectance Spectroscopy (NIRS)

Near-infrared reflectance spectroscopy is a tool that relies on light distribution and absorbance on different wavelengths. Faecal samples are scanned, and the NIR spectra of the faecal samples are used to determine the dry matter intake and the botanical composition, as well as the digestibility of the diet consumed by ruminants [67–69]. Plant samples harvested from the grazing area are collected and scanned to create the library, which is then subsequently used by the machine to compare the hydroxyl (OH), amine (NH), and methylene (CH) groups found in faeces arising from the consumed diet. The drawback associated with this procedure is the requirement of large data sets to establish calibration values based on plant species, growth stages, and climatic regions [67]. Nevertheless, once calibrations exist, the NIRS technique is easy to use to determine the proximate composition of the diet selected by animals in a specifically defined area [70]. Furthermore, NIRS can be used to predict the stem-to-leaf ratio in consumed diets [71], and this is important in determining nutrients derived from the selected plant parts. The robustness of the calibrations is directly related to their accuracy [72], and achieving this is the major limitation of the use of NIRS, especially in many countries. Furthermore, to ensure high predictive accuracy from faecal NIRS equations, it is recommended that the database be situation specific such that most of the factors that can influence spectra diversity are incorporated for each farm/research station, such as plant species in the pasture, geographical location, soil type, management regime, season and year [68]. Nevertheless, many research stations across climatic regions have been calibrated to meet the analysis required for different

feeding systems. Additionally, the use of NIRS can be extended to the estimation of in vivo nutrient digestibility.

3.4. Estimating Feed Intake in Grazing Ruminants Using Diet Digestibility

The relationship between digestibility and subsequent faecal output has been effectively used to estimate intake. This is possible where the diet consumed is homogenous, its digestibility is known, and equally, the faecal output can be quantitatively determined. The most commonly used equation in the estimation of intake comprises:

$$I = F/(1 - D) \quad (4)$$

where (I) intake, (D) diet digestibility and (F) faecal output.

However, digestibility and faecal output are variables that are difficult to accurately determine in grazing animals [50]. Due to difficulties associated with measuring total faecal output and digestibility in grazing animals, digestibility is usually measured in animals under confinement, where total faecal collection and feed intake can be quantified for subsequent estimation of digestibility. This digestibility value is then used in predicting the intake of a larger group of such animals when grazing. However, representative estimates of digestibility can be erroneous, especially when animals in confinement have higher levels of diet intake compared to grazing animals [50]. The predicted intake value of grazing animals will be inflated. Therefore, the level of supplementation might be less than required due to the overestimated value of intake.

3.5. Estimation of Intake in Grazing Animals Using Double n-alkanes

When animals consume feedstuff containing naturally occurring odd-numbered alkane and then simultaneously dosed with synthetic even-numbered alkane of adjacent chain length, the intake of the feedstuff can be estimated [73]. This is because both n-alkane types can be recovered to a similar extent and errors arising from incomplete recoveries cancel out when using Equation (5). Plant *n-alkane* (generally C_{31} and/or C_{33}) measures digestibility as an internal marker (i), while synthetic orally dosed even-numbered (C_{32} or C_{36}) alkane measures faecal output as an external marker (j). Digestibility and faecal output measured by the alkane pair (i and j) are subsequently used to calculate intake, as follows:

$$I = \frac{Dj}{\left(\frac{Fj \times Rj}{Fi \times Ri}\right) \times (Hi - Hj)} \quad (5)$$

where Dj is the maker dose rate of synthetic alkane j; Fi and Fj are the concentration of *n-alkanes* i and j in faeces; Hi and Hj are marker concentrations of *n-alkanes* i and j in herbage; Ri and Rj are faecal recoveries of *n-alkanes* i and j. Absolute concentrations are not important, but the ratio of alkane faecal concentrations is. This equation also accounts for the possibility of readily having orally dosed synthetic alkane j in herbage consumed by the animal (Hj).

Dosing with short-chain saturated hydrocarbons results in the excretion curves of *n-alkanes* with low amplitude, consequently reducing the precision and accuracy of developing meaningful faecal n-alkane parameters to determine faecal output and digestibility of diet consumed [74]. These errors could be carried over to the estimation of intake [75]. *N-alkanes* with long C-chain lengths result in more accurate estimations approaching the true value of diet consumed than *n-alkanes* with short C-chain lengths [74]. When these hydrocarbons are used as markers to estimate intake, GC-MS allows both plant and dosed markers to be analysed simultaneously, which circumvents bias and other analytical errors [76].

Conclusively, errors arising from the incorrect estimation of digestibility can reduce the accuracy of intake estimation (Equation (4)), especially when the diet digestibility is overestimated. One of the possibilities arising from the overestimation of forage digestibility is the provision of less than sufficient quantity of supplements as a result of overestimated

intake resulting in reduced animal performance such as lower milk yield, poorer carcass yield, lower conception rate, or lower weight gain [76].

3.6. Estimating Diet Intake Using Supplement Proportion

The cheapest source of nutrients available for herbivores grazing in rangelands is browse and herbage. However, supplements are provided to mitigate nutritional stress and ensure that animals reach the production target. The understanding of the associative nature of supplementing the diet with available forage in the natural grasslands is a key consideration in improving the voluntary dry matter intake of grazing animals. The proportion of supplements in the total diet consumed can be estimated by dosing supplements with plant cuticular wax compounds (synthetic *n-alkanes*) with a distinctive profile compared to grazed dietary components [77]. As such, whole diet intake can be estimated using Equation (5) if animals are dosed with an even-chain hydrocarbon, commonly dotriacontane (C_{32}). Equally, when the supplement diet lacks sufficient n-alkane concentration to be detected as a distinct feed source which could allow for the estimation of proportions of diet components, it can be mixed with beeswax before being fed to animals [50,78]. For example, cereal-based supplements generally contain low levels of cuticular *n-alkanes* [49], whereas roughage-based supplementary feeds often contain distinct and known alkane profiles which easily permit the estimation of diet composition [77]. When diet composition is determined, diet intake can be partitioned into its constituents. However, errors in estimating whole diet intake and supplement intake can arise if diet composition is not accurately estimated.

In the study by Dove and Olivan [78], the supplement is coated with 1.22% of finely grated beeswax previously dissolved in 2.5 L n-heptane with moderate heating to synthesise the n-alkane while Octacosane (C_{28}) is added to the solution to make a final concentration of 250–300 mg/kg of dry matter. A known quantity of the marker-containing supplement is then offered to sheep alongside grazed forage. The proportions of diet components consumed by each sheep are then used to calculate the intake of each diet component in the total consumed diet. The intake for a given dietary component is calculated as:

$$\text{Idc} = \text{Is} \times (\text{Pdc}/\text{Ps}) \quad (6)$$

where Idc is the intake for a given dietary component, Is is the intake of a supplement, Pdc is the proportion of a given dietary component, and Ps is the proportion of supplement with the known amount in the diet. The advantage of this approach is that it does not require the dosing of animals, which disturbs the natural foraging behaviour of animals. The details of this approach have been extensively reported elsewhere [48], and satisfactory results have been observed in several subsequent trials [79].

4. Estimating Nutrient Digestibility in Grazing Animals

In typical on-farm conditions, forage digestibility is one of the most important factors affecting productivity. The "conventional method" of determining the digestibility involves collecting samples (randomly hand-clipped) of the grazed forage and feeding them to another set of confined animals for several days; data are collected on feed consumed and faecal output, which is subsequently used to estimate digestibility [48]. The constraint of estimating intake, the principal variable required to estimate digestibility, remains daunting. Furthermore, the assumption that the randomly hand-clipped plant species are the true reflection of what grazing animals select in their respective situation-specific conditions exacerbates the predictions of in vitro or in vivo digestibility as it is based on the assumption that all plant species are available ad libitum as opposed to varying availability between the most preferred and less preferred plant species in the grazed area. To circumvent this, the use of OF animals was employed, which allows periodic sampling of consumed forage. Results obtained with OF animals yielded better estimates than the predictions from hand-clipped samples of diets selected, but the grazing pattern of fistulated animals was noted to be different from intact animals [48]. Samples from oesophageal fistulated

(OF) animals are collected within a short period (minutes) of grazing, whereas the total grazing time of rangeland animals may take from a few days up to weeks before sample collection [8,48]. Rumen fistulated animals have equally been very valuable with the nylon bag in situ technique which involves incubating forage samples collected by hand-clipping inside the rumen followed by 48 h acid–pepsin treatment [80].

One other approach to estimating digestibility is the faecal nitrogen index approach which can be used as a proxy to estimate diet quality and digestibility [81]. This is premised on the relationship between the quality of faeces and the quality and quantity of diet consumed and its digestibility [82]. Several studies showed that total faecal nitrogen concentration is associated with various measures of nutritional status (diet quantity and quality, diet digestibility and weight changes) in a wide variety of herbivore species [81]. The ease of collection of faecal material also makes this approach attractive.

One major limitation to the use of total faecal nitrogen concentration as a proxy to estimate diet digestibility is that many range forage species, particularly forbs and shrubs, contain high levels of soluble phenolic compounds with protein complexing capabilities that elevate faecal nitrogen concentrations relative to those in the diet [83]. Equally, nitrogen digestibility would expectedly vary significantly from species to species.

The difficulty associated with in vivo digestibility estimation, as well as the need to minimise the use of live animals, necessitated the development of in vitro digestibility procedures. Digestibility of the selected (hand-clipped) plant species can be analysed using any of the several in vitro procedures, such as the Tilley and Terry method [84], or more recent modifications, such as the DAISY incubator technique [85]. The digestibility value is then used to estimate the intake of animals grazing in pastures. In vitro digestibility does not account for animal–animal variation, while the results from in vitro procedures can be greatly influenced by sample size, sample processing, and incubation conditions [86].

4.1. Estimating Digestibility Using Biomarkers

Internal markers have been reported to have low accuracy in digestibility estimation due to low recoveries in faeces [79]. However, subsequent reports have shown that AIA [5,51], *n-alkanes* [47], and INDF [87] have been used to estimate nutrient digestibility with high accuracy ($R^2 > 0.75$). Diet digestibility is calculated from the concentration of an internal marker in the diet and faeces, as follows:

$$D = 1 - Cd/Cf \quad (7)$$

where D is diet digestibility, Cd is the concentration of marker in the diet, and Cf is the concentration of the marker in faeces.

The technique of analysing *n-alkanes* in faeces and forage has been used in sheep [52] and cattle [46,47,88] to determine nutrient digestibility and dry matter intake. Andriarimalala et al. [47] reported average faecal recoveries of 0.83 (C_{31}) and 0.89 (C33) in cattle, while in sheep, average recoveries were 0.91 (C_{31}) and 0.95 (C_{33}). Conventional internal markers in sheep are reported by Pepeta et al. [5] to have average faecal recoveries of 0.88 (AIA), 0.50 (ADL), and 0.34 (MADF). Therefore, AIA can be used to accurately estimate the digestibility of a diet selected by grazing ruminants. Results obtained from AIA, as well as those obtained from *n-alkanes*, can be regressed against observed (directly measured) data sets in obtaining standard ranges of the degree of error. This will facilitate the applicability of AIA markers in the study of free-ranging animals with minimal errors. Nevertheless, the analysis of AIA concentration is relatively cheap and easy to analyse compared to *n–alkanes*.

When the least square optimisation procedure, such as the Excel-based Solver programme, is utilised to estimate dietary contributions in a selected diet, the whole diet digestibility can then be estimated. The dietary proportions can be denoted as; a, b, c . . . to z,

which, summed together, would produce a total of one kilogram of faeces. This information can also be used to determine whole diet digestibility [89], as follows:

$$\text{Diet digestibility} = ((a + b + c + \ldots\ z)\text{-}1)/(a + b + c + \ldots\ z) \quad (8)$$

The absolute concentrations of biomarkers are important in the quantitative estimation of the contribution of each forage component in the animal's diet that is reflected in the faecal dry matter and consequently in estimating diet dry matter digestibility using Equation (8). Where only proportional concentrations of biomarkers relative to total biomarker concentration are used, the proportion of each diet component is estimated, not allowing the estimation of diet digestibility.

4.2. Using Faecal Output to Estimate Digestibility in Grazing Animals

Faecal collection from grazing herbivores is difficult to undertake because animals can be disturbed during sample collection. Harness faecal bags are mostly limited to males because samples from females are often contaminated with urine leading to increased irritation to animals due to increased faecal weight. As such, in most cases, only data from males is used for both sexes, and that could introduce bias due to differences in physiological and reproductive status. A metabolic harness specifically for separate collection of faecal and urinary excretions in female sheep was developed in the late 1970s but was found to be prone to blockage by faeces, necessitating design modifications. Therefore, external markers have an advantage because there is no need for total faecal collection. These markers are consistently administered for 12–14 days twice a day, and faecal collection is conducted on the last 4–6 days when the biomarker should have reached a steady concentration in faeces [75]. Van Wyngaard et al. [87] found 6 days post marker (titanium dioxide) administration sufficient to give accurate predictions of total faecal output in grazing dairy cows. On the other hand, Ferreira et al. [90] discovered that 3 to 5 days are enough for administered commercial alkane (C_{32}) to become uniformly distributed inside the faeces. Therefore, faecal sampling either by rectal grab or by picking it up from the ground is efficient after 3 to 6 days have elapsed since the commencement of dosing to predict diet intake, digestibility, and composition using the *n-alkanes* technique. The identity of the animals defecating needs to be known for the latter method to be applied and needs to be taken into consideration as a limiting factor. The rate of passage of a diet consumed dictates the time for markers stability in faeces. Furthermore, sampling should preferably be done in the morning post-introduction of animals to the diet when digesta from all feeds consumed previously should have reached the end of the GIT.

Another important factor is the dosing method, such as the use of frequency (once or more), with or without the 'wall materials' such as paper pellets, gelatine capsules, and controlled release devices, which could influence marker distribution in the gut and subsequent recovery in faecal samples [91–93]. Alkane dosing frequency and faecal sampling frequency both affect the validity of rectal grab samples as representative of total faeces voided [94]. To overcome these, three samples for three days for each animal provide enough information to determine across- and within-day variations [95]. The study by Dove et al. [96] shows that sampling or dosing frequency (twice a day) did not have any effect on the diurnal variation in faecal alkane ratios, as shown in Table 3.

Table 3. Regression relationship for concentrations of various components dosed twice a day in rectal grab samples of faeces (y) from grazing sheep in relation to the concentrations in total faeces (x).

Component	Regression Slope [1]	Different from y = x?
Nitrogen	0.997 ± 0.011	NS
Ash	0.985 ± 0.074	NS

Table 3. *Cont.*

Component	Regression Slope [1]	Different from y = x?
Herbage *n-alkanes*		
C_{27}	0.990 ± 0.012	NS
C_{29}	1.004 ± 0.010	NS
C_{31}	1.003 ± 0.005	NS
C_{33}	0.939 ± 0.041	NS
Dosed *n-alkanes*		
C_{28}	0.968 ± 0.024	NS
C_{32}	0.975 ± 0.022	NS
Chromium	0.904 ± 0.023	$p < 0.01$
Chromium [2]	0.879 ± 0.019	$p < 0.001$

[1] All regressions were constrained through zero since the y-intercept of unconstrained regression did not differ significantly from zero. [2] Chromium concentration in the rectal grab samples against expected chromium concentration in total faeces based on chromium dose and known faecal output (the relationship between actual and expected chromium concentration in total faeces did not differ (y = x). (NS) Not significant (Adopted from Dove et al. [96]).

Further studies involving the use of external dosed n-alkane markers to estimate faecal output in ruminants are warranted. However, faecal concentrations of chromium from grab samples were lower than in total faeces, although the dosing frequency for chromium was the same as for *n-alkanes* [97]. Using this method, the faecal output is determined using the following equation:

$$\mathrm{FO}\left(\mathrm{kg\ DM\ day^{-1}}\right) = \frac{\mathrm{mr}\left(\mathrm{mg\ day^{-1}}\right)}{\mathrm{fc}\left(\mathrm{mg\ kg^{-1}\ DM}\right)} \quad (9)$$

where FO is the faecal output, mr is the marker dose rate, and fc is the faecal marker concentration.

5. Application of Dietary Component Predictions

Wild ruminants in tropical and subtropical grasslands occupy a significant ecological niche. However, the scanty literature about the global greenhouse gaseous emission potential of wild ruminants due to insufficient information about the botanical composition and nutritional aspects of diets selected justifies further research. The faecal analysis technique can be used to obtain insight into the botanical proportions of the diet consumed by wild ruminants [37,79]. Furthermore, in vitro gas production analysis of simulated selected diets determined through faecal analysis can be used to predict greenhouse gas emissions (i.e., methane) by wild ruminants. In agreement with the proposed idea, Bois et al. [98] successfully used the in vitro methane production values multiplied by the corresponding dry matter intake values of selected diet estimates obtained from the NIRS to quantify the daily methanogenic potential of grazing Sahelian zebu cattle. Therefore, the contribution of domesticated and wild ruminants can be incorporated into mechanistic models used to estimate greenhouse gas emissions from ruminants grazing rangelands. Hence, it can be speculated that national inventories and emission factors of greenhouse gas emissions from ruminants can be improved by adopting the application of these approaches. Also, national greenhouse gas emission mitigating strategies and grazing calendars can be prudently formulated from such knowledge.

Equally, in vitro studies could be used prudently as the first step toward gaining an insight into the digestibility of a wide array of forage species, a key variable in predicting the intake of selected diets. Thereafter, the use of the combination of approaches, such as the use of internal markers and supplement intake to estimate faecal output and forage intake, respectively, could be used to estimate, validate, and calibrate in vitro digestibility estimates to improve the accuracy of predictions values of digestibility.

The development of mathematical models that rely on easy-to-collect data and can accurately predict intake and diet composition remains a challenge. A model that can iterate animal characteristics, grazing and rumination behaviour, as well as pasture/range characteristics based on large data sets, may offer such commercial applications [13]. Such models could potentially be utilised in estimating residual feed intake of grazing animals, genetic selection, and estimation of feed efficiency in grazing systems.

6. Conclusions

The use of internal markers with subsequent results analysed using the Excel Solver software is one effective tool to get a better insight into diet botanical composition, digestibility, dry matter and nutrient intake in grazing animals. The complexity of typical rangelands means that there are limited distinctive *n-alkanes* with numbers greater or equal to the number of plant species to be used in the estimation of the botanical composition of diets selected by grazing ruminants. Conventional markers such as AIA, ADL, INDF, IADF, and MADF can be combined with plant cuticular compounds (*n-alkanes*, LCOHs, and LCFAs) in estimating diet composition, intake, and digestibility in grazing ruminants. For more accurate results, it is advisable to calibrate and validate results obtained using the internal marker method with the data obtained from other methods, such as faecal NIRS and plant cuticular compounds analysis.

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