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*Research Article*

# Evaluation of N-alkanes as Faecal Markers to Estimate Diet Composition, Feed Intake, and Digestibility in European Bison (*Bison bonasus*)

**Luis Miguel Mendes Ferreira**,[1] **Daniela Pereira**,[2] **Ana Lourenço**,[2] **and Miguel Rodrigues**[1]

[1]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro (UTAD-CITAB), Inov4Agro (Institute for Innovation, Capacity Building and Sustainability of Agri-Food Production), Vila Real, Portugal*
[2]*Animal and Veterinary Research Center, University of Trás-os-Montes and Alto Douro (UTAD-CECAV), Vila Real, Portugal*

Correspondence should be addressed to Luis Miguel Mendes Ferreira; lmf@utad.pt

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There is a lack of knowledge on the European bison (*Bison bonasus L.* 1758) foraging behavior that is essential to develop an appropriate feeding strategy for each specific situation (captive or wild). Utilization of faecal markers may allow us to fill this gap, accommodating animal welfare and intensive labour issues that are major limitations of traditional techniques. This study aimed to evaluate the potential of *n*-alkane markers ($C_{25}$ to $C_{33}$) to estimate diet composition, feed intake its digestibility on three captive male*Bison bonasus* fed on known amounts of straw, and a beeswax labelled concentrate feed. Feeds and faecal samples were taken daily during 10 days. Prior to calculations, faecal concentrations were corrected for incomplete faecal recovery (FR). Results indicated that 4–5 days were sufficient for these markers to reach a steady concentration in bison faeces. Accurate estimates of diet composition and feed intake were obtained not differing from known values. Results suggest that faecal recovery of *n*-alkanes in bison are incomplete and tend to increase with carbon-chain length. Apparent dry-matter digestibility (DMDap) estimates were affected by the n-alkane ($C_{27}$, $C_{29}$,$C_{31}$, and $C_{33}$) used in the calculations. Estimates of DMDap obtained with application of FR corrections were 6.3% higher than those without correction. Results indicate that feeding a known amount of beeswax labelled supplement can be successfully used to estimate composition, feed intake, and its digestibility, requiring the application of n-alkane FR data.

## 1. Introduction

The European bison (*Bison bonasus L.* 1758) is considered to be the largest terrestrial mammal of Europe. Given as extinct in the wild during the mid-20$^{th}$ century, several groups of animals were maintained in captivity in zoos and breeding centers (Linnell et al., 2020). After its reintroduction in Europe, the European bison population has been consistently growing and its impact on vegetation communities has been shaping several ecosystems as its foraging behaviour can regulate the composition of Europe's temperate forests [1], modulating the structure and functioning of the ecosystems [2].

The knowledge about herbivores feeding habits, diet selection, and feed intake is essential to develop an appropriate feeding strategy, i.e. covering their nutritional needs for each specific situation (captive or wild) and providing efficient and sustainable management of the existing vegetation [3–5]. This information is required to match areas of foraging with different types of herbivores and, as stated by Holechek et al. [3], to select species compatible with the forage resource, to choose species to reseed, to predict the effect of overgrazing by different animals, to identify new species on which to base management, as well as helping to preserve species balance and achieving better animal performance [6].

The use of markers allows the estimation of diet selection, feed intake, and its digestibility, when logistical constraints preclude one or more direct determinations on either captive or wild animals [7]. Indigestible markers remain with feed residues throughout the digestive tract and emerge in the faeces, representing the fraction of the diet that is not absorbed by the animal. This indigestible fraction is therefore most representative of feeds with a large proportion of refractory material such as plant fibre consumed by herbivores [8]. Nowadays, the n-alkanes are the wax components most widely studied and used in herbivore nutrition studies as faecal indigestible markers [9] (Dove and Mayes, 2005). The use of these markers allows to overcome accuracy and animal welfare issues and intensive labour that are major limitations identified in the traditional techniques, and present more extended results [6].

However, for wild ruminants, information on this technique is still scarce and, as a consequence, its potential is still far from being fulfilled. Furthermore, its application needs validation from trials conducted with captive animals and, in the case of animals maintained in zoos, measurements of digestibility and feed intake may be difficult to accomplish, as these animals are difficult to handle and controlled feeding and sampling are rather complicated. The utilization of markers can overcome these difficulties, enabling data collection for future studies.

The objective of this study was to evaluate the potential of the n-alkane markers to estimate diet composition, voluntary feed intake, and apparent dry-matter digestibility (DMDap) in three captive European bison (*Bison bonasus*) fed on straw and a known amount of a beeswax labelled supplement.

## 2. Materials and Methods

This study was performed at Parque Biológico de Gaia, Portugal, between the end of July and the beginning of August 2021. It was only possible due to the availability of Parque Biológico de Gaia and Câmara Municipal of Vila Nova de Gaia that granted access to their animals, park, and facilities and to the adaptation of their daily routine to accommodate this study. These entities also provided their bison biometric data.

*2.1. Animals and Diet.* Three male European bison (*Bison bonasus*) housed at Parque Biológico de Gaia with estimated weights ranging between 650 and 900 kg were used in the present study. Animals age range between 11 and 16 years old. Animals received once daily a diet composed of straw supplied *ad libitum* and a known amount (5.8 kg ± 0.20 per day) of a concentrate feed that was labelled with beeswax. Animals were housed in an area of approximately 3500 $m^2$ that was divided into three separated enclosures; enclosure A was approximately 557 $m^2$, the maternity (C) was 181 $m^2$ and enclosure B was 2790 $m^2$, all enclosures were used during the experiment. The marked concentrate feed was given individually to the animals, and the straw and water were provided at all times in all enclosures. After the concentrate feed intake, animals stayed in one of the smaller enclosures (enclosure A or C) to enable the collection of faecal samples. The faecal samples were collected from the ground, avoiding any type of contamination with soil particles, and were immediately frozen for subsequent n-alkane analysis. After the consumption of the concentrate feed, animals re-entered the bigger enclosure (enclosure B).

*2.2. Experimental Design.* The experimental study (10 days) consisted of a preliminary period of 5 days for adaptation of the animals to the diet, experimental conditions, and their new routine, followed by a 5-day period for collection of representative samples of the straw, labelled concentrate feed, and faeces. Feed intake of both straw and concentrate feed were calculated daily by measuring the offered and refused feed. It should be noted that all concentrate feed offered was consumed by the animals. Representative samples of the provided and refused straw, concentrate feed, and faeces were daily sampled and then frozen at −20°C for subsequent chemical analysis. Prior to the analysis samples were dried using a forced-air oven at 50°C and milled through a 1 mm screen.

*2.3. Preparation of Labelled Supplement.* Beeswax was added to the concentrate feed as described by Charmley and Dove [10]. The addition of beeswax to the concentrate feed was made to ensure an n-alkane profile that was markedly different from that of the straw. Briefly, 45 kg of supplement was labelled with 0.550 kg of beeswax dissolved in 2 L of n-heptane, with gentle heating. The mixture was then poured into a container with the concentrate feed and well mixed with the help of a drill with a paint mixer, to produce the marked concentrate feed containing 12.2 g of beeswax per kg concentrate feed. This procedure was repeated throughout the experiment every two days (5 times during the study), with 25 or 50 kg of supplement each time. Each batch of labelled concentrate feed produced was not used until at least the next morning, to allow the n-heptane to evaporate from the concentrate feed at ambient temperature.

*2.4. n-Alkane Analysis.* The *n*-alkane concentrations of individual samples of diet components (straw and concentrate feed) and faeces were analysed in duplicate using direct saponification procedure and quantified by gas chromatography according to the methods of Dove and Mayes [7], using a Perkin Helmer Clarus 580, equipped with flame ionization detector (FID) and an autosampler. First, saponification of 0.2 g of faeces and 0.5 g of feed samples was made for 16 h using an ethanolical solution of 1M KOH at 90°C in a dry-block heater. Subsequently, it was performed a hot extraction with n-heptane at 65°C. After the extraction, samples were filtered through a silica-gel column with a bed volume of 5 ml, to isolate the n-alkanes from pigments, sterols, and long-chain alcohols. The resulting elutes were evaporated and then redissolved in 200 or 300 $\mu$l (depending on the sample) of n-heptane for chromatographic analysis. The *n*-alkane extracts were then injected by on-column

injection on a 30-m column BP1 of 0.530 mm internal diameter and 0.5 $\mu$m film thickness. The injector was maintained at 280°C. The oven was maintained at 170°C for 4 min after injection, ramped at 30°C/min up to 215°C, 1 min hold, and then ramped at 6°C/min up to 300°C, where it was held for 8 min. The FID detector was maintained at 310°C. Helium was the carrier gas used at a constant flow of 20 ml $min^{-1}$. Gas chromatographic method was calibrated with a standard solution containing a mixture of synthetic n-alkanes ($C_{22}$ to $C_{36}$). Peak areas and known concentrations were used to calculate the response factors for individual n-alkanes. The n-alkane concentrations were quantified relative to known amounts of the internal standards $C_{22}$ (n-docosane) and $C_{34}$ (n-tetratriacontane), added at the beginning of the extraction process. The use of two internal standards enabled the evaluation of the effectiveness of the extraction process and the correction of the peak areas for any discrimination detected during the solvent extraction step.

*2.5. Calculations.* Proportions of straw and concentrate feed in the diet were estimated by comparing their n-alkane ($C_{25}$ to $C_{33}$) profiles with those found in the faeces of the animals. The *n*-alkanes concentrations in faeces were corrected for their incomplete faecal recovery using data obtained from cattle (*Bos taurus* Linnaeus, 1758) in metabolic cages by [11]. Calculations were carried out using the computer package Eat What [12] that uses a non-negative least-squares method to estimate the combination of n-alkane patterns of diet components that best matches the pattern of recovery-corrected n-alkane concentrations observed in faeces. Diet composition estimates given by Eat What were then compared with known consumed proportions of each feed.

Individual total feed intake was also estimated, based on the estimated proportions of concentrate feed and straw in the animals' diet, and on previous knowledge on the amount of concentrate feed supplied to the animals, according to the equation:

$$\text{Feed Intake}\,(kgDM) = Is \times (Pf/Ps), \quad (1)$$

where Is being the intake of the labelled concentrate feed, Ps is its proportion in the diet and Pf is the proportion of the straw in the diet [10, 13].

Additionally, apparent dry matter digestibility (DMDap) was estimated using the *n*-alkanes $C_{27}$, $C_{29}$, $C_{31}$, and $C_{33}$ as internal markers using the equation:

$$\text{DM Dap}\,(g/kgDM) = (1 - Ci/Cf) \times 1000, \quad (2)$$

where Ci and Cf are the concentrations of the *n*-alkanes $C_{27}$, $C_{29}$, $C_{31}$, and $C_{33}$ in the diet and faeces, respectively. For the calculation of the n-alkane concentrations in the diet, the proportions of straw and concentrate feed in the animals' diet used in the calculations were the ones previously estimated by the n-alkane markers. For this calculation, concentrations of the *n*-alkanes $C_{27}$, $C_{29}$, $C_{31}$, and $C_{33}$ were corrected or not with faecal recovery correction (FRC) using data obtained by Ferreira et al. [11] in cattle.

*2.6. Chemical Analyses.* Samples of the diet components were analysed for the ash (642.05) and nitrogen (N; 654.01) following the procedures of the Association of Official Analytical Chemists [14]. Crude protein (CP) was calculated as $N \times 6.25$. Neutral detergent fibre (NDF) was determined according to the procedures proposed by Mertens [15]. Acid detergent fibre (ADF) was determined following the procedures of AOAC [14] (973.18), and expressed inclusive of residual ash. Lignin (sa) was analysed by solubilizing cellulose with sulphuric acid [16]. All contents are given as concentrations on dry matter (DM) basis. The difference between NDF and ADF was used to calculate hemicellulose concentration and the difference between ADF and ADL to calculate the cellulose concentration.

*2.7. Statistics Analysis.* The statistical analysis was carried out with JMP PRO software version 16 [17]. To determine when the excretion of n-alkanes reached a steady state their concentration in each day was compared with the mean for subsequent days [18] using analysis of variance. The effect of calculation method for diet composition estimates and feed intake (i.e. measured vs. estimated) was explored by analysis of variance. The effect of using different *n*-alkanes ($C_{27}$, $C_{29}$, $C_{31}$, and $C_{33}$), FRC (with or without FRC) and their interaction on the estimates of the apparent dry matter digestibility (DMDap) were analysed by two-way analysis of variance (ANOVA). Tukey's test was used for multiple comparisons among means, and differences among the means with a $P$-value $< 0.05$ were considered significant.

## 3. Results and Discussion

The chemical composition of the feeds used in the experiment is presented in Table 1. As expected, cell wall components (NDF) made up the highest fraction in the straw. The NDF content was lower for both the labelled and nonlabelled concentrate feed (<210 gNDF/kg) compared to the straw (>800 gNDF/kg). It should be noted that all chemical fractions analysed in the straw had a high variation between days, possibly due to variations in its botanical composition. The nutritive value of straw can be considered low with high levels of ADF (550 g/kg DM) and low CP content (36 g/kg DM). Still, the low nutritive value of the straw was counterbalanced with the high nutritional value of the concentrated feed with high CP and starch content (>125 and > 445 g/kg DM) and low ADF content (85 g/kg DM). The chemical composition of the labelled and nonlabelled concentrate feed was very similar in all chemical parameters analysed.

The n-alkane content of the straw and labelled and nonlabelled concentrate feed is presented in Table 2. The n-alkanes $C_{25}$ to $C_{33}$ were chosen due to their association with plant epicuticular waxes and usually they are present in high concentrations [9]. The supplement was labelled with beeswax to provide a distinct n-alkane profile due to the original low levels of n-alkanes in the concentrate feed, being insufficient to allow the estimation of diet composition [9, 10, 19, 20]. Labelling of the supplement with beeswax

Table 1: Chemical composition of the feeds used in this experiment.

| Parameters (g/kg DM) | OM | CP | CF | Starch | NDF | ADF | ADL | Cellulose | Hemicellulose |
|---|---|---|---|---|---|---|---|---|---|
| Straw | 966 ± 2.2 | 35 ± 3.5 | — | — | 821 ± 6.2 | 550 ± 6.0 | 66 ± 1.2 | 484 ± 4.9 | 762 ± 5.0 |
| CF | 943 ± 0.1 | 129 ± 0.4 | 34 ± 1.1 | 454 ± 1.5 | 189 ± 0.3 | 85 ± 0.2 | 11 ± 0.2 | 73 ± 0.1 | 181 ± 0.4 |
| BCF | 937 ± 0.4 | 126 ± 0.7 | 45 ± 2.0 | 449 ± 15.1 | 201 ± 1.0 | 95 ± 0.3 | 15 ± 0.2 | 80 ± 0.3 | 187 ± 0.8 |

CF, concentrate feed. BCF, beeswax labelled concentrate feed. OM, organic matter. CP, crude protein. CF, crude fat. NDF, neutral detergent fibre ash free. ADF, acid detergent fibre. ADL, lignin detergent fibre.

resulted in high (>135 mg/kg DM) concentrations of *n*-alkanes ranging from $C_{25}$ to $C_{33}$ ($C_{27}$ with >350 mg/kg DM). Straw n-alkane concentrations for several *n*-alkanes ($C_{26}$, $C_{28}$, $C_{30}$ and $C_{32}$) were very low (<10 mg/kg DM), which is consistent with the results obtained in previous studies [10, 21]. These authors observed the highest concentrations on *n*-alkanes $C_{29}$ and $C_{31}$ (<80 mg/kg DM) while the remainder *n*-alkanes were present below 25 mg/kg DM.

As expected, the nonlabelled concentrate feed (NCF) showed a low concentration on all *n*-alkanes analysed, with the highest concentration being the $C_{31}$ alkane (25 mg/kg DM). The beeswax labelled concentrate feed (BCF) was characterized by high concentrations of $C_{27}$ and $C_{25}$n-alkanes, with other *n*-alkanes being present at below 300 mg/kg DM, with the lowest concentration being of 137 mg/kg DM for *n*-alkane $C_{33}$. In the BCF, no single n-alkane comprised more than 20% of the total n-alkane content. The odd-chain *n*-alkanes represented the highest fraction in all feeds, varying between 60.5 (BCF) and 93.1% (straw) of the total n-alkane content. The n-alkane profile of the BCF in the present study was similar to that observed by Elwert and Dove [22] in solvent-extracted cottonseed meal, also labelled with beeswax.

The total mean n-alkane concentrations (sum of concentration of *n*-alkanes from $C_{25}$ to $C_{33}$) varied between a minimum of 73.7 (NCF) to a maximum of 2205.3 mg/kg DM (BCF). By analysing the n-alkane profile of the BCF and comparing it with the NCF, it can be concluded that the concentrate feed was marked properly with a low coefficient of variation (below 10%) between samples ($n = 30$) for all n-alkane concentrations. Also, its n-alkane profile was markedly different from the NCF and straw.

The evolution of *n*-alkanes mean concentrations ($C_{25}$ to $C_{33}$) in animal faeces along the study is presented in Figure 1, and was studied to detect the minimum time required to reach a steady excretion pattern of the markers. Comparing the faecal excretion of markers for each day with the mean for subsequent days [18], we were able to determinate when the excretion of *n*-alkanes reached a steady state. The n-alkanes $C_{28}$ to $C_{33}$ reached steady excretion on day 4 as faecal n-alkane concentrations on day 4 were not different ($P > 0.05$) from those of day 5 to 10. By contrast, 5 days were necessary for n-alkanes $C_{25}$ to $C_{27}$ to reach a steady concentration in faeces. These results are consistent with those previously obtained results by Oliván et al. [23] and Molina et al. [18] who found that in cattle, 4 to 5 days were necessary for n-alkanes to reach a "plateau." It should be noted that these authors used a different marker administration method (paper pellet matrix, controlled release device, or gelatine capsules) than the one used in the present study. A shorter period (3 days) was observed by Ferreira et al. [11] to n-alkanes to reach a steady concentration in faeces of cattle dosed with a paper pellet matrix. Although the animal species used in those studies were different (*Bos taurus*), they belong to the same family and subfamily as the species used in our experiment (*Bison bonasus*). The equilibrium concentration of the markers in faeces coincided with the adaptation period of this experiment, allowing to conclude that, in the conditions of the present experiment, a 5-day period of adaptation was enough for the bison. Although the "plateau" of all *n*-alkanes was reached on day 5, animals continued to receive BCF at a constant dosing for an equal period of 5 days of experimental period, to allow the study of feed intake, digestibility, and diet composition [9, 10, 13].

The comparison of measured proportions of dietary components and those estimated using the *n*-alkanes is presented in Table 3. Many authors that studied the faecal recovery of n-alkanes in cattle [11], goats [24], sheep [10, 25–28], and other wild ruminant species as the moose [29] and fallow deer [30] suggested that *n*-alkanes are incompletely recovered in their faeces. For that reason, it is recommended that the application of correction factors is to adjust for incomplete faecal recovery prior to diet composition calculations [9, 26, 29, 31]. Hence, a correction of faecal n-alkane concentrations for their incomplete recovery was applied to avoid potential bias when estimating diet composition. The faecal correction data used was obtained from a study performed on metabolic cages with cattle [11] as they belong to the same family (Bovidae) and subfamily (Bovinae) of the European bison (*Bison bonasus*). This data was also applied due to the absence of faecal recovery data in bison. In an ideal situation, faecal recovery data based on bison fed with a similar diet should have been applied. Dove and Mayes [7] recommend the use of precise n-alkane faecal-recovery corrections to obtain accurate estimates of diet composition. Nevertheless, the use of accurate recovery corrections is less relevant when the diet components have similar proportions of long and short carbon-chain lengths (chain-length bias) and have distinct n-alkane profiles [9, 24]. When comparing the accuracy of diet composition estimates using recovery rate from individual animals, mean or grand mean recoveries for a certain dietary treatment on *n*-alkanes faecal concentrations, the most accurate diet composition estimation was obtained when the faecal corrections from individual animals were applied [10].

Estimates of diet composition (proportions of straw and BCF) were similar to the measured ones (Table 3). The estimated proportions of the straw differed from the known ones only by 2% (i.e. straw intake was underestimated and BCF intake was overestimated), being these differences not

Table 2: Mean *n-alkane* concentrations (mg/kg DM) of the straw, non-labelled and beeswax labelled concentrate feed used in this experiment.

| n-alkanes | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | Total | Even-chain | Odd-chain |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Straw | 11 ± 5.1 | 2 ± 1.1 | 21 ± 7.4 | 5 ± 1.9 | 74 ± 24.6 | 5 ± 1.6 | 79 ± 26.1 | 3 ± 1.3 | 13 ± 7.0 | 214 ± 58.6 | 15 ± 5.1 | 199 ± 55.3 |
| CF | 2 ± 0.6 | 2 ± 0.3 | 5 ± 0.8 | 9 ± 3.0 | 18 ± 0.9 | 2 ± 0.1 | 25 ± 1.0 | 3 ± 1.3 | 6 ± 0.1 | 74 ± 1.8 | 17 ± 2.7 | 57 ± 3.4 |
| BCF | 313 ± 30.1 | 277 ± 26.2 | 364 ± 32.9 | 252 ± 22.0 | 294 ± 22.7 | 190 ± 15.0 | 225 ± 13.2 | 153 ± 10.5 | 137 ± 8.6 | 2205 ± 173.3 | 872 ± 71.3 | 1333 ± 102.2 |

CF, concentrate feed. BCF, beeswax labelled concentrate feed.

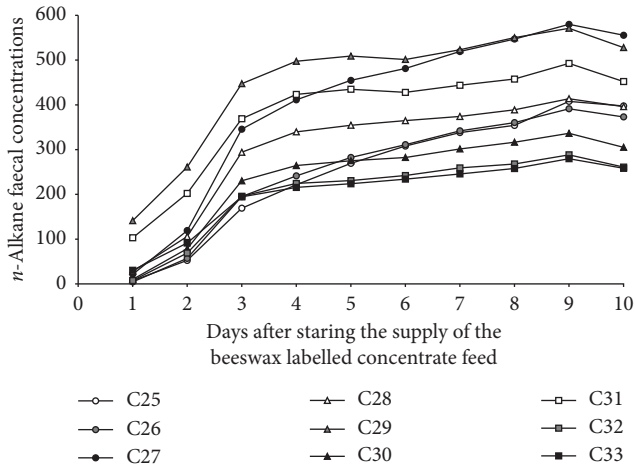


Figure 1: Evolution of the *n-alkane* faecal concentrations (mg/kg DM) of the bison along the experimental period.

Table 3: Comparison of known and estimated measured proportions of dietary components and dry matter feed intake of bison fed on straw and beeswax labelled concentrate feed.

| Diet component | Known (K) | Estimated (E) | E–K | SEM | Effect |
|---|---|---|---|---|---|
| Straw | 0.453 ± 0.0088 | 0.473 ± 0.0105 | +0.020 | 0.0056 | 0.0654 |
| BCF | 0.547 ± 0.0088 | 0.527 ± 0.0105 | −0.020 | 0.0053 | 0.0550 |
| DMI (kg DM/day) | 9.34 ± 0.179 | 9.69 ± 0.284 | +0.35 | 0.137 | 0.1404 |

BCF, beeswax labelled concentrate feed. DMI, dry matter intake. K, known proportions of dietary components. *E*, estimated proportions of dietary components. SEM, standard error of mean.

statistically significant ($P > 0.05$). These results suggest that the rates of n-alkane faecal recovery for bison and cattle were possibly similar for the *n*-alkanes' range used, considering that the accuracy of diet composition estimates reflects the representativeness of the recovery rates used in faecal corrections [11]. Also, Elwert et al. [22] was able to obtain accurate estimates of diet composition in sheep fed on different proportions of chaffed *Trifolium subterraneum* hay and beeswax-labelled cottonseed meal (in 4 mixtures of 7 : 1, 6 : 2, 5 : 3, and 4 : 4). Similar levels of accuracy were observed by Charmely and Dove [10] in sheep fed on 4 diets composed by equal amounts beeswax-labelled cottonseed meal and different proportions of subterranean clover, phalaris, annual ryegrass, and wheat straw. However, it should be highlighted that the accuracy of estimates declined with the utilization of less precise faecal recovery corrections (i.e. individual, mean treatment and mean for all four treatments). In a subsequent study, Dove and Charmley [32] were able to improve the diet composition estimated by combining *n*-alkanes with long-chain alcohols. According to Dove and Mayes [7], the combination of different marker types allows greater accuracies of estimations diet composition and the discrimination of more diet components in situations where the complexity (i.e. in terms of feed items) of the diet is higher.

One of the factors that may have contributed to an accurate estimation of the labelled concentrate feed in the bison's diet was its high proportion in the diet (>50% of the diet). It might be expected that at lower levels of marked concentrate incorporation, the accuracy of the estimates would decrease. Nevertheless, accurate estimates of beeswax-labelled cottonseed meal incorporation in sheep diets (i.e. varying from 12.5 to 50%) were obtained by Elwert and Dove [22] in lower proportions than that observed in this study. Also, Maxfield et al. [33] was able to estimate accurately the proportion of beeswax labelled concentrate feed in the diet of equines (i.e. varying from 5 to 20%).

The average daily feed intake of the bison observed in this study was of 9.34 kg DM/day. Although the aim of this study was not to characterize DMI of this animal species, the values obtained were very close to the estimations obtained by Borowski et al. [34] for adult bison (10 kg DM/day) to meet their daily amount of feed requirements. The utilization of estimated proportions for each diet component resulted in an overestimation of DMI of only +350 g/day (+3.6%) than the known DMI (9.69 vs. 9.34 kg DM/day, respectively). These results indicate that we were able to estimate with high accuracy the DMI, as the difference between known and estimated DMI values were not significant ($P > 0.05$). Using the same "labelled supplement method," Elwert et al. [25] observed similar differences between estimated of measured daily intake of *T. subterraneum* hay (between −3.7 and + 7.2% when using less accurate recovery data, i.e. mean faecal recovery across diets) and lower ones (−0.3 to +0.9%) when using more accurate data (i.e. mean faecal recovery of each diet). Charmley and Dove [10] compared the "labelled supplement method" or daily dosing with external even-chain n-alkane ($C_{32}$ and $C_{36}$) and observed similar and precise estimates of feed intake. As pointed out by Dove and Charmley [32], the

Table 4: Estimates of apparent dry matter digestibility (DMDap) in bison fed on straw and beeswax labelled concentrate feed, using different long-chain *n*-alkanes.

| | Faecal recovery correction (FRC) | | | | | | | | Effects | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Without FRC | | | | With FRC | | | | | | | |
| n-alkane | 27 | 29 | 31 | 33 | 27 | 29 | 31 | 33 | FRC | M | FRC × M | SEM |
| DMDap (g/kg DM) | 583 ± 5.4 | 615 ± 6.1 | 635 ± 3.2 | 671 ± 0.1 | 682 ± 4.1 | 665 ± 5.3 | 667 ± 3.0 | 657 ± 0.1 | <0.001 | <0.001 | <0.001 | 2.33 |

DMD ap, apparent dry matter digestibility. FRC, faecal recovery correction. *M*, *n-alkane* marker. SEM, standard error of mean.

"labelled supplement method" has several advantages over the traditional dosing methods in terms of animal welfare, i.e. obviating the need to marker administration that is quite important in wild herbivorous. Less handling is a major advantage when working with wild herbivores, such as the European bison, as this species tends to be unstable and have low tolerance to handling and human close contact. They can also be aggressive and unpredictable; thus the ability to reduce contact time and handling with animals improves safety for the person performing the handling and decreases the amount of labour needed to implement a successful nutritional study.

Results obtained in metabolic cages studies indicate an incomplete recovery of n-alkanes in the faeces of ruminant species, pointing out to a possible positive association between their carbon-chain length and their faecal recovery [6, 11, 30]. Due to this association, it is expected that only the long-chain n-alkanes achieve high levels of faecal recovery and can provide accurate estimates of digestibility. For this reason, only the n-alkanes $C_{27}$, $C_{29}$, $C_{31}$, and $C_{33}$ were used to estimate DMDap (Table 4). As for the feed intake estimations, it was expected that estimations of DMDap reflect the good accuracy of diet composition estimates used in the DMDap calculations, as diet composition estimates are known to affect the estimation of DMD. It should be pointed out that the lack of known DMDap values did not allow an accurate evaluation of DMDap obtained by the n-alkane markers.

The discrepancies between DMDap estimated with FRC were 6.3% higher ($P < 0.05$) than that obtained when FRC was not applied to the faecal n-alkane concentrations. Moreover, DMDap values obtained without FRC increased with carbon-chain length of the n-alkane (*M*) used in the estimations. Although total faecal collection was not performed and, for that reason, faecal recovery data was not calculated, this increase indicates that faecal recovery of n-alkanes in bison increased with the increase of carbon-chain length of the markers. This tendency was also reported by Morais et al. [35] when estimating DMDap in beef cattle using n-alkanes $C_{31}$, $C_{33}$, and $C_{35}$ and assuming 100% n-alkane faecal recoveries. A similar association was also observed in other ruminant animal species and is consistent with the general observations that, in ruminant species, are partially lost in their loss gastrointestinal tract [24]. The degree of this loss varies between species, diet composition, and its digestibility [24, 29]. It should be pointed out that DMDap estimation using $C_{33}$*n*-alkanes without FRC was higher than that with FRC (671.1 vs. 657.3 g/kg DM), due to the fact that FRC used for this n-alkane, based on the recovery data obtained by Ferreira et al. [11], was higher than 1.0 (1.042), contrastingly with the other markers ($C_{27}$, $C_{29}$, $C_{31}$) with FRC inferiors to 1.0. Results obtained suggest that the application of *n*-alkanes as internal markers to estimate DMDap should be preceded by the correction of their faecal concentrations for their incomplete faecal recovery.

## 4. Conclusions

Data obtained in this study suggest that the beeswax labelled method can be used to estimate accurately diet composition and voluntary feed intake of European bison (*Bison bonasus*) limiting the handling and work with these wild herbivores. Results showed that the utilization of n-alkane faecal recovery obtained in cattle can be used in the bison, without comprising the accuracy of diet composition and voluntary feed intake estimates. Similarly to what was previously observed in domestic herbivore species, results suggest that the faecal recovery of *n*-alkanes in bison is incomplete and tends to increase with carbon-chain length. Thus, the application of these markers to estimate DMDap should be preceded by the corrections of *n*-alkanes concentrations to their incomplete faecal recovery.

## Data Availability

The data that support the findings of this study are available within the article.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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