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1 Running title: N-alkanes as fecal markers of intake

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3 **Use of n-alkanes to estimate feed intake in ruminants: a meta-analysis**

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5 **Jose Herilalao Andriarimalala,* Jose Carlos B. Dubeux Jr.,† Nicolas DiLorenzo,† David**
6 **Mirabedini Jaramillo,† Jean de Neupomuscène Rakotozandriny,* Paulo Salgado‡**

7

8 *High School of Agricultural Sciences, University of Antananarivo, 101 Antananarivo,
9 Madagascar

10 †North Florida Research and Education Center, University of Florida, 3925 Highway 71,
11 Marianna, FL, USA, 32446

12 ‡CIRAD, UMR SELMET, 110 Antsirabe, Madagascar, / SELMET, University of Montpellier,
13 CIRAD, INRA, Montpellier SupAgro, Montpellier, 34398, France

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20 ²Corresponding author: Jose Herilalao Andriarimalala, andriari.jose@gmail.com

21 ABSTRACT

22 Precise techniques to estimate feed intake by ruminants are critical to enhance feed
23 efficiency and to reduce greenhouse gas emissions and nutrient losses to the environment. Using
24 a meta-analysis, we evaluated the accuracy of the n-alkane technique to predict feed intake in
25 cattle and sheep, and assessed the relationships between feed intake and fecal recovery of n-
26 alkanes. The database was composed of 28 studies, including 129 treatments (87 and 42 for
27 cattle and sheep, respectively) and 402 animals (232 cattle and 170 sheep) fed at troughs, from
28 published studies. Relationships between observed (*in vivo* measurement) and predicted feed
29 intake by C31:C32 and C32:C33 n-alkane pairs were evaluated by regression. Meta-regression
30 addressed the relationships between the difference in fecal recovery of n-alkane pairs and the
31 error in intake estimation, as well as the amount and duration of C32 n-alkane dosing.
32 Regression of observed intake on n-alkane-based estimates revealed good relationships in cattle
33 (adjusted $R^2 = 0.99$ for C31:C32, and adjusted $R^2 = 0.98$ for C32:C33; $P < 0.0001$) and in sheep
34 (adjusted $R^2 = 0.94$ for C31:C32, and adjusted $R^2 = 0.96$ for C32:C33; $P < 0.0001$). Fecal
35 recovery of natural n-alkanes showed a coefficient of variation about 15% and 16% for C31 and
36 C33, respectively in cattle. In sheep, the coefficient of variation was 8% and 14% for C31 and C33,
37 respectively. The relationships between the difference of fecal recovery of n-alkane pairs and
38 the error in feed intake estimation in cattle were characterized by an adjusted $R^2 = 0.83$ for
39 C31:C32 ($P < 0.0001$) and adjusted $R^2 = 0.93$ for C32:C33 ($P < 0.0001$). In sheep, they were
40 characterized by an adjusted $R^2 = 0.69$ for C31:C32 ($P < 0.001$) and adjusted $R^2 = 0.76$ for
41 C32:C33 ($P < 0.001$). The n-alkane technique provided the reliability for estimating feed intake in
42 cattle and sheep in barn experiments. The present meta-analysis demonstrated that without
43 correction for differences in fecal recovery of n-alkane pairs, deviation in feed intake prediction
44 would occur. However, further research is necessary to determine the relationship between the

45 n-alkane dosing procedure (daily amount and duration of dosing) and fecal recovery of n-
46 alkane.

47 **Key words:** cattle, feed intake, markers, recovery, sheep.

48 *List of Abbreviations:* BW, body weight; DM, dry matterFR, fecal recovery; RMSE, root mean square
49 error.

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INTRODUCTION

Having accurate techniques to estimate feed intake is critical to evaluate the nutritive value of feed and the nutritional status of livestock. Additional benefit includes the selection for feed efficiency and reduction of greenhouse gas emissions by selecting more efficient livestock. However, individual feed intake is difficult to measure accurately in group-housed and grazing animals because of the lack of reliable methodologies (Penning, 2004). The n-alkane technique was used for estimating the herbage intake in grazing ruminants (Mayes et al., 1986). Errors due to incomplete recovery of n-alkanes would cancel out in intake calculations, when using consecutive pair of n-alkanes with similar fecal recovery rates. Nevertheless, the results from numerous studies were highly variable when the n-alkane techniques were used to estimate feed intake (Azevedo et al., 2014). Meta-analysis is a useful tool that can be used to both summarize the effects of treatment across studies and investigate factors explaining potential heterogeneity of response (Duffield et al., 2008). Mixed model regression methods allow for data from various experiments to be adjusted for random effects associated with trials and weighted for differences in variability associated with a particular study (St-Pierre, 2001; Arelovich et al., 2008). Very few studies have summarized the accuracy of intake estimation by n-alkanes in sheep and cattle, under variable experimental conditions. The hypothesis of the present study was that the accuracy of feed intake estimation is influenced by differences in fecal recovery of n-alkanes. The present study aimed to evaluate the accuracy of the n-alkane technique for estimating the feed intake in cattle and sheep fed at a trough, using a meta-analysis. The second objective was to analyze the relationships between the discrepancies of intake estimation and fecal recovery of n-alkanes, and the effect of the dosing procedure on fecal recovery of n-alkanes.

MATERIAL AND METHODS

75 *Literature review and dataset construction*

76 A literature search was carried out using two search engines: (1) the online databases
77 Agricola (National Agricultural Library, U.S Department of Agriculture) and (2)
78 CAB Abstracts and Global Health on Web of Science (Centre for Agriculture and Bioscience
79 International). Candidate publications were selected using the following keywords: n-alkanes,
80 intake, fecal recovery, ruminant, cattle, and sheep. The initial dataset had 62 studies focusing
81 on feed intake estimation by n-alkane pairs C31:C32 and C32:C33. Thirty-four studies were
82 excluded because they had no information about the observed feed intake (22 studies) and the
83 dosing procedure (7 studies). Five studies were also rejected because they have been carried
84 out with animal species other than cattle and sheep. After discarding these manuscripts, 28 studies
85 were selected (19 for cattle and 9 for sheep), including 129 treatments (87 for cattle and 42 for
86 sheep) and 402 animals (232 cattle and 170 sheep). Each study and treatment were coded by
87 a number. The number of replicates within an experiment and standards errors of responses
88 were included in the database. A template for data extraction was drafted, which included
89 information about the number of replicates within an experiment, animals
90 characteristics (species, sex, number of animals within the study, body weight [BW], age, and
91 performance), diet characteristics (type, number of forage species, feeding levels, crude protein,
92 digestibility, and n-alkane concentration), the procedure of synthetic n-alkane dosing (amount
93 and duration) and fecal collection method. The trial number, literature references, and the
94 number of treatments for the database were recorded (Table 1).

95 *Data processing*

96 For both animal species, the relationships between the observed feed intake (kg DM/d)
97 and predicted feed intake from C31:C32 and C32:C33 n-alkane pairs (kg DM/d) were studied
98 by linear regression. The relationship between the fecal recovery of n-alkanes and the error in

99 feed intake estimation was also considered. For that purpose, the difference of fecal recovery
100 for each pair of n-alkanes was calculated, as follows:

$$101 \quad \text{DFR (\%)} = \frac{(\text{FR dosed} - \text{FR natural}) \times 100}{\text{FR dosed}}$$

102 Where DFR = difference in fecal recovery (%); FR dosed = fecal recovery of dosed n-alkane;
103 and FR natural = fecal recovery of naturally occurring odd-chain n-alkane.

104 The discrepancies between the observed and predicted feed intake were calculated, as
105 follows:

$$106 \quad \text{DI (\%)} = \frac{(\text{obs intake} - \text{pred intake}) \times 100}{\text{obs intake}}$$

107 Where DI = discrepancies in intake estimation; obs intake = observed intake (kg DM/d); and pred
108 intake = predicted intake (kg DM/d)

109 *Statistical analyses*

110 The meta-regression between the independent and dependent variables was analyzed
111 using the Mixed procedure of Statistical Analysis System (SAS Inc., Cary, NC). The independent
112 variable was the observed feed intake (kg DM/d) while the predicted feed intake by n-alkane pairs
113 (kg DM/d) was the response variable. For other relationships, the fecal recovery of each n-alkane and the
114 differences in fecal recovery between the n-alkane pairs (%) were used as independent
115 variables. They were studied with other responses such as: (1) the discrepancies in observed vs.
116 marker-predicted feed intake estimates (%); (2) the daily amount of C32 n-alkane dosing
117 (mg·kg of BW⁻¹·d⁻¹); and (3) the duration of the dosing (days). The effect of animal species on
118 the fecal recovery of each n-alkane, the discrepancies in intake estimates and the differences in
119 fecal recovery of n-alkane pairs were analyzed by analysis of variance. The average and
120 standard error (SE) for each quantitative variable were recorded. If the SE was not reported, it
121 was calculated by using the standard deviation and the number of replicates. In order to consider
122 relative contributions of individual studies to the total effect estimate, each study was weight

123 according to its number of replicates and SE (St-Pierre, 2001; Arelovich et al., 2008). Briefly,
124 the optimal weight (w_2) was calculated as $w_1/\sqrt{\text{avg}(w)}$, where $w_1 = 1/\text{SE}$ for a set of means in
125 a study, and $\text{avg}(w) = \text{mean } w_1$ value. As described by St-Pierre (2001), this offers the
126 advantage that the sum of $w_2 = 1$, and thus variance and covariance components are on the same
127 scale as the original data.

128 First, trial-adjusted dependent variable means were computed, using a mixed model that
129 included the random effect of study. Those variables were weighted using the “weight”
130 statement in the Mixed procedure, as described above. Moreover, all independent variables were
131 fitted to a model that first included a fixed slope and intercept and subsequently was corrected
132 by a generated random slope and intercept clustered by study to yield trial-adjusted data. Once
133 determined from mixed model analyses and weighted, trial-adjusted values of dependent
134 variables were regressed between the considered independent variables.

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RESULTS

Database characteristics

138 In the cattle database, each study included on average 11 animals with 5 replicates
139 per treatment, a live BW that averaged 425 kg and a coefficient of variation (CV) of 45% (Table 2). On
140 average, three different forages species were used for each trial, with average crude protein concentration and
141 DM digestibility of 150 and 627 g/kg DM, respectively. The synthetic C32 n-alkane
142 was dosed to animals during 1 to 20 d. The amount of dosed n-alkane averaged 540 mg/d and
143 ranged between 177 and 1,122 mg/d with a CV of 52%. The n-alkane concentrations of the
144 forage diet for cattle averaged 251, 10, and 91 mg/kg DM for C31, C32 and C33, respectively.

145 On average, 12 sheep were used for each trial and five replicates per treatment, with
146 a live BW that averaged 39 kg, and ranged from 30 to 65 kg, with a CV of 27% (Table 3).

147 Two forage species were on average fed to sheep, with 169 and 590 g/kg DM of crude protein and

148 DM digestibility, respectively. Forage DM digestibility ranged from 573 to 608 mg/kg DM
149 with a CV of 3%. The synthetic C32 n-alkane was administrated to sheep on average
150 116 mg/d for 14 d. In sheep, the forage diet contained on average 209, 8 and 68 mg/kg DM for
151 C31, C32, and C33, respectively.

152 *Accuracy of the n-alkanes techniques for estimating feed intake*

153 In cattle, observed feed intakes were correlated with estimated feed intakes for both n-
154 alkane pairs (Figure 1). The adjusted coefficient of determination (adjusted R^2) was 0.99 and the root mean
155 square error (RMSE) was 0.65 kg DM/d ($P < 0.0001$) for C31:C32 while adjusted $R^2 = 0.98$ and RMSE
156 = 0.77 kg DM/d ($P < 0.0001$) for C32:C33. In cattle, the observed feed intake ranged from 2.7 to
157 24.7 kg DM/d (6.3 to 58.1 g DM·kg of $BW^{-1} \cdot d^{-1}$) across various experiments. The estimated
158 feed intake ranged from 2.6 to 24.7 kg DM/d (6.0 to 58.1 g DM·kg of $BW^{-1} \cdot d^{-1}$) and from 2.5
159 to 24.3 kg DM/d (5.9 to 57.2 g DM·kg of $BW^{-1} \cdot d^{-1}$), when using C31:C32 and C32:C33 ratios,
160 respectively. In cattle, the intercept was estimated at 0.45 ± 0.15 for C31:C32 which was
161 different from 0 ($P < 0.01$). For C32:C33, the intercept was estimated at 0.11 ± 0.15 which was
162 not different from 0 ($P = 0.48$). The slope estimates of 0.90 ± 0.01 and 0.97 ± 0.01 for the n-alkane
163 pairs C31:C32 and C32:C33, respectively were different from 1 ($P < 0.0001$).

164 In sheep, there were linear regressions between actual and estimated feed intakes (Figure 2)
165 The adjusted R^2 and RMSE were respectively 0.94 and 0.13 kg DM/d for C31:C32 ($P <$
166 0.0001) while adjusted $R^2 = 0.96$ and RMSE = 0.14 kg DM/d for C32:C33 ($P < 0.0001$). In
167 sheep, actual feed intake ranged from 0.4 to 2.4 kg DM/d (8.9 to 60.1 g DM·kg of $BW^{-1} \cdot d^{-1}$).
168 The feed intake estimated by C31:C32 pairs ranged from 0.3 to 2.3 kg DM/d (8.5 to 58.5 g
169 DM·kg of $BW^{-1} \cdot d^{-1}$), while the C32:C33 pairs ranged from 0.4 to 2.4 kg DM/d (9.0 to 60.8 g
170 DM·kg of $BW^{-1} \cdot d^{-1}$). In sheep, the intercept for C31:C32 was estimated at 0.90 ± 0.04 and had a $P <$
171 0.05 of being different from 0. Instead, the intercept was estimated at 0.04 ± 0.04 for C32:C33
172 which was not different from 0 ($P = 0.29$). Likewise, the slope estimates of 0.92 ± 0.04 and

173 0.98±0.03 for the n-alkane pairs C31:C32 and C32:C33, respectively also had a $P < 0.0001$ of
174 being different from 1.

175 ***Fecal recovery of n-alkanes***

176 There was no significant difference between animal species on the fecal recovery of C31
177 ($P = 0.09$), C32 ($P = 0.20$) and C33 ($P = 0.37$) n-alkanes (Table 4). In cattle, fecal recovery
178 ranged from 0.60 to 1.04, from 0.80 to 1.06, and from 0.63 to 1.26, for C31, C32, and C33 n-
179 alkanes, respectively. In cattle, the fecal recovery had a CV of 15 and 16% for C31 and C33,
180 respectively. In sheep, fecal recovery ranged from 0.79 to 1.04, from 0.75 to 0.97, and from
181 0.72 to 1.16, for C31, C32, and C33 n-alkanes, respectively. In sheep, the fecal recovery had a
182 CV of 8 and 14% for C31 and C33, respectively. The fecal recovery of synthetic C32 n-alkane
183 showed a similar CV of about 6% among animal species. The difference in fecal recovery in n-
184 alkane pairs was 30% ($P < 0.001$) and 32% ($P < 0.1$) lower in sheep than in cattle for C31:C32
185 and C32:C33, respectively.

186 ***Relationship between the fecal recovery and feed intake***

187 The relationships between the difference of fecal recovery of n-alkane pairs and the
188 error in feed intake estimation in cattle were characterized by an adjusted $R^2 = 0.83$ and adjusted
189 $R^2 = 0.93$, $P < 0.0001$ for C31:C32 and C32:C33, respectively (Figure 3). The RMSE was about
190 2.74% and 1.75% for C31:C32 and C32:C33, respectively. In cattle, every percentage unit
191 difference in fecal recovery between the n-alkane pair resulted in an error of 2.6% and 0.4% in
192 estimated feed intake by C31:C32 and C32:C33, respectively. In sheep, the relationship had an
193 adjusted $R^2 = 0.69$ ($P < 0.001$) and adjusted $R^2 = 0.76$ ($P < 0.0001$) for C31:C32 and C32:C33,
194 respectively (Figure 4). In sheep, every percentage unit difference in fecal recovery between the
195 n-alkane pair resulted in an error of 0.8% and 2.1% in estimated feed intake by C31:C32 and
196 C32:C33, respectively.

197 ***Relationship between the fecal recovery and the procedure of dosing (amount and duration***
198 ***of dosing)***

199 There was no relationship observed between the amount of C32 n-alkane dosing and the
200 fecal recovery of C32 n-alkanes ($P = 0.17$) or between the day of dosing and the fecal recovery
201 of C31 ($P = 0.71$), C32 ($P = 0.15$) and C33 ($P = 0.12$) in cattle (Table 5). The only exception
202 occurred for the linear regression between the amount of C32 n-alkane dosed and the fecal
203 recovery of C31 and C33 by cattle, which had an adjusted $R^2 = 0.69$ and adjusted $R^2 = 0.52$ (P
204 < 0.0001), respectively. The relationships between the amount of C32 n-alkane dosed and the
205 fecal recovery of C31 and C33 by cattle were negative. For each unit of C32 dosing ($1 \text{ mg} \cdot \text{kg}$
206 of $\text{BW}^{-1} \cdot \text{d}^{-1}$), 85% and 92 % of fecal recovery of C31 and C33 were affected, respectively.

207 In sheep, there was no relationship between the day of dosing and the fecal recovery of
208 C31 ($P = 0.26$), C32 ($P = 0.08$) and C33 ($P = 0.81$) n-alkanes (Table 6). The negative
209 relationship between the amount of C32 n-alkane dosing and the fecal recovery in sheep had an
210 adjusted $R^2 = 0.25$ for C31 ($P < 0.01$), an adjusted $R^2 = 0$ for C32 ($P = 0.95$) and an adjusted R^2
211 $= 0.34$ for C33 ($P < 0.001$). For each unit of C32 dosing ($1 \text{ mg} \cdot \text{kg}$ of $\text{BW}^{-1} \cdot \text{d}^{-1}$), 92% and 97%
212 of fecal recovery of C31 and C33 were affected, respectively.

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DISCUSSION

215 The advantage of meta-analytic methods is the ability to integrate smaller studies using
216 effect-size metrics and enhance the statistical power over that of any single study. Therefore,
217 they provide the potential to explore new hypotheses (Rodney et al., 2015). This study was
218 designed to explore the effects of fecal recovery of n-alkanes on the accuracy of feed intake
219 estimation in cattle and sheep. The estimation of feed intake by using n-alkane techniques
220 involved simultaneous computing of digestibility, from natural odd-chain n-alkane and fecal
221 output, from dosed even-chain n-alkane, as follows:

$$222 \quad \text{Feed intake (kg DM/day)} = \frac{D_j}{\left(\frac{F_j * R_i}{F_i * R_j}\right) * (H_i - H_j)}$$

223 where H_i and F_i are the respective concentrations of natural odd-chain alkanes in diet and feces
 224 (mg/kg DM); H_j and F_j are the respective concentrations of even chain n-alkanes in diet and
 225 feces (mg/kg DM); D_j is the dose rate of even chain n-alkane to animals (mg/day); R_i and R_j
 226 are the respective fecal recoveries of natural odd-chain and even chain n-alkanes. In principle,
 227 errors arising from the incomplete fecal recovery of used n-alkanes are canceled out in the
 228 calculation, if the fecal recoveries of adjacent n-alkanes are similar. N-alkane pairs C31:C32
 229 and C32:C33 were generally proposed to estimate feed intake because they had the lowest
 230 discrepancy in fecal recovery and gave better estimation of intake (Dove et al., 2002). Natural
 231 n-alkanes C31 and C33 can be found in greater quantities in the cuticular and epicuticular wax
 232 of plants, while n-alkane C32, a synthetic compound, is administrated to animals. The high n-
 233 alkane concentrations of natural odd-chain alkanes (C31 and C33) and low concentrations of
 234 even-chain alkanes (C32) in forage diet in our database are in agreements with the literature
 235 (Hu et al., 2014).

236 *Fecal recovery of n-alkanes and accuracy of feed intake estimates*

237 The significant linear regressions between the estimated and measured feed intake in
 238 cattle and sheep, indicated the accuracy of n-alkane techniques to estimate this parameter. All
 239 the models had adjusted coefficients of determination greater than 0.94 for both pairs of n-
 240 alkanes (C31:C32 and C32:C33). Azevedo et al. (2014) reported that there was a difference in feed
 241 intake estimation depending on the pair of n-alkane used and generally, n-alkane techniques
 242 overestimated the actual feed intake. The accuracy of feed intake estimation relies on the
 243 similarity in fecal n-alkane recovery of the dosed and herbage odd-chain alkanes. Our findings
 244 are in agreements with Dove and Mayes (1996) who reported a linear relationship between the
 245 difference in fecal recoveries of n-alkanes and errors in the estimated feed intake. Every

246 percentage unit difference in fecal recovery between the alkane pair resulted in an error of
247 1.25% in estimated intake (Dove and Mayes, 1996). However, this proportion is slightly lower
248 than our findings, which were on average 1.52 and 1.45% in cattle and sheep, respectively.
249 Errors in intake estimates were dependent upon animal species and natural n-alkane used. In
250 cattle, the n-alkane ratio C32:C33 seemed to be better while C31:C32 was better in sheep.

251 *Fecal recovery and procedure of n-alkane dosing*

252 The double n-alkane ratio technique involves dosing the known quantities of synthetic n-
253 alkanes of a chain-length adjacent to natural n-alkanes present in plants. It requires an accurate
254 administration method related to the amount and timing of n-alkane dosing. Several carrier
255 matrices were used for synthetic n-alkanes administration to ruminants, such as paper pellets,
256 controlled release capsule and devices, bolus, gelatin capsules, labeled feedstuffs (roughages
257 or concentrates) or feed pellets, and alkane suspensions or oil-in-water. Paper pellets have been the
258 most commonly used method in experiments with cattle and sheep (Giráldez et al., 2004). Smith
259 et al. (2007) reported that the carrier material used, the frequency of dosing, and fecal sampling
260 schedules are factors that may have an influence on the pattern of fecal concentration of dosed
261 n-alkanes. The carrier matrices (method of administration of n-alkanes) were not quantitative
262 variables, unlike the amount and duration of dosing. Therefore, in the present study, they were
263 not analyzed by regression with the intake or fecal recovery of n-alkanes.

264 The average amount of dosed n-alkanes in this meta-analysis is relatively lower than the
265 amount of C32 n-alkane reported by other studies. Indeed, to have a good accuracy of feed
266 intake estimates, more than 700 to 800 mg/d must be dosed to cattle (Smit et al., 2005). Ferreira
267 et al. (2007) reported a 5-day equilibrium period for n-alkane dosing involving paper pellets
268 and controlled release devices to be adequate. After this period of equilibrium, feces collection
269 has a reduced diurnal variation of n-alkanes in feces. In most cases, the animals were dosed
270 once or twice daily for several days to achieve a steady state situation (Giráldez et al., 2004).

271 However, when using controlled release capsule and devices, a single administration of C32 n-
272 alkane followed by repeated sampling of feces can be applied.

273 *Fecal recovery and digestive kinetics of n-alkanes*

274 Even as markers, long-chain n-alkanes are not totally recoverable in the feces but can
275 be absorbed endogenously. It has been shown that they are probably taken up by the liver and
276 metabolized mainly to phospholipids or broken down to carbon dioxide (Hargrove et al., 2004).
277 Some authors have reported losses of n-alkanes along the gastro-intestinal tract (Hendricksen
278 et al., 2003). However, the digestive kinetics of n-alkane and their loss through metabolism and
279 absorption merits further study. The passage and kinetics of long-chain n-alkanes into the
280 animal digestive tract are variable according to their source. Indeed, the dosed even-chain n-
281 alkanes are linked with the liquid phase of digesta and pass more rapidly along the digestive
282 tract while natural odd-chain n-alkanes are associated with the particulate phase of digesta
283 (Dove and Mayes, 1991). Therefore, the dosed n-alkane C32 can be recovered in feces in a
284 greater proportion than natural n-alkanes C31 or C33 (Elwert et al., 2008). These are in
285 agreement with our observations in cattle, in which the fecal recovery of C32 n-alkanes had
286 been greater than C31 and C33. Such was not the case in sheep when fecal recovery of alkanes
287 tended to be similar. Many authors reported that the recovery of n-alkanes in sheep is greater and
288 less variable compared with cattle (Dove and Mayes, 1991).

289 *Validation of the models into grazing conditions*

290 Models of feed intake estimation by n-alkane technique were developed in cattle and
291 sheep under stall-feeding conditions. In grazing conditions, studies have been conducted using
292 the n-alkane technique to measure feed intake but either actual feed intake had not been
293 measured or fecal sampling had not been considered (Mann and Stewart, 2003). Indeed, most
294 methods for measuring feed intake in grazing animals presented lack of precision (Smit et al.,

295 2005). Moreover, as the total fecal collection is difficult to perform in grazing animals, literature
296 values of n-alkane fecal recovery have been often used.

297 Despite these challenges, the developed models in barn experiments merit to be
298 validated under grazing conditions. Some existing methods for measuring feed intake were
299 proposed, such as the method of herbage mass difference before and after grazing, animal live
300 weight difference, comparison of animal requirements and performance, empirical models,
301 animal grazing behavior, and fecal near infrared reflectance spectrometry. However, the choice
302 of the method depends on the objective and the duration of the study. Furthermore, the intra-
303 ruminal controlled-release device technique can be used, which limits animal manipulation.
304 This technique gives less diurnal variability of marker excretion allowing the validity of grab
305 or spot fecal sampling in grazing animals. Grazing ruminants in tropical conditions have a diet
306 based on heterogeneous grasslands, mostly with low nutritive value and large seasonal
307 variations in quantity and quality. It is obvious that feed intake by grazing animals would be
308 very different than in barn experiments. Before using the models to estimate the feed intake of
309 animals on pasture, it is recommended to add more variability in data, such as animals breed,
310 physiological status, body weight, forage species and climatic conditions (temperature and
311 humidity) to be able to get closer to the reality in grazing conditions.

312

313

CONCLUSIONS

314 For more than 30 years, the n-alkane technique provided a valid alternative and reliable
315 method for estimating feed intake in cattle and sheep in barn experiments. The feed intake
316 estimated by both n-alkane pairs C31:C32 and C32:C33 were highly correlated with the
317 observed feed intake. All the models were robust and the coefficients of determination (adjusted
318 R^2) were similar for both pairs of n-alkanes (C31:C32 and C32:C33) and animal species.
319 A linear relationship was observed between the difference in fecal recovery rates of n-

320 alkane pairs used and the error in feed intake prediction. Without correction for differences in
321 recovery of n-alkane pairs, deviation in feed intake prediction will be observed. Fecal recovery
322 rates of n-alkanes were affected by animal species and types of n-alkanes. Effect of the dosing
323 procedure, as the amount and the duration of n-alkane dosing on the fecal recovery rates of n-
324 alkanes merits further study for validation. Validation of the method in grazing animals needs
325 further reflection with the challenge that there is no reliable method as reference. The accurate
326 measurement of feed intake enables to increase pasture management practices, which enhances
327 the animal productivity and therefore, ensures a sustainable food security.

328

329 **Conflict of interest statement**

330 No actual or potential conflicts of interest which may affect the submission or reviewing this
331 research article or its data were perceived.

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333

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477

FIGURE LEGENDS

478 **Figure 1.** Relationships between observed and estimated feed intake in the cattle database.
479 Regressions between observed intake and n-alkane-based estimates were expressed in kg DM/d
480 in A and B for C31:C32 and C32:C33, respectively, and in g DM·kg of BW⁻¹·d⁻¹ in C and D
481 for C31:C32 and C32:C33, respectively. As actual intake increased, n-alkane-based estimates
482 of intake increased for A (Adjusted R² = 0.99, RMSE = 0.65, *P* < 0.0001), B (Adjusted R² =
483 0.98, RMSE = 0.77, *P* < 0.0001), C (Adjusted R² = 0.56, RMSE = 0.00, *P* < 0.0001) and D
484 (Adjusted R² = 0.71, RMSE = 0.00, *P* < 0.0001). The continuous line represented the linear
485 trend line of all data, the discontinuous line represented the *y* = *x* line.

486

487 **Figure 2.** Relationships between observed and estimated feed intake in the sheep database.
488 Regressions between observed intake and n-alkane-based estimates were expressed in kg DM/d
489 in A and B for C31:C32 and C32:C33, respectively, and in g DM·kg of BW⁻¹·d⁻¹ in C and D
490 for C31:C32 and C32:C33, respectively. As actual intake increased, n-alkane-based estimates
491 of intake increased for A (Adjusted R² = 0.94, RMSE = 0.13, *P* < 0.0001), B (Adjusted R² =
492 0.96, RMSE = 0.14, *P* < 0.0001), C (Adjusted R² = 0.90, RMSE = 0.01, *P* < 0.0001) and D
493 (Adjusted R² = 0.89, RMSE = 0.01, *P* < 0.0001). The continuous line represented the linear
494 trend line of all data, the discontinuous line represented the *y* = *x* line.

495

496 **Figure 3.** Relationships between the difference in fecal recovery and error in feed intake
497 estimates in cattle for C31:C32 (A) and C32:C33 (B) n-alkane pairs. Every percentage unit
498 difference in fecal recovery between the n-alkane pair resulted to an error of 2.6% in estimated
499 feed intake by C31:C33 (Adjusted R² = 0.83, RMSE = 2.74, *P* < 0.0001) and 0.4% by C32:C33
500 (Adjusted R² = 0.93, RMSE = 1.75, *P* < 0.0001). The continuous line represented the linear
501 trend line of all data, the discontinuous line represented the *y* = *x* line.

502

503 **Figure 4.** Relationships between the difference in fecal recovery and error in feed intake
504 estimates in sheep for C31:C32 (A) and C32:C33 (B) n-alkane pairs. Every percentage unit
505 difference in fecal recovery between the n-alkane pair resulted to an error of 0.8% in estimated
506 feed intake by C31:C33 (Adjusted $R^2 = 0.69$, RMSE = 1.48, $P < 0.001$) and 2.1% by C32:C33
507 (Adjusted $R^2 = 0.76$, RMSE = 3.18, $P < 0.0001$). The continuous line represented the linear
508 trend line of all data, the discontinuous line represented the $y = x$ line.

509 **Table 1.** Summary of the databases including the number of treatments

Database and trial	Reference	No. of treatments
Cattle		
1	Bani et al., 2014	5
2	Berry et al., 2000	4
3	Bezabih et al., 2012	7
4	Chavez et al., 2011	8
5	Chopa et al., 2012	2
6	Ferreira et al., 2004	4
7	Hameleers and Mayes, 1998	5
8	Hendricksen et al., 2003	5
9	Hofstetter et al., 2011	2
10	Molina et al., 2004	2
11	Morais et al., 2011	2
12	Moshtaghi Nia and Wittenberg, 2002	4
13	Oliván et al., 2007	2
14	Ouellet et al., 2004	8
15	Pérez-Ramírez et al., 2011	8
16	Premaratne et al., 2005	1
17	Richmond et al., 2015	8
18	Unal and Garnsworthy, 1999	6
19	Wright et al., 2018	4
Sheep		
1	Amaral et al., 2013	2
2	Charmley and Dove, 2007	4
3	Dove and Oliván, 1998	2
4	Keli et al., 2008	4
5	Lewis et al., 2003	7
6	Lin et al., 2007	6
7	Mayes et al., 1986	12
8	Sibbald et al., 2000	4
9	Vulich et al., 1991	1
	Total	129

510 **Table 2.** Number of replications and animals per treatment, animal body weight, forage
 511 nutritional and n-alkanes composition, procedure of dosing, observed and predicted intake in
 512 cattle

Item	Mean	Median	Mode	Range	SD
Replications/treatment	5	4	4	[2 – 16]	4
No. of animal/treatment	11	8	8	[3 – 32]	9
Animal BW ¹ , kg	424.9	422.0	422.0	[160.0 – 675.0]	191.3
No. of forage species	3	2	1	[1 - 12]	3
Crude protein, g/kg DM ²	149.6	127.0	121.0	[68.0 – 254.0]	58.7
DM Digestibility, g/kg	627.4	610.0	596.0	[437.0 – 810.0]	104.4
N-alkanes concentrations in the forage					
C31, mg/kg DM	251.3	203.2	-	[28.1 – 625.5]	181.4
C32, mg/kg DM	9.95	8.25	-	[0.10 – 21.3]	6.64
C33, mg/kg DM	90.7	56.9	-	[3.20 – 422.2]	107.3
Procedure of dosing ³ (synthetic C32 n-alkane)					
Amount, mg/d	539.5	400.0	400.0	[176.8 – 1,122.0]	279.5
Duration, d	12	12	6	[1 - 20]	5
DMI ⁴ , kg DM/d					
Observed	9.06	6.33	4.10	[2.69 – 24.7]	5.95
C31:C32	9.40	7.01	15.7	[2.55 – 24.7]	6.36
C32:C33	9.07	6.41	2.65	[2.52 – 24.3]	6.28

513 ¹BW: body weight

514 ²DM: dry matter

515 ³The daily amount of synthetic C32 n-alkanes administered to the animal during the experiment,
 516 expressed in mg/d, and the duration of administration, expressed in d.

517 ⁴DMI: Dry matter intake

518 **Table 3.** Number of replications and animals per treatment, animal body weight, forage
 519 nutritional and n-alkanes composition, procedure of dosing, observed and predicted intake in
 520 sheep

Item	Mean	Median	Mode	Range	SD
Replicates/treatment	5	6	6	[2 – 12]	3
No. of animal/treatment	12	12	12	[4 – 24]	6
Animal BW ¹ , kg	39.3	38	30	[30.0 – 65.0]	10.8
No. of forage species	2	2	2	[1 - 4]	1
Crude protein, g/kg DM ²	169.4	201	201	[93.0 – 201.0]	40.5
DM Digestibility, g/kg	590.0	598	573	[573.0 – 608.0]	15.1
N-alkanes concentrations in the forage					
C31, mg/kg DM	209.0	207.8	-	[137.0 – 272.3]	58.8
C32, mg/kg DM	7.66	8.03	-	[3.90 – 10.7]	2.92
C33, mg/kg DM	67.7	45.6	-	[26.7 – 142.3]	49.5
Procedure of dosing ³ (synthetic C32 n-alkane)					
Amount, mg/d	116.0	130	130	[50.0 – 200.0]	50.1
Duration, d	14	12	12	[6 – 21]	5
DMI ⁴ , kg DM/d					
Observed	1.03	0.70	2.16	[0.35 – 2.36]	0.63
C31:C32	0.95	0.72	1.15	[0.34 – 2.30]	0.54
C32:C33	1.05	0.72	0.73	[0.35 – 2.39]	0.68

521 ¹BW: body weight

522 ²DM: dry matter

523 ³The daily amount of synthetic C32 n-alkanes administered to the animal during the experiment,
 524 expressed in mg/d, and the duration of administration, expressed in d.

525 ⁴DMI: Dry matter intake

526 **Table 4.** Effect of animal species on fecal recovery¹ of C31, C32 and C33 n-alkanes and
 527 difference in fecal recovery of n-alkane pairs

Item	Mean	Range	SE	<i>P</i> -value
C31				
cattle	0.83	[0.60 - 1.04]	0.05	0.09
sheep	0.91	[0.79 - 1.04]		
C32				
cattle	0.93	[0.80 - 1.06]	0.02	0.20
sheep	0.90	[0.75 - 0.97]		
C33				
cattle	0.89	[0.63 - 1.26]	0.06	0.37
sheep	0.95	[0.72 - 1.16]		
Difference of fecal recovery in n-alkane pairs ² (%)				
C31:C32				
cattle	16.10 ^a		3.05	< 0.001
sheep	4.81 ^b			
C32:C33				
cattle	10.12 ^a		3.57	< 0.1
sheep	3.27 ^b			

528 ^{a,b}The values of mean fecal recovery in the same column with different letters are significantly
 529 different ($P < 0.05$).

530 ¹The fecal recovery is the proportion of ingested n-alkanes recovered in feces

531 ²The difference of fecal recovery for each pair of n-alkanes is the ratio between the difference
 532 of fecal recovery for dosed and naturally occurring odd-chain n-alkanes and the fecal recovery
 533 of dosed n-alkanes, as follows:

534 Difference in fecal recovery (%) = $(FR_{\text{dosed}} - FR_{\text{natural}}) * 100 / FR_{\text{dosed}}$

535 Where FR_{dosed} = fecal recovery of dosed n-alkane; FR_{natural} = fecal recovery of naturally

536 occurring odd-chain n-alkane.

537 **Table 5.** Regression relationships between fecal recovery of n-alkanes and procedure of dosing
 538 in cattle¹

Alkane	Intercept	SE	Slope	SE	Adjusted R ²	P-value	RMSE ²	CV (%)
C32 n-alkane dosing (mg·kg of BW ⁻¹ ·d ⁻¹) ³								
C31	0.95	0.01	-0.10	0.01	0.69	< 0.0001	0.04	5.36
C32	0.94	0.01	-0.10	0.01	0.02	0.17	0.04	4.47
C33	1.05	0.02	-0.14	0.02	0.52	< 0.0001	0.08	8.74
days of dosing								
C31	0.81	0.02	0.00	0.00	0.00	0.71	0.05	5.99
C32	0.91	0.01	0.00	0.00	0.02	0.15	0.04	4.47
C33	0.84	0.03	0.00	0.00	0.03	0.12	0.08	9.66

539 ¹The daily amount of synthetic C32 n-alkanes administered to the animal during the experiment,
 540 expressed in mg·kg of BW⁻¹·d⁻¹, and the duration of administration, expressed in d.

541 ²RMSE: root mean square error, which is the standard deviation of the residuals or prediction
 542 errors

543 ³BW: body weight

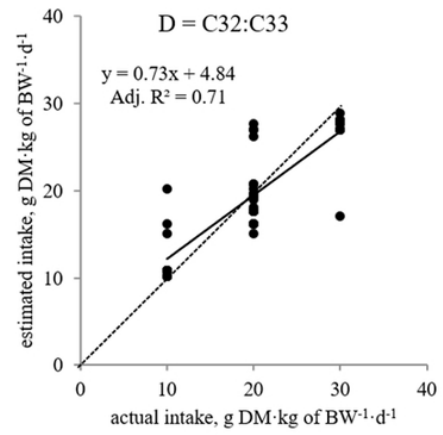
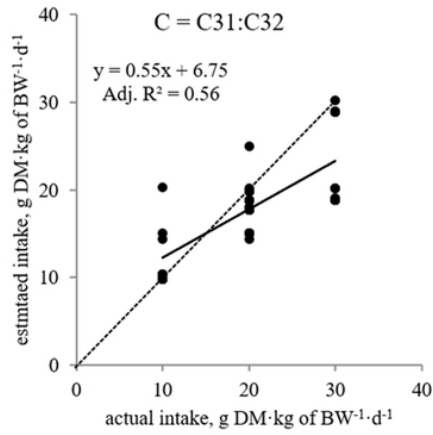
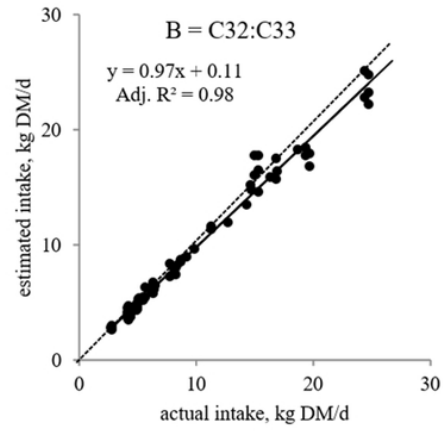
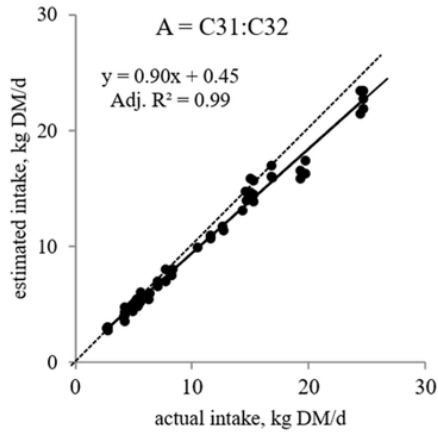
544 **Table 6.** Regression relationships between fecal recovery of n-alkanes and procedure of dosing
 545 in sheep¹

Alkane	Intercept	SE	Slope	SE	Adjusted R ²	P-value	RMSE ²	CV (%)
C32 n-alkane dosing (mg·kg of BW ⁻¹ ·d ⁻¹) ³								
C31	0.94	0.01	-0.01	0.00	0.25	<0.01	0.03	2.84
C32	0.90	0.02	0.00	0.01	0.00	0.95	0.04	4.37
C33	0.99	0.02	-0.02	0.01	0.34	<0.001	0.04	4.54
days of dosing								
C31	0.93	0.01	0.00	0.00	0.01	0.26	0.03	2.87
C32	0.93	0.02	0.00	0.00	0.07	0.08	0.04	4.62
C33	0.94	0.02	0.00	0.00	0.00	0.81	0.04	4.55

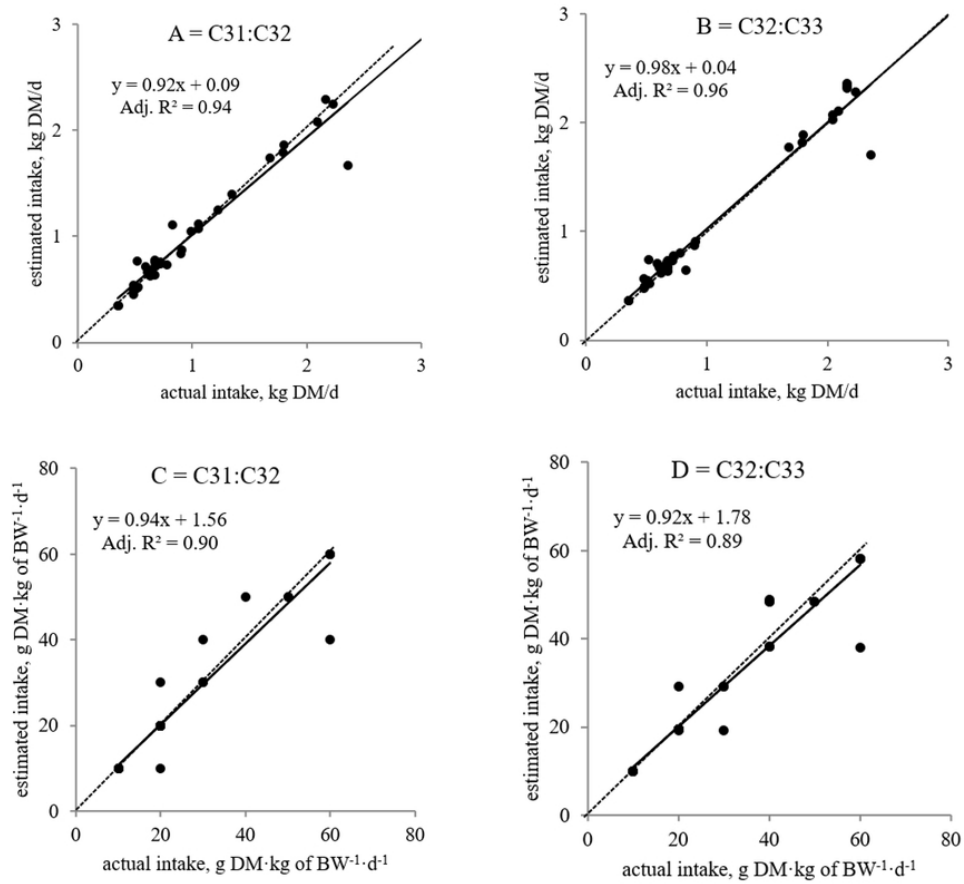
546 ¹The daily amount of synthetic C32 n-alkanes administered to the animal during the experiment,
 547 expressed in mg·kg of BW⁻¹·d⁻¹, and the duration of administration, expressed in d.

548 ²RMSE: root mean square error, which is the standard deviation of the residuals or prediction
 549 errors.

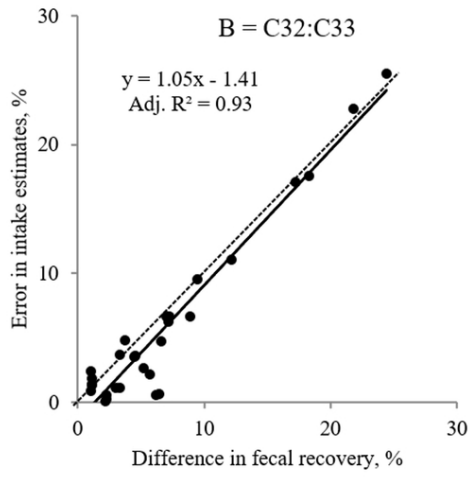
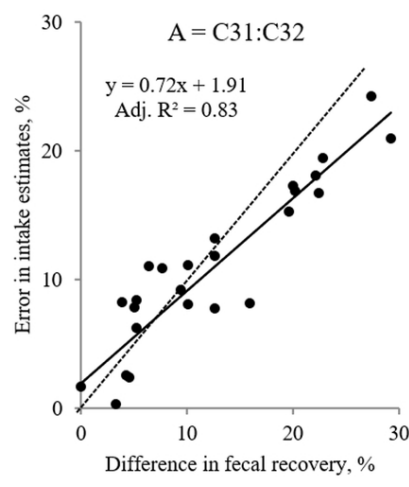
550 ³BW: body weight



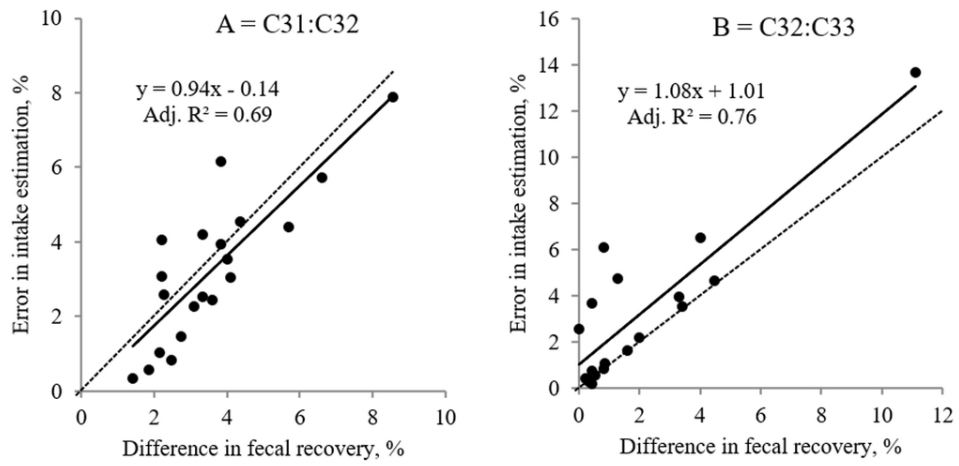
71x67mm (300 x 300 DPI)



76x69mm (300 x 300 DPI)



89x45mm (300 x 300 DPI)



90x44mm (300 x 300 DPI)