

*Assessing the accuracy of current near  
infra-red reflectance spectroscopy  
analysis for fresh grass-clover mixture  
silages and development of new equations  
for this purpose*

Article

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1 **Assessing the accuracy of current near infra-red reflectance spectroscopy**  
2 **analysis for fresh grass-clover mixture silages and development of new**  
3 **equations for this purpose**

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13

14

15

16 **Abstract**

17 The purpose of this study was to ascertain whether Near Infra-Red Reflectance  
18 Spectroscopy (NIRS) prediction equations calibrated on grass silage samples, could  
19 accurately predict the chemical composition of mixed grass-clover silage samples, and  
20 furthermore, to develop and calibrate new grass-clover equations should the grass-  
21 based equations be insufficiently accurate for these silages. A set of 94 silage samples  
22 from mixed grass-clover swards (clover concentration (CC) ranging from 4 to 1000  
23 g/kg as fed; determined manually) were analysed for chemical composition using  
24 reference laboratory techniques, *in vivo* digestible organic matter in the dry matter  
25 (DOMD, in sheep), and *in situ* degradability of dry matter and crude protein (in cows).  
26 The same samples were scanned fresh (undried and unmilled, as is standard practice  
27 for silage analysis within UK laboratories) using NIRS (at AFBI, Northern Ireland) and  
28 grass-based prediction equations applied. Predicted and observed results were  
29 compared. Of 15 chemical components that were tested for prediction accuracy, only  
30 volatile-corrected dry matter and nitrogen were well predicted (RPD values of 4.9 and  
31 2.4 respectively, with low root mean square errors of prediction (RMSEP)). Neutral  
32 detergent fibre and DOMD showed low RPD values, however the predicted and  
33 observed datasets had no significant bias between them and were therefore also  
34 considered as fit for purpose. Variables with significant bias between predicted and  
35 observed datasets that were not considered suitably accurate included crude protein,  
36 acid detergent fibre, microbial dry matter yield and the effective degradability of  
37 protein. For many components, bias could be attributed at least in part to CC and  
38 changes in the fractionation of nutrients present. For some variables such as crude  
39 protein, grass-based equations were sufficiently accurate at low CCs but became  
40 inaccurate as CC increased, as expected. In response to inadequate prediction

41 accuracy of certain nutrients, new grass-clover equations were calibrated using the  
42 obtained spectra. These were validated and results indicated that the grass-clover-  
43 based equations outperformed their grass-based counterparts. The adoption of new  
44 grass-clover equations, or alternatively, with further development, the use of a CC  
45 correction factor to the existing grass-based equations, is recommended for  
46 commercial laboratories offering undried and unmilled silage analysis on samples  
47 containing clover.

48

49 **Keywords:** Grass, Clover, silage, mixtures, NIRS, calibration,

50

51 **Abbreviations:** ADF, acid detergent fibre; aNDF, neutral detergent fibre; CC, clover  
52 concentration; CP, crude protein; VCODM, volatile corrected oven dry matter; EDN,  
53 effective degradable nitrogen; EDDM, effective degradability of dry matter; EE, ether  
54 extract; FIM, Feed into Milk; OM, organic matter; LA, lactic acid; MDM, microbial dry  
55 matter; N, nitrogen; NH<sub>3</sub>-N, ammonia nitrogen; NIRS, near infrared reflectance  
56 spectroscopy; NMSC, normal multiplicative scatter correction;  $r^2$ , coefficient of  
57 determination of cross validation; RMSEP, root mean standard error of prediction;  
58 RPD, ratio of standard deviation of the measured population to the standard error of  
59 prediction; SEC, standard error of calibration; SECV, standard error of cross  
60 validation; SEP, standard error of prediction; SNVD, standard normal variate de-  
61 trending; TMR, total mixed ration; TVC, total volatile content; TVFA, total volatile fatty  
62 acids; WMSC, weighted multiplicative scatter correction; WSC, water soluble  
63 carbohydrate.

64

65 **1. Introduction**

66 Near Infra-Red Reflectance Spectroscopy (**NIRS**) is a relatively rapid and inexpensive  
67 technique, routinely used to provide nutritional analysis of silage and other livestock  
68 feeds in the dairy and beef industries. However, obtaining accurate results requires  
69 robust prediction equations. This is particularly relevant to the UK where most silages  
70 are analysed 'fresh' (i.e. undried and unmilled) for rapid through-put in comparison to  
71 Europe where analysis of dried, ground samples is more common. Dry analysis  
72 requires lengthy sample preparation but has the benefit of increased precision of NIRS  
73 prediction, partly explained by the increased homogeneity of ground samples as well  
74 as the stability of the feedstuff after the removal of water (Sorensen, 2004). Currently  
75 UK laboratories do not offer NIRS equations for grass-legume mixtures, instead, a  
76 prediction equation with a monoculture grass-based calibration is used for a number  
77 of different grass and legume-based forages.

78         This study focusses on NIRS analysis for grass-clover silages, since clover is  
79 thought to be present within grass swards on 70% of UK dairy farms, and therefore is  
80 likely to be the most widely-grown forage legume in the UK (DEFRA, 2015).  
81 Furthermore, clover-containing forages are thought to be a promising feed to increase  
82 sustainability on farms due to reduced inorganic fertiliser required for growth in  
83 comparison to ryegrasses (Elgersma *et al.*, 2000), while maintaining high yields of milk  
84 or meat due to a fast rate of passage promoting intake (Dewhurst *et al.*, 2009; Copani  
85 *et al.*, 2016). A preliminary study has shown that the current NIRS analysis available  
86 for use on grass silages in the UK has poor prediction accuracy of crude protein, pH  
87 and lactic acid when used on mixtures containing both clover and grass (Davies *et al.*,  
88 2012). However, Davies *et al.* (2012) did not evaluate the degradability of dry matter  
89 (**DM**), nitrogen (**N**), or the apparent total tract digestibility of organic matter (**OM**; from  
90 which metabolisable energy (**ME**) is calculated) for prediction accuracy, despite these

91 nutrient fractions being very important for diet formulation when balancing the ratio of  
92 metabolisable protein to metabolisable energy supply. Imbalances in the degradable  
93 protein to fermentable energy ratio will result in poor N use efficiency. Creating  
94 calibration equations for grass-clover silages poses a challenge because these silages  
95 are a mixture of two (or more) forage species, meaning that any resulting equation  
96 must be able to deal with a broad spectrum of sample composition. To date, the  
97 majority of forage-based NIRS calibrations have focussed on predicting the nutritional  
98 composition of just one species, and moreover, in a few instances where mixtures  
99 were analysed using NIRS, typically the focus of the study was on predicting botanical  
100 composition rather than chemical composition (Wachendorf *et al.*, 1999; Cougnon *et*  
101 *al.*, 2014; Karayilanli *et al.*, 2016).

102 The objective of this study was primarily to assess the adequacy of a grass  
103 silage-based prediction equation, commonly used in the UK for predicting chemical  
104 composition, when it was applied to grass silage samples that contained clover in  
105 varying concentrations. Subsequently, a secondary objective was to investigate  
106 whether using grass-clover based prediction equations could improve accuracy of  
107 predicted chemical composition.

108

## 109 **2. Material and methods**

### 110 *2.1 Experimental design*

111 In total, 94 grass-clover silages were sourced from commercial farms and transported  
112 to the Centre for Dairy Research (**CEDAR**), (Arborfield, Reading, UK) for processing.  
113 Samples were acquired from a diverse range of UK farms to ensure maximum  
114 variation within the sample set, in line with the findings of Cougnon *et al.* (2014) for  
115 sourcing robust calibration data. Silage was collected over three consecutive years

116 (2012/13, 2013/14, and 2014/15). The clover content range of greatest importance  
117 was deemed to be 300 - 600 g/kg DM as a more even distribution of grass-clover  
118 within a ley has been shown to create the most advantageous conditions for growth  
119 and promote symbiotic N fixation (Nyfeler *et al.*, 2011; Luescher *et al.*, 2014); although  
120 samples containing < 300 and > 600 g/kg DM clover were also included to provide  
121 sufficient range for statistical analysis and equation evaluation.

122

## 123 *2.2 The silage sample set*

124 *2.2.1 Sample description.* The set of 94 silage samples consisted of 58 bales and 36  
125 samples from clamps which were collected from 50 different locations distributed  
126 across the UK. Of the samples where the clover variety was known (n=65) 66 % were  
127 red clover, 20 % were white clover and 14 % were a mixture of both. Different cuts  
128 were also represented within the set with 36 first, 20 second, 16 third and 4 fourth cut  
129 silages (harvest number not reported for 22 samples). The mean CC within the set  
130 was 310 g/kg DM (Table 1). The sample containing the least clover contained 4 g/kg  
131 DM clover and two samples contained 1000 g/kg DM clover, however all samples  
132 originated from swards that were grass-clover mixtures. Twenty-three of the 94  
133 samples contained < 70 g/kg DM CC and were considered a 'minimal' clover group  
134 for which we hypothesised prediction accuracy would be similar to that of a pure grass  
135 silage. The measured concentration of weed species within samples (any species  
136 other than grass or clover) ranged from 0 - 380 g/kg DM with a mean of 50 g/kg DM.

137 *2.2.2. Sample processing.* Samples sourced were either unchopped bales or chopped  
138 clamped material. If in the form of an unchopped bale, it was mixed and chopped in a  
139 feeder wagon (Hi-Spec Mix Max, Hi-Spec Engineering, Co. Carlow, Ireland) for 45  
140 minutes to minimize variability in chop length. Clamp silages that were already



141 chopped, were mixed in a DataRanger diet mixer which did not contain knives  
142 (American Calan, Northwood, NH, USA). The DM content of the silage was estimated  
143 from the loss in weight of a subsample after it has been repeatedly placed in a  
144 microwave oven (Belling 384TC, 850 Watts) until a constant weight was achieved.  
145 From this determination, the amount of silage (fresh weight) required for feeding an  
146 individual sheep for 63 days was calculated. This amount was then weighed into  
147 polythene bags with one days' feed per bag, the air was removed under vacuum, and  
148 the bags were sealed and stored frozen (-20°C) until required. Frozen subsamples of  
149 each silage were stored separately for future analysis of chemical and botanical  
150 composition.

151

## 152 *2.3 Nutritional analysis*

153 *2.3.1 NIRS analysis* A 2 kg frozen subsample of each silage was sent to the Agri-Food  
154 and Biosciences Institute (**AFBI**; Hillsborough, Northern Ireland) where the reference  
155 chemical composition of the silages was determined using UKAS accredited methods  
156 and NIRS spectra were obtained. Before scanning, all samples were further chopped  
157 by hand to approximately 2.5 cm lengths and then thoroughly mixed. Two separate  
158 packages were prepared by wrapping approximately 100 g of fresh sample in non-  
159 PVC cling film (Park *et al.*, 1999). These packages were then placed in a rectangular  
160 coarse transport cell and scanned through a Foss NIRSystems 6500 instrument (Foss,  
161 Hillerød, Denmark). The optical values for each scan were recorded as Log  
162 1/Reflectance over the range 400-2498 nm at 2 nm gaps using the ISI v3.10 (Infrasoft  
163 International, Port Matilda, PA, USA) software.

164 *2.3.2 Laboratory reference analyses* Dry matter was determined in a forced-air oven  
165 and corrected for the loss of VFAs, lactic acid (**LA**), alcohols and ammonia (Porter and

166 Murray, 2001) and reported as volatile-corrected oven dry matter (**VCODM**). Ash was  
167 measured through combustion in a muffle oven at 550°C for 18 h. Lactic acid and other  
168 volatile compound measurements (total volatile fatty acids (**TVFA**) were determined  
169 using gas chromatography following extraction of representative samples in distilled  
170 water (Erwin *et al.*, 1961; Givens *et al.*, 2009). Nitrogen (**N**) was measured using the  
171 macro Kjeldahl method 954.01 (AOAC, 2000) and Ammonia-N (**NH<sub>3</sub>-N**) was  
172 determined using a calibrated ammonia ion selective electrode, which required 30 g  
173 silage soaked in 150 ml of purified water for 18 h at 4°C. (McDonald *et al.*, 1981; Orion  
174 Research, 1990). Both ether extract (**EE**) and water soluble carbohydrate (**WSC**) were  
175 measured on dried and ground samples: EE according to AOAC method 920.29  
176 (AOAC, 1990), and WSC as described previously (Fuller, 1967). Dried and ground  
177 samples were subsequently passed on to Trouw Nutrition (Ashbourne, Derbyshire)  
178 who performed analyses for neutral detergent fibre (**aNDF**) and acid detergent fibre  
179 (**ADF**) both inclusive of residual ash using Fibrecap equipment (Foss, Hillerod,  
180 Denmark) (Robertson and Van Soest, 1981; Kitcherside *et al.*, 2000; Mertens *et al.*,  
181 2002). A further 200 g of silage was manually separated into clover, grass and other  
182 species to determine the CC of the silage. This procedure was predominantly  
183 performed by the same individual to minimise human error. Resulting fractions were  
184 then dried to determine species composition on a DM basis. *In vivo* reference methods  
185 were performed at CEDAR to determine silage digestibility and degradability.

186

#### 187 2.4. *In vivo* analyses.

188 2.4.1 *In vivo* Digestibility Eighteen Mule x Texel wether sheep originating from a local  
189 breeder were used to measure *in vivo* silage digestibility using a series of 3 x 3 Latin  
190 square design experiments so that the final digestibility values comprised the mean of

191 measurements from three different animals. Each sheep was fed a silage sample *ad*  
192 *libitum* (with 10% refusals) for 16 d adaption followed by a 5 d sampling period during  
193 which sheep were placed in a metabolism crate for faeces and urine collection as  
194 described previously (Givens *et al.*, 1989; Bratzler, 1951). All *in vivo* procedures were  
195 licensed and monitored by the UK government Home Office under the Animal  
196 (Scientific Procedures) Act 1986.

197         Sheep were enrolled on the study when they reached adult weight at > 30 kg.  
198 Their diet was supplemented with 20 g/d of a general purpose vitamin/mineral mixture  
199 for sheep (Countrywide, Evesham, Worcestershire, UK) and the weights of feed  
200 offered and refused was recorded each day during the collection period. A subsample  
201 of feed was taken and analysed for DM and ash to calculate OM content. Refused  
202 feed was also corrected for DM. Out of the 94 samples, 4 were excluded from *in vivo*  
203 analysis as there was insufficient material for the 9 week feeding schedule, but were  
204 still used for all other analyses. Complete collections of faeces were taken for each  
205 sheep. Each days' faecal material from the 5 d collection period was refrigerated at <  
206 4°C until bulked together on d 5, thoroughly mixed and three 200 g subsamples  
207 obtained. These subsamples were immediately placed in a forced air oven at 60°C for  
208 72 h to determine DM content. Dried samples were then bulked, ground and a further  
209 subsample was placed in a muffle oven for combustion at 500°C for 16 h for  
210 determination of OM content. Digestibility results have been presented as digestible  
211 organic matter in total dry matter (**DOMD**, g/kg DM).

212 *2.4.2 In situ degradability.* Degradability values were obtained using an *in situ* method  
213 with rumen cannulated Holstein-Friesian dairy cattle. These cattle were housed in a  
214 dedicated metabolism unit, fed a commercial grass-maize based total mixed ration  
215 (**TMR**) diet once daily and milked twice daily at 0600 h and 1600 h approximately.

216 Fresh samples of each silage were placed in porous (43  $\mu\text{m}$  pore size) bags that were  
217 sequentially incubated in the rumen for six time intervals (3, 6, 12, 24, 48, and 72 h)  
218 using a complete exchange method as described previously (Lovett *et al.* 2004).  
219 Replicates were obtained by repeating the procedure with three different animals. To  
220 quantify '0' hour washing loss, three further bags per silage were placed in a tub of  
221 cold tap water and swirled for 5 minutes. All bags were washed (Zanussi SupeLuxe,  
222 Electrolux plc, Luton, UK) on a 53 min cold wash cycle, dried (at 60°C), and weighed  
223 for the determination of DM degradability, then further analysed for N (as described  
224 previously). The solubility (S) of DM and N was determined by adding 1 g of DM to 30  
225 ml of water and stirring for 5 minutes every half hour for a period of 2 h, the insoluble  
226 material was then filtered (Whatman filter paper grade 4, Sigma-Aldrich, MO, USA)  
227 (Hvelplund and Weisbjerg, 2000). The filter paper and substrate was then dried and  
228 weighed to determine DM solubility by difference and residual N was measured as  
229 described previously.

230 The percentage of material degraded at each time-point was used to plot a  
231 degradation curve as described by Ørskov and McDonald (1979). Degradability  
232 fractions termed 'a', 'b' and 'c' were obtained from the intercept, asymptote and slope  
233 of the curve. Fraction 'a' contained material that is apparently degraded almost  
234 immediately upon ingestion and 'b' contained the remaining insoluble but degradable  
235 material with 'c' being the rate of degradation of 'b'. Two different approaches were  
236 used to calculate effective degradability (**ED**) based on the above fractions. To ensure  
237 the best comparison with predicted data, the ED of nitrogen (**EDN<sub>FIM</sub>**) and of dry matter  
238 (**EDDM<sub>FIM</sub>**) were calculated using the 'Feed into Milk' (**FIM**) rationing software  
239 equations (Equation 1). In this equation the outflow rate of small ( $k_{\text{liq}}$ ) and large ( $k_f$ )  
240 particles was standardised at 0.075 and 0.045 respectively to fairly compare against

241 predicted data.  $EDDM_{FIM}$  was converted to microbial dry matter ( $MDM_{FIM}$ , g/kg DM)  
242 using standard equations to convert EDDM into ATP supply as described previously  
243 (FiM consortium, 2004).

244

245 Equation 1. 
$$ED_{FIM} = (0.9s/(0.9+k_{liq}))+(b_D c/(c+k_{liq}))+bc/(c+k_f)$$

246 Where  $s$  is the soluble proportion,  $k_{liq}$  is the fractional outflow rate of the liquid pool (0.075),  $b_D$   
247 is the degradable small particle proportion,  $b$  is the degradable large particle proportion,  $c$  is  
248 the fractional degradation rate of  $b$ , and  $k_f$  is the fractional outflow rate of the large particle  
249 pool (0.045).  
250

251 A second, simpler, approach was also tested simultaneously to calculate the  
252 ED of N and DM using 0.08 as the standard outflow rate ( $k$ ) of all particles ( $EDN_{0.08}$ ,  
253 and  $EDDM_{0.08}$ ) (Equation 2; Ørskov and McDonald, 1979).

254

255 Equation 2. 
$$ED = a+bc/(c+k)$$

256 Where  $a$  is the rapidly degraded,  $b$  is the slowly, potentially degradable proportion,  $c$  is the  
257 fractional rate of degradation of  $b$ , and  $k$  is the fractional outflow rate of material (0.08  $h^{-1}$ ).  
258

## 259 2.5 Statistical analysis

260 2.5.1 Tests of relationships and trends within the measured dataset. Statistical  
261 analysis was conducted using Genstat 16<sup>th</sup> Edition (VSNI, Hemel Hempstead, UK).  
262 Composition of the silages was predicted from NIRS spectra using equations  
263 developed for the UK Forage Analysis Assurance (FAA) group ([www.faagroup.co.uk](http://www.faagroup.co.uk))  
264 initially using 136 grass silage calibration samples from the studies reported by Park  
265 *et al.* (1997, 1998) which were regularly updated with new spectra over time for most  
266 chemical component variables other than those requiring in vivo reference analyses.  
267 The measured dataset has been presented as maximum, minimum, mean and  
268 coefficient of variation (CV%) values for each measured variable. The effect of CC on  
269 each of the other variables was tested by grouping samples into minimal, low, medium

270 and high groups (which are equal quartiles of the dataset; representing samples within  
271 the ranges of < 70, 70 - 250, 250 - 500 and > 500 g/kg DM CC respectively) which  
272 were compared using analysis of variance (**ANOVA**). A post hoc Tukey test was  
273 performed to determine whether there were significant differences between the means  
274 of the 4 groups. The means of the observed and NIRS predicted datasets were  
275 compared using a student's t-test to determine significance. Crude protein (**CP**) was  
276 not directly measured or predicted but calculated using either measured or predicted  
277 N and VCODM values (6.25 x Total N on a DM basis). For all dry matter values  
278 throughout this study, VCODM has been used rather than DM, in accordance with the  
279 industry standard used by UK laboratories. For ash, EE, WSC, ADF, and aNDF  
280 (variables where the measured concentration is produced from a dry sample)  
281 equations were produced that predicted concentrations on both a fresh basis and  
282 directly on a DM basis.

283 *2.5.2 Tests of prediction accuracy during validation.* For the grass-based prediction  
284 equation results, the difference between laboratory assays and NIRS predicted values  
285 was calculated using measured minus predicted values and is henceforth termed  
286 'bias'. Relative root mean square standard error of prediction (**RMSEP** as a  
287 percentage of the measured mean), ratio of the standard error of prediction to the  
288 standard deviation of the measured dataset (**RPD**) as recommended by Williams  
289 (2014), and the R-squared value of the relationship between observed and predicted  
290 data ( $r^2$ ) were used to measure prediction accuracy.

291 *2.5.3 Calibration of new NIRS equations.* To create new grass-clover prediction  
292 equations, different data pre-treatment methods were first assessed by varying use of  
293 derivatives, gap, smoothing and scatter correction. All calibrations were performed  
294 using the WinISI III v1.50 (Infrasoft International, Port Matilda, PA, USA) software.

295 They were carried out as Modified Partial Least Squares regressions over the range  
296 1100-2498 nm using a 2 nm gap. To account for any sub-sampling error the root mean  
297 square difference of each sub-sample was calculated using the WinISI III v1.50  
298 software. An upper limit of 5000 was used to judge poor replication meaning any  
299 sample with a root mean square greater than 5000 would be removed. None of the  
300 samples in the calibration set were above this limit. Raw data and two derivatives  
301 were tested in the process (Raw (0,0,1,1), 1st Derivative (1,4,4,1) and 2nd Derivative  
302 (2,10,5,1)) and three scatter corrections (Standard Normal Variate Detrending  
303 (**SNVD**), Normal Multiplicative Scatter Correction (**NMSC**) and Weighted Multiplicative  
304 Scatter Correction (**WMSC**)) for each of the derivatives. The maximum number of  
305 terms set for each equation was 11. There were three elimination passes carried out  
306 and the cross validation value was set at 6 in which the calibration set was divided into  
307 six groups with one group removed sequentially and predicted using a calibration  
308 formed using the remaining samples. The validation errors were combined to give a  
309 standard error of cross validation (**SECV**). The optimal equations were those with the  
310 lowest SECV. The combination of data pre-treatment giving the optimal prediction  
311 model is shown in supplementary table 1 for each variable. The optimal equation was  
312 compared against the industry standard method, based on the study of Park *et al.*  
313 (1997), which was taking the first derivative (1,4,4,1) with SNVD scatter correction and  
314 a repeatability file (a file containing multiple spectra from the same sample measured  
315 under different conditions, designed to reduce the variability caused by differing  
316 environmental conditions and instruments). Differences between the optimal  
317 equations and the industry standard equations were small, therefore further validation  
318 was performed using the industry standard equations as these were the most likely to  
319 be utilised commercially. For the purposes of a validation test, 10 samples were

320 removed from the dataset and tested using the remaining equation. These samples  
321 were chosen by including the very first sample to be collected and then every tenth  
322 sample in order of their arrival at CEDAR for processing.

323

### 324 **3. Results**

#### 325 *3.1 Sample chemical composition*

326 The silages contained a wide range of chemical composition with LA, WSC and TVFA  
327 being the nutritional characteristics with the greatest variance of those measured.  
328 Volatile corrected dry matter of the silages was evenly distributed with a mean of 395  
329 g/kg. Measured CP concentration (calculated from N and VCODM) ranged from 57 to  
330 215 g/kg DM and with a mean of 138 g/kg DM.

331 With the exception of ash, aNDF, and WSC, the concentration of all other  
332 measured variables were affected by the CC of the sample when grouped into  
333 minimal, low, medium, and high clover groups (Table 2). VCODM and N were  
334 significantly increased in the high clover group (>500 g/kg DM CC) relative to the other  
335 three groups (both  $P < 0.001$ ), as was CP with the exception of the medium group  
336 which contained an intermediary CP concentration ( $P < 0.001$ ). Degradability  
337 parameters calculated using the Ørskov and McDonald (1979) model and DOMD were  
338 lowest in the high clover group (all  $P < 0.04$ ) and numerically highest in the low clover  
339 group (60-240 g/kg DM CC), however, when degradability parameters were calculated  
340 using FiM equations, differences between clover groups were non-significant.  
341 Fermentation end products (LA, TFVA and TVC) decreased in concentration  
342 sequentially as CC increased (all  $P < 0.003$ ) while pH was similar for minimal, low and  
343 medium groups and higher for the high clover group ( $P < 0.001$ ). NH<sub>3</sub>-N was also



344 highest in the high clover group in comparison to the minimal clover group while the  
345 other two groups contained intermediate concentrations of NH<sub>3</sub>-N ( $P < 0.02$ ).

346

### 347 *3.2 Validation of current grass-based NIRS equations*

348 Using data from the present study grass-clover sample set to verify the accuracy of  
349 the current grass-based prediction equations, a wide range of prediction accuracy was  
350 observed depending on the chemical component tested (Table 3). Volatile corrected  
351 dry matter and N showed good prediction accuracy with RPD values of 4.92 and 2.35  
352 respectively, and no significant difference between observed and predicted means.  
353 Furthermore, the relationship between the observed and predicted data for both these  
354 variables closely followed a line of parity (Figure 1) especially at low concentrations.  
355 However, all other variables led to RPD values that were  $<2$  denoting inadequate  
356 performance. Digestible organic matter in total dry matter, and aNDF, had low relative  
357 RMSEP (both  $<10\%$  of the observed mean) and no significant difference between the  
358 observed and predicted means which could be considered acceptable despite having  
359 an RPD value  $<2$ . For these variables the slope of the relationship between observed  
360 and predicted data followed a line of parity however there was greater variability in the  
361 relationship than was seen for VCODM and N (Figure 1). Crude protein prediction  
362 showed a relatively high RPD value (1.58) and good correlation between predicted  
363 and observed data ( $r^2=0.75$ ) however the slope of the relationship did not follow a line  
364 of parity (Figure 1) leading to a significant bias ( $P < 0.005$ ) for under-estimation at  
365 higher concentrations with the average under-estimation being 12.4 g/kg DM.

366 Fermentation characteristics (LA, pH, TVC and TVFA) all showed intermediate  
367 prediction accuracy with RPD values ranging from 1.15 to 1.22. Of these variables, LA  
368 in particular had a very high relative RMSEP at 71% of the observed mean as a result

369 of high variability in prediction accuracy where concentration was low (Figure 1). For  
370 both TVC and TVFA there was a significant bias towards over-estimation (both  $P <$   
371 0.001). Poor prediction accuracy (RPD value  $<1$ ) was observed for  $\text{NH}_3\text{-N}$ , ADF, EE,  
372  $\text{EDN}_{\text{FIM}}$ , and  $\text{MDM}_{\text{FIM}}$  all of which showed a significant bias between the predicted and  
373 observed means (all  $P < 0.001$ ). Of special note,  $\text{EDN}_{\text{FIM}}$  and  $\text{MDM}_{\text{FIM}}$  showed the least  
374 prediction accuracy of all the variables tested with a significant over-estimation for  
375  $\text{EDN}_{\text{FIM}}$  of 139 g/kg N and an under-estimation for  $\text{MDM}_{\text{FIM}}$  of 17 g/kg DM. Moreover,  
376 predicted and observed data showed little correlation (Figure 1) indicated by  $r^2$  values  
377 of 0.01.

378         The degree of variation and the magnitude of bias in relation to sample CC is  
379 illustrated in Figure 2 using CP and  $\text{EDN}_{\text{FIM}}$  as examples which are crucial to diet  
380 formulation. In the case of CP, prediction bias in samples containing 800-1000 g/kg  
381 DM CC is greater than 30 g/kg DM (Figure 2a), and similarly for  $\text{EDN}_{\text{FIM}}$ , a prediction  
382 bias greater than 200 g/kg N was observed in this very high CC range. Meanwhile,  
383 bias was comparatively lower in the minimal clover group ( $< 70$  g/kg DM CC) at 6 g/kg  
384 DM for CP and 103 g/kg N for  $\text{EDN}_{\text{FIM}}$  reflecting the degree of bias that might be  
385 expected for a pure grass sample.

386

### 387 *3.3 Validation of new grass-clover equations*

388 Following production of new equations using the NIRS spectra from the grass-clover  
389 silages in the sample set, a cross validation test indicated 12 out of 21 new equations  
390 had a relative SECV of 10% of the observed mean, suggesting a good calibration was  
391 achieved for these variables (Table 4).  $\text{VCODM}$ , pH, aNDF, ADF, and  $\text{EDDM}_{0.08}$  were  
392 amongst the strongest calibrations according to cross validation while TVC, WSC,  
393 TVFA, Alcohol and LA were the least robust. For variables where both a fresh and a

394 DM basis equation were produced, the equation that predicted on a fresh basis gave  
395 the more accurate result for ash, EE and WSC, whereas the opposite was true for  
396 ADF and aNDF, where the equation that predicted concentration on a DM basis was  
397 more accurate.

398 A validation test was also applied to the new grass-clover prediction equations  
399 through removal of 10 samples from the calibration data-set (Table 5). Seven variables  
400 gave an RPD value > 2 denoting good accuracy including VCODM, ADF, aNDF, EDN  
401 and N. Additionally the RPD score of all values were improved relative to prediction  
402 accuracy using the grass-based equations, which was reflected in greatly reduced  
403 bias, for example, new equations reduced crude protein mean bias from -12.4 to -0.82  
404 g/kg DM and EDN mean bias improved from 139 to 12 g/kg N on average. The new  
405 alcohol and EE (DM basis) equations gave a low RPD value (>1) suggesting these  
406 equations are unlikely to be suitable for use without further improvement.

407

## 408 **4. Discussion**

### 409 *4.1 Chemical composition and clover concentration*

410 The wide range of samples collected in this study provided a robust test for the current  
411 grass-based prediction equations. The sample set was dominated by samples  
412 containing predominantly grass with only a quarter of the samples obtained containing  
413 > 500 g/kg DM CC. Roughly half the total number of samples obtained were below the  
414 minimum optimum clover inclusion rate of 300 g/kg DM suggested by Nyfeler *et al.*  
415 (2011). This may be due to the sample set comprising a greater number of first cut  
416 silage samples than second, third or fourth cuts in which CC would have been greater  
417 due to warmer and drier conditions in the latter half of the year (Chmelikova *et al.*,  
418 2015).

419 Crude protein concentration (ranging from 57 to 215 g/kg DM with a mean  
420 concentration of 138g/kg DM) indicated that, although some of the samples contained  
421 very high levels of crude protein, mean concentration was similar to that expected for  
422 well fertilised modern grass silages which have been observed ranging from 120-270  
423 g/kg DM (Burns *et al.*, 2015). This mean is also significantly lower than those reported  
424 in published feed composition tables for crude protein concentration of grass-clover  
425 silages e.g. 173 g/kg DM; AFRC (1993).

426 The supply of effective degradable N (EDN) is another important factor in diet  
427 formulation. High concentrations (>700 g/kg N) of rapidly degraded protein in the  
428 rumen can be wasteful as there is insufficient time for bacterial N capture, which is  
429 often a characteristic of legume silages (Coblentz and Grabber, 2013; Dewhurst,  
430 2013). In this study, average EDN<sub>FIM</sub> was 623 g/kg N so within the optimal range and  
431 lower than values cited in other studies, for example, in another study, measured  
432 average EDN of grass-clover silages was 880 g/kg N at an assumed passage rate of  
433 0.05/hr (Hvelplund and Weisbjerg, 2000). The discrepancy may be due to clover  
434 varieties in this sample set being predominantly comprised of red clovers containing  
435 the enzyme poly-phenol oxidase which is thought to reduce proteolysis in the rumen  
436 (Lee *et al.*, 2009). Digestibility, EDDM<sub>0.08</sub>, and EDN<sub>0.08</sub> all showed a similar pattern  
437 where the low group (70 – 250 g/kg DM CC) gave the highest value and the high group  
438 (> 500 g/kg DM CC) the lowest suggesting inclusion of clover between 70 - 250 g/kg  
439 DM is an optimal range for digestibility and degradability. Poor digestibility in the high  
440 group may relate to an increased maturity of clover and grass with higher lignification  
441 in samples in that range (Nousiainen *et al.*, 2009). Increasing ratios of ADF:aNDF in  
442 samples with a higher CC indicates the differing fractions of fibre present in legumes  
443 in comparison to grasses, especially red clover which is largely comprised of stem

444 where ADF concentration is higher than in leaves (Alstrup *et al.*, 2016). There was a  
445 notable decrease in volatiles content (LA, TVC and TVFA) and an increase in pH in  
446 the high group relative to the other quartiles where values were generally similar. This  
447 suggests samples with a very high CC were more difficult to ensile, perhaps due to  
448 reduced availability of sugar to fuel bacterial activity that may also indicate an  
449 increased maturity. Using a factor of 0.0157 of DOMD, mean ME within the sample  
450 set was predicted as 9.9 MJ/kg DM which is considerably lower than recently  
451 measured values for modern monoculture grass silages which ranged from 12-13  
452 MJ/kg DM (Burns *et al.*, 2015).

453

#### 454 *4.2 Using grass-based NIRS equations for clover containing samples*

455 The key objective of this project was to determine whether the current grass NIRS  
456 equations could be applied to grass-clover samples and predict the concentrations of  
457 chemical components with good accuracy. Variates that were considered most  
458 important for correct diet formation included CP, EDN, MDM and DOMD as these are  
459 the variables involved in balancing rumen degradable protein and energy supply.  
460 Whilst the prediction accuracy of DOMD and some other variables (including VCODM,  
461 N and aNDF) could be considered suitably accurate with relative RMSEPs of <10 %  
462 of the measured mean, CP, MDM, and EDN were amongst the variables with high  
463 relative RMSEPs and RPD values of less than 2.0 combined with a significant bias.  
464 Similar results were seen in a smaller preliminary study of 58 grass-clover silages in  
465 which the same equations were tested and crude protein was significantly under-  
466 estimated by 22 g/kg DM on average (Davies *et al.*, 2012). The consequence of this  
467 bias would be an imbalance in microbial N supply in the ration which is likely to lead  
468 to reduced N use efficiency in cattle resulting in higher levels of N excretion in urine

469 and faeces contributing to environmental loading (Kebreab *et al.*, 2002). Under-  
470 estimation of CP in silage samples could result in farmers under-valuing grass-clover  
471 silages as protein sources, and compensating through an oversupply of expensive  
472 bought-in protein within the concentrate portion of the diet. For CP and EDN,  
473 increasing bias correlated with increasing CC. This might be explained by samples  
474 containing a high concentration of grass being more similar in composition to the  
475 calibration samples used to create the current grass-based equations. Also, bias may  
476 be created due to different N fractionation within clover, with some fractions present  
477 that are absent (or present in different concentrations) in grass, such as the  
478 concentration of non-protein N (Chrenkova *et al.*, 2014).

479         When considering the impact of the observed inaccuracies on diet formulation  
480 it is estimated metabolisable protein, and not crude protein that is often the protein  
481 fraction used for diet formulation. Crude protein multiplied by EDN (as a proportion of  
482 total N) is used to calculate effective rumen degradable protein (**eRDP**) which is one  
483 of the factors that determines metabolisable protein (alongside digestible undegraded  
484 true protein, **DUP**) in diet formulation software. The effect of CC on calculated eRDP  
485 bias is shown in Figure 2c. The opposing bias in EDN and CP cancel out to some  
486 extent at low CC however the overall effect is an over-estimation of eRDP that  
487 increases at higher CC. This may lead to an oversupply of fermentable energy in  
488 relation to available protein for microbial N capture, creating an imbalance that could  
489 reduce the efficiency of dietary nutrient utilisation. This would only be further  
490 compounded by the inaccuracy seen in MDM prediction which is used to determine  
491 the requirement for fermentable energy.

492

493 *4.3 Performance of new grass-clover-based equations*

494 Comparing the performance of new grass-clover equations with the grass-based  
495 equations for use on clover-containing samples, and using relative SECV as a  
496 measure of potential performance of the calibration, some of the new grass-clover  
497 equations produced in this study are likely to perform well (including important variates  
498 such as VCODM, N, EDN, and DOMD) whereas others have very high errors  
499 (particularly the volatile compounds) and would require further development. The  
500 accuracy of prediction for volatile compounds (LA, TVC, and TVFA) is notable in all  
501 equations tested (both grass and grass-clover based) for producing poor reliability,  
502 and volatile concentrations showed some of the greatest variation within the measured  
503 sample set. Some of the lack of reliability in these equations could be due to the  
504 variability of scanning undried and unmilled material rather than presenting the sample  
505 in an homogenous, dried form (Sorensen, 2004).

506 For variates where the measured value was calculated on a dry sample (ash,  
507 EE, aNDF, ADF and WSC) equations have been calibrated to give both a fresh and a  
508 DM basis value. In most instances, the calibration for the fresh value was more robust,  
509 however, because presenting information on a DM basis is widely practiced, fresh  
510 values would be transformed based on VCODM values which would introduce further  
511 error. Overall however, in a small validation test, new equations were better able to  
512 predict all variables when compared to the accuracy of grass-based prediction  
513 equations. The prediction of  $EDN_{0.08}$  and  $EDDM_{0.08}$  showed marked improvement over  
514 the previous prediction accuracy for  $EDN_{FIM}$  and  $MDM_{FIM}$  using the grass-based  
515 equations perhaps due to the reduced complexity of calculating these variables from  
516 measured data. For example, calculating reference values for  $MDM_{FIM}$  from measured  
517 degradability at different timepoints *in vivo* is a multi-step process involving many  
518 different variables (such as corrections for solubility, fatty acid content and crude

519 protein concentration) and therefore it may not be feasible to predict such a value  
520 based on NIRS spectra alone. These improvements would have a significant impact  
521 on the accuracy of rumen degradable protein and fermentable energy prediction.

522

#### 523 *4.4 Implementation of new equations*

524 The implementation of new grass-clover equations requires that nutritionists, feed  
525 company representatives, and farmers are widely aware of the new option, and that  
526 samples are correctly identified as containing a viable quantity of clover. Additionally,  
527 grass-clover mixtures are just one example of alternative forages that are currently  
528 gaining popularity, and it is unlikely that new equations can be created and  
529 implemented for all of them due to the time needed to collect a sufficiently large group  
530 of calibration samples. Therefore, it would be more convenient if one equation (such  
531 as the current grass-based equation or alternatively a separate general 'legume'  
532 equation) could be adapted to analyse many different grass and legume based  
533 forages. Another solution would be to use a two step process in which the CC of the  
534 sample is predicted using NIRS, and then used to apply a correction to nutritional  
535 predictions. A number of previous studies have used NIRS to determine the botanical  
536 composition of a mixed sample (containing two species) with success for both grass-  
537 clover (Cougnon *et al.*, 2014) and lucerne-grass silages (Karayilanli *et al.*, 2016)  
538 however in all instances the calibration was performed on dry samples and therefore  
539 further work is required to create an analysis for fresh samples that would be  
540 appropriate for use in the UK.

541

## 542 **5. Conclusions**



543 For some variables, notably VCODM, N, DOMD and aNDF, current UK grass-based  
544 equations were able to be applied to clover-containing samples with adequate  
545 accuracy. However, in general, it was concluded from the evidence observed in this  
546 study that the NIRS calibration equations developed for use on grass silages, could  
547 not predict a number of key chemical components (including CP and EDN) with  
548 sufficient accuracy, when used for grass-clover mixture silages. This was consistent  
549 with the findings of a previous study (Davies *et al.*, 2012). Therefore, we suggest two  
550 possible solutions that would be appropriate for uptake by UK laboratories: (i) the  
551 introduction of new grass-clover prediction equations calibrated using the sample set  
552 obtained for this study or (ii) the use of a correction factor that could be applied based  
553 on the CC of the sample. Furthermore, in a wider sense, this study provides some  
554 evidence that caution should be used whenever NIRS equations are applied to forage  
555 mixtures where only one component of the mixture was represented within the  
556 equation calibration set. Where possible, using an equation based on a specific  
557 calibration set that is very similar to the material requiring analysis is likely to produce  
558 the most accurate predictions.

559

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569

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715  
716

717 **Table 1** The means, ranges and variation coefficients (CV) of chemical components  
 718 measured in a set of 94 diverse grass-clover silages from UK farms (in g/kg DM  
 719 unless otherwise stated).

Item	Min	Max	Mean	CV, %
ADF	229	513	335	10.2
Ash	58	158	97	20.6
aNDF	299	585	447	10.0
CC	4	1000	310	91.3
CP	57	215	138	24.7
Degradability				
EDDM <sub>0.08</sub> †	217	626	472	16.4
EDN <sub>0.08</sub> , g/kg N†	55	821	625	18.0
MDM <sub>FIM</sub> ‡	60	274	146	34.8
EDN <sub>FIM</sub> , g/kg N‡	297	811	623	14.3
DOMD	400	766	632	10.6
EE	14.6	42.9	26.6	26.0
LA, g/kg	0.0	64.4	13.4	91.5
pH	3.6	6.7	4.6	13.5
N, g/kg	3.6	17.7	8.8	42.2
NH <sub>3</sub> -N, g/kg DM*100	17.5	203	62.5	42.2
TVC, g/kg§	2.3	76.1	23.6	57.8
TVFA, g/kg¶	1.1	74.3	19.7	66.0
VCODM, g/kg	182	793	395	33.4
WSC	3.9	164	41.4	86.3

720 CC = clover concentration; EDDM = effective degradability of dry matter; EDN = effective degradable  
 721 nitrogen; DOMD = digestible organic matter in total dry matter; EE = ether extract; LA = lactic acid;  
 722 MDM = microbial dry matter yield; NH<sub>3</sub>-N = ammonia nitrogen; TVC = total volatile content, TVFA =  
 723 total volatile fatty acids; WSC = water soluble carbohydrates.

724 † Degradability parameters determined by *in situ* incubation in the rumen, using the model of Ørskov  
 725 and McDonald (1979)  $ED = a + b[c/(c+k)]$  where a = rapidly soluble material; b = non-soluble but  
 726 degradable material; c = rate of degradation of b; and k = an assumed outflow rate of 0.08/hr.

727 ‡ Degradability parameters determined by *in situ* incubation in the rumen, using the Feed Into Milk  
 728 (FIM) Consortium (2004) model  $ED_{FIM} = (0.9s/(0.9+k_{liq})) + (b_D c/(c+k_{liq})) + (bc/(c+k_r))$  where s = soluble  
 729 proportion,  $k_{liq}$  = fractional outflow rate of the liquid pool (0.075/hr),  $b_D$  = degradable small particle  
 730 proportion, b = degradable large particle proportion, c = fractional degradation rate of b, and  $k_r$  is the  
 731 fractional outflow rate of the large particle pool (0.045/hr).

732 § TVC is the sum of acetic, butyric, lactic, propionic and valeric acids plus ethanol and propanol.

733 ¶ TVFA is calculated as for TVC but excluding ethanol and propanol.

734



735 **Table 2** Differences in chemical components in 94 grass-clover silages grouped into  
 736 four quartiles (Minimal (Mi), Low (L), Medium (M) and High (H)) according to their  
 737 clover concentration (mean of each quartile, in g/kg DM unless otherwise stated).

Item	Clover concentration quartiles†				SED	P value‡
	Mi	L	M	H		
CC	34 <sup>a</sup>	145 <sup>b</sup>	335 <sup>c</sup>	743 <sup>d</sup>	28.9	0.001
<i>n</i>	23	24	24	23		
<i>Chemical components</i>						
ADF	311 <sup>a</sup>	329 <sup>ab</sup>	345 <sup>b</sup>	356 <sup>b</sup>	12.5	0.003
Ash	91.2	94.9	103.4	96.8	5.84	0.201
aNDF	465	452	443	432	16.5	0.229
CP	122 <sup>a</sup>	130 <sup>a</sup>	143 <sup>ab</sup>	158 <sup>b</sup>	9.3	0.001
<i>Degradability</i>						
EDDM <sub>0.08</sub> §	470 <sup>ab</sup>	501 <sup>b</sup>	478 <sup>ab</sup>	436 <sup>a</sup>	21.7	0.032
EDN <sub>0.08</sub> , g/kg N§	643 <sup>b</sup>	682 <sup>b</sup>	640 <sup>b</sup>	531 <sup>a</sup>	28.6	0.001
MDM <sub>FIM</sub> ¶	130	135	127	122	6.3	0.218
EDN <sub>FIM</sub> , g/kg N¶	627	645	629	589	25.9	0.182
DOMD	647 <sup>b</sup>	668 <sup>b</sup>	631 <sup>b</sup>	581 <sup>a</sup>	18.5	0.001
EE	26.7 <sup>ab</sup>	28.7 <sup>b</sup>	27.4 <sup>ab</sup>	23.2 <sup>a</sup>	1.99	0.044
LA, g/kg	17.6 <sup>b</sup>	16.2 <sup>b</sup>	14.0 <sup>ab</sup>	5.6 <sup>a</sup>	3.42	0.003
pH	4.45 <sup>a</sup>	4.41 <sup>a</sup>	4.44 <sup>a</sup>	5.23 <sup>b</sup>	0.155	0.001
N, g/kg	7.8 <sup>a</sup>	7.3 <sup>a</sup>	7.9 <sup>a</sup>	12.3 <sup>b</sup>	0.93	0.001
NH <sub>3</sub> -N, g/kg DM*100	48.3 <sup>a</sup>	56.3 <sup>ab</sup>	68.0 <sup>ab</sup>	77.5 <sup>b</sup>	9.71	0.018
TVC, g/kg	28.4 <sup>b</sup>	26.8 <sup>b</sup>	24.0 <sup>ab</sup>	14.3 <sup>a</sup>	3.74	0.001
TVFA, g/kg ¥	23.3 <sup>b</sup>	22.4 <sup>b</sup>	20.8 <sup>ab</sup>	11.6 <sup>a</sup>	3.65	0.001
VCODM, g/kg	397 <sup>a</sup>	350 <sup>a</sup>	347 <sup>a</sup>	498 <sup>b</sup>	35.1	0.001
WSC	56.6	39.6	32.9	38.6	10.37	0.125

738 CC = clover concentration; EDDM = effective degradability of dry matter; EDN = effective degradable  
 739 nitrogen; DOMD = digestible organic matter in total dry matter; EE = ether extract; LA = lactic acid;  
 740 MDM = microbial dry matter yield; NH<sub>3</sub>-N = ammonia nitrogen; SED = standard error of the difference  
 741 between means; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble  
 742 carbohydrates.

743 † The 94 samples were sorted by ascending clover concentration and divided into four evenly sized  
 744 quartiles: 0-6%DM clover (Mi); 6-24% clover (L); 25-49% clover (M); and 50-100% clover (H).

745 ‡ The probability of there being no significant difference between treatment means determined using  
 746 Analysis of Variance (ANOVA).

747 § Degradability parameters determined by *in situ* incubation in the rumen, using the model of Ørskov  
 748 and McDonald (1979)  $ED = a + b[c/(c+k)]$  where a = rapidly soluble material; b = non-soluble but  
 749 degradable material; c = rate of degradation of b; and k = an assumed outflow rate of 0.08/hr.

750 ¶ Degradability parameters determined by *in situ* incubation in the rumen, using the Feed Into Milk  
 751 (FIM) Consortium (2004) model  $ED_{FIM} = (0.9s/(0.9+k_{liq})) + (b_D c/(c+k_{liq})) + (bc/(c+k_f))$  where s = soluble  
 752 proportion,  $k_{liq}$  = fractional outflow rate of the liquid pool (0.075/hr),  $b_D$  = degradable small particle  
 753 proportion, b = degradable large particle proportion, c = fractional degradation rate of b, and  $k_f$  is the  
 754 fractional outflow rate of the large particle pool (0.045/hr).

755 || TVC is the sum of acetic, butyric, lactic, propionic and valeric acids plus ethanol and propanol.

756 ¥ TVFA is calculated as for TVC but excluding ethanol and propanol.

757 <sup>a,b</sup> Values within a row with different superscripts differ significantly at  $P < 0.05$ .

758

759 **Table 3** The results of a validation in which 94 grass-clover silages were used to test  
 760 the prediction accuracy of grass-based NIRS equations for chemical composition  
 761 when used on clover-containing samples (in g/kg DM unless otherwise stated).

Item	Measured mean	Predicted mean	Bias†	P value‡	r <sup>2</sup> §	Relative RMSEP, %¶	RPD
ADF	336	292	43.0	0.001	0.61	17.6	0.87
aNDF	448	438	9.65	0.209	0.56	8.9	1.45
Ash	96.6	91.6	5.0	0.033	0.52	16.5	1.32
CP	138	126	12.4	0.005	0.75	17.1	1.58
DOMD	632	645	-13.0	0.195	0.64	6.7	1.56
EDN <sub>FIM</sub> , g/kg N	623	762	139	0.001	0.01	24.5	0.48
EE	26.5	30.1	-3.6	0.001	0.25	25.9	0.89
LA, g/kg	13.4	14.3	-0.9	0.622	0.48	70.6	1.22
MDM <sub>FIM</sub>	129	146	-17	0.003	0.01	38.1	0.39
N, g/kg	8.8	8.1	0.7	0.187	0.86	19.4	2.35
NH <sub>3</sub> -N, g/kgDM*100	62.5	85.2	-22.6	0.001	0.34	45.0	0.89
pH	4.6	4.8	-0.1	0.122	0.48	10.8	1.21
TVC, g/kg ¥	23.4	30.2	-6.8	0.001	0.52	39.3	1.15
TVFA, g/kg #	19.6	25.6	-5.9	0.001	0.51	43.5	1.17
VCODM, g/kg	397	409	-12.0	0.558	0.98	6.6	4.92
WSC	41.8	48.8	-7.0	0.113	0.40	58.4	1.25

762 DOMD = digestible organic matter in total dry matter; EDN = effective degradable nitrogen; EE = ether  
 763 extract; LA = lactic acid; MDM = microbial dry matter yield; NH<sub>3</sub>-N = ammonia nitrogen; RMSEP = root  
 764 mean standard error of prediction; RPD = ratio of standard deviation of the measured population to  
 765 the standard error of prediction; TVC = total volatile content, TVFA = total volatile fatty acids; WSC =  
 766 water soluble carbohydrates.

767 † Bias is the measured mean minus the predicted mean, therefore minus values indicate over-  
 768 estimation and positive values indicate under-estimation of the equation.

769 ‡ The probability of there being no significant difference between the measured mean and the  
 770 predicted mean analysed using student's t-test.

771 § Simple linear regression coefficient

772 ¶ Root mean square error of prediction presented as a percentage of the measured mean for  
 773 standardisation

774 || Degradability parameters determined by *in situ* incubation in the rumen, using the Feed Into Milk  
 775 (FIM) Consortium (2004) model  $ED_{FIM} = (0.9s/(0.9+k_{liq})) + (b_D c/(c+k_{liq})) + (bc/(c+k_f))$  where s = soluble  
 776 proportion,  $k_{liq}$  = fractional outflow rate of the liquid pool (0.075/hr),  $b_D$  = degradable small particle  
 777 proportion, b = degradable large particle proportion, c = fractional degradation rate of b, and  $k_f$  is the  
 778 fractional outflow rate of the large particle pool (0.045/hr).

779 ¥ TVFA is calculated as for TVC but excluding ethanol and propanol.

780 # TVC is the sum of acetic, butyric, lactic, propionic and valeric acids plus ethanol and propanol.

781

782

783 **Table 4** Indicators of calibration strength and prediction accuracy using cross-  
 784 validation for a range of optimised new NIRS equations calibrated on spectra from  
 785 95 diverse grass-clover silages.

Item†	n‡	SEC	r <sup>2</sup> §	Relative SECV, % ¶
ADF (DM)	183	13.4	0.90	4.49
ADF (Fresh)	181	6.22	0.98	5.71
Alcohol	178	1.08	0.83	37.1
aNDF (DM)	183	18.5	0.89	4.80
aNDF (Fresh)	182	7.79	0.98	5.26
Ash (DM)	185	10.4	0.70	12.5
Ash (Fresh)	179	3.30	0.91	11.1
DOMD	172	3.10	0.83	5.47
EDDM <sub>0.08</sub> ¥	174	2.15	0.88	5.28
EDN <sub>0.08</sub> ¥	174	3.93	0.79	7.03
EE (DM)	180	2.67	0.83	11.2
EE (Fresh)	179	0.94	0.90	10.8
LA	173	4.76	0.81	41.5
N	180	0.65	0.97	8.33
NH <sub>3</sub> -N	176	0.01	0.88	18.8
pH	180	0.16	0.93	4.18
TVC #	185	5.39	0.82	27.9
TVFA ††	183	5.17	0.81	31.8
VCODM	181	7.17	1.00	2.10
WSC (DM)	180	10.1	0.92	31.4
WSC (Fresh)	181	4.62	0.93	29.6

786 EDDM = effective degradability of dry matter; EDN = effective degradable nitrogen; DOMD =  
 787 digestible organic matter in total dry matter; EE = ether extract; LA = lactic acid; NH<sub>3</sub>-N = ammonia  
 788 nitrogen; SEC = standard error of calibration; SECV = standard error of cross-validation; TVC = total  
 789 volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

790 † For variables that are measured on a dry sample (Ash, ADF, aNDF and WSC) two equations were  
 791 produced, one predicting on a fresh basis and one on a DM basis.

792 ‡ The number of spectra that were included in the prediction equation.

793 § Simple linear regression coefficient

794 ¶ Standard error of cross validation presented as a percentage of the measured mean for  
 795 standardisation

796 || Alcohol is the sum of ethanol and propanol

797 ¥ Degradability parameters determined by *in situ* incubation in the rumen, using the model of Ørskov  
 798 and McDonald (1979) where a = rapidly soluble material; b = non-soluble but degradable material; c =  
 799 rate of degradation of b; effective degradability =  $a+b[c/(c+k)]$  where k = an assumed outflow rate of  
 800 0.08/hr.

801 # TVC is the sum of acetic, butyric, lactic, propionic and valeric acids plus ethanol and propanol.

802 †† TVFA is calculated as for TVC but excluding ethanol and propanol.

803

804 **Table 5** The results of a validation in which 10 grass-clover silages were used to test  
 805 the prediction accuracy of new clover/grass-based NIRS equations generated from  
 806 the spectra of 85 other grass-clover silages (in g/kg DM unless otherwise stated).  
 807 Industry standardised data pre-treatment methods were used (1st derivative and  
 808 SNVD scatter correction) in the calibration of these equations.

Item	Measured mean	Predicted mean	Bias†	$r^2$ ‡	Relative RMSEP, % §	RPD
ADF	343	352	-9.28	0.93	7.31	2.94
ADF, g/kg	162	159	1.93	0.99	6.17	8.66
Alcohol, g/kg ¶	3.5	4.9	-1.41	0.19	75.7	0.93
aNDF	459	479	-20.6	0.85	8.02	2.15
aNDF, g/kg	212	215	-2.54	0.98	5.81	7.87
Ash	88.5	87.0	1.49	0.26	16.5	1.19
Ash, g/kg	39.2	40.6	-1.38	0.73	18.8	1.82
CP	120	120	-0.82	0.74	12.3	1.92
DOMD	637	637	-0.2	0.71	9.18	1.76
EDDM <sub>0.08</sub>	452	438	14.7	0.68	16.3	1.58
EDN <sub>0.08</sub> , g/kg N	600	588	11.9	0.92	6.74	3.43
EE	22.8	21.6	1.22	0.46	23.2	0.95
EE, g/kg	9.9	9.8	0.15	0.67	16.9	1.82
LA, g/kg	15.1	12.7	2.41	0.72	49.0	1.74
N, g/kg	8.9	9.0	-0.02	0.92	13.4	3.76
NH <sub>3</sub> -N, g/kg DM*100	104	112	-8.01	0.64	25.0	1.64
pH	4.7	4.6	0.06	0.70	10.8	1.86
TVC, g/kg ¥	22.9	21.7	1.20	0.76	33.5	1.85
TVFA, g/kg #	19.5	18.4	1.03	0.73	37.0	1.86
VCODM, g/kg	451	448	2.87	0.99	2.46	14.2
WSC	51.7	58.7	-7.01	0.69	35.3	1.76
WSC, g/kg	22.4	27.2	-4.84	0.69	44.4	1.57

809 DOMD = digestible organic matter in total dry matter; EDN = effective degradable nitrogen; EE = ether  
 810 extract; LA = lactic acid; NH<sub>3</sub>-N = ammonia nitrogen; RMSEP = root mean standard error of  
 811 prediction; RPD = ratio of standard deviation of the measured population to the standard error of  
 812 prediction; SNVD, standard normal variate de-trending; TVC = total volatile content, TVFA = total  
 813 volatile fatty acids; WSC = water soluble carbohydrates.

814 † Bias is the measured mean minus the predicted mean, therefore minus values indicate over-  
 815 estimation and positive values indicate under-estimation of the equation.

816 ‡ Simple linear regression coefficient

817 § Root mean square error of prediction presented as a percentage of the measured mean for  
 818 standardisation

819 ¶ Alcohol is the sum of ethanol and propanol

820 || Degradability parameters determined by *in situ* incubation in the rumen, using the model of Ørskov  
 821 and McDonald (1979) where a = rapidly soluble material; b = non-soluble but degradable material; c =  
 822 rate of degradation of b; effective degradability =  $a+b[c/(c+k)]$  where k = an assumed outflow rate of  
 823 0.08/hr.

824 ¥ TVC is the sum of acetic, butyric, lactic, propionic and valeric acids plus ethanol and propanol.

825 # TVFA is calculated as for TVC but excluding ethanol and propanol.

826

827 **Figure captions**

828 **Figure 1** The relationship between predicted and measured values where 94 grass-  
829 clover silages were utilised to assess prediction accuracy of grass-based near infra-  
830 red reflectance spectrometry (NIRS) equations for 15 chemical components when  
831 used on clover-containing samples. Graphs for each chemical component show a line  
832 of parity. VCODM = volatile corrected oven dry matter; TVFA = total volatile fatty acids;  
833 TVC = total volatile content; DOMD = digestible organic matter in total dry matter; ADF  
834 = acid detergent fibre; aNDF neutral detergent fibre; MDM = microbial dry matter yield;  
835 NH<sub>3</sub>-N = ammonia nitrogen; EDN = effective degradable nitrogen; WSC = water  
836 soluble carbohydrate.

837 **Figure 2** The relationship between bias and sample clover concentration in a  
838 validation test where 94 grass-clover silages were utilised to assess prediction  
839 accuracy of grass-based near infra-red reflectance spectroscopy (NIRS) equations  
840 for a) crude protein b) effective degradable nitrogen (EDN<sub>FIM</sub>) and c) calculated  
841 effective rumen degradable protein (eRDP) concentration ( $eRDP = CP * (0.8 * EDN_{FIM})$ ).  
842 Linear lines of best fit are shown for measured (—) and NIRS predicted  
843 (— —) data.  
844