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Abstract

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

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

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

Abbreviations: ADF, acid detergent fibre; aNDF, neutral detergent fibre; CC, clover concentration; CP, crude protein; VCODM, volatile corrected oven dry matter; EDN, effective degradable nitrogen; EDDM, effective degradability of dry matter; EE, ether extract; FiM, Feed into Milk; OM, organic matter; LA, lactic acid; MDM, microbial dry matter; N, nitrogen; NH_3-N, ammonia nitrogen; NIRS, near infrared reflectance spectroscopy; NMSC, normal multiplicative scatter correction; r^2, coefficient of determination of cross validation; RMSEP, root mean standard error of prediction; RPD, ratio of standard deviation of the measured population to the standard error of prediction; SEC, standard error of calibration; SECV, standard error of cross validation; SEP, standard error of prediction; SNVD, standard normal variate de-trending; TMR, total mixed ration; TVC, total volatile content; TVFA, total volatile fatty acids; WMSC, weighted multiplicative scatter correction; WSC, water soluble carbohydrate.

1. Introduction

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

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

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

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

2. Material and methods

2.1 Experimental design

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

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

2.2 The silage sample set

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

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

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

2.3 Nutritional analysis

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

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

Murray, 2001) and reported as volatile-corrected oven dry matter (**VCODM**). Ash was measured through combustion in a muffle oven at 550°C for 18 h. Lactic acid and other volatile compound measurements (total volatile fatty acids (**TVFA**) were determined using gas chromatography following extraction of representative samples in distilled water (Erwin *et al.*, 1961; Givens *et al.*, 2009). Nitrogen (**N**) was measured using the macro Kjeldahl method 954.01 (AOAC, 2000) and Ammonia-N (**NH_3-N**) was determined using a calibrated ammonia ion selective electrode, which required 30 g silage soaked in 150 ml of purified water for 18 h at 4°C. (McDonald *et al.,* 1981; Orion Research, 1990). Both ether extract (**EE**) and water soluble carbohydrate (**WSC**) were measured on dried and ground samples: EE according to AOAC method 920.29 (AOAC, 1990), and WSC as described previously (Fuller, 1967). Dried and ground samples were subsequently passed on to Trouw Nutrition (Ashbourne, Derbyshire) who performed analyses for neutral detergent fibre (**aNDF**) and acid detergent fibre (**ADF**) both inclusive of residual ash using Fibrecap equipment (Foss, Hillerod, Denmark) (Robertson and Van Soest, 1981; Kitcherside *et al.*, 2000; Mertens *et al.*, 2002). A further 200 g of silage was manually separated into clover, grass and other species to determine the CC of the silage. This procedure was predominantly performed by the same individual to minimise human error. Resulting fractions were then dried to determine species composition on a DM basis. *In vivo* reference methods were performed at CEDAR to determine silage digestibility and degradability.

2.4. In vivo analyses.

2.4.1 In vivo Digestibility Eighteen Mule x Texel wether sheep originating from a local breeder were used to measure *in vivo* silage digestibility using a series of 3 x 3 Latin 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.

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

The percentage of material degraded at each time-point was used to plot a degradation curve as described by Ørskov and McDonald (1979). Degradability fractions termed 'a', 'b' and 'c' were obtained from the intercept, asimptote and slope of the curve. Fraction 'a' contained material that is apparently degraded almost immediately upon ingestion and 'b' contained the remaining insoluble but degradable material with 'c' being the rate of degradation of 'b'. Two different approaches were used to calculate effective degradabilitiy (**ED**) based on the above fractions. To ensure the best comparison with predicted data, the ED of nitrogen (**EDN_{FIM}**) and of dry matter (**$EDDM_{FIM}$**) were calculated using the 'Feed into Milk' (**FiM**) rationing software equations (Equation 1). In this equation the outflow rate of small (k_{liq}) and large (k_f) 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 16th 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)
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

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

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

2.5.3 Calibration of new NIRS equations. To create new grass-clover prediction equations, different data pre-treatment methods were first assessed by varying use of derivitives, gap, smoothing and scatter correction. All calibrations were performed 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

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

3. Results

3.1 Sample chemical composition

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

With the exception of ash, aNDF, and WSC, the concentration of all other measured variables were affected by the CC of the sample when grouped into minimal, low, medium, and high clover groups (Table 2). VCODM and N were significantly increased in the high clover group (>500 g/kg DM CC) relative to the other three groups (both $P < 0.001$), as was CP with the exception of the medium group which contained an intermediary CP concentration ($P < 0.001$). Degradability parameters calculated using the Ørskov and McDonald (1979) model and DOMD were lowest in the high clover group (all $P < 0.04$) and numerically highest in the low clover group (60-240 g/kg DM CC), however, when degradability parameters were calculated using FiM equations, differences between clover groups were non-significant. Fermentation end products (LA, TFVA and TVC) decreased in concentration sequentially as CC increased (all $P < 0.003$) while pH was similar for minimal, low and medium groups and higher for the high clover group ($P < 0.001$). NH_3-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_3-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

of high variability in prediction accuracy where concentration was low (Figure 1). For both TVC and TVFA there was a significant bias towards over-estimation (both $P < 0.001$). Poor prediction accuracy (RPD value <1) was observed for NH_3-N, ADF, EE, EDN_{FIM}, and MDM_{FIM} all of which showed a significant bias between the predicted and observed means (all $P < 0.001$). Of special note, EDN_{FIM} and MDM_{FIM} showed the least prediction accuracy of all the variables tested with a significant over-estimation for EDN_{FIM} of 139 g/kg N and an under-estimation for MDM_{FIM} of 17 g/kg DM. Moreover, predicted and observed data showed little correlation (Figure 1) indicated by r^2 values of 0.01.

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

3.3 Validation of new grass-clover equations

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

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

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

4. Discussion

4.1 Chemical composition and clover concentration

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

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

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

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

4.2 Using grass-based NIRS equations for clover containing samples

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

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

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

4.3 Performance of new grass-clover-based equations

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

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

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

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Table 1 The means, ranges and variation coefficients (CV) of chemical components measured in a set of 94 diverse grass-clover silages from UK farms (in g/kg DM 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				
$EDDM_{0.08}$†	217	626	472	16.4
$EDN_{0.08}$, g/kg N†	55	821	625	18.0
MDM_{FIM}‡	60	274	146	34.8
EDN_{FIM}, 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_3-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

CC = clover concentration; EDDM = effective degradability of dry matter; EDN = effective degradable nitrogen; DOMD = digestible organic matter in total dry matter; EE = ether extract; LA = lactic acid; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

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

‡ Degradability parameters determined by *in situ* incubation in the rumen, using the Feed Into Milk (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 proportion, k_{liq} = fractional outflow rate of the liquid pool (0.075/hr), b_D = degradable small particle proportion, b = degradable large particle proportion, c = fractional degradation rate of b, and k_f is the fractional outflow rate of the large particle pool (0.045/hr).

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

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

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

	Clover concentration quartiles†					
Item	Mi	L	M	H	SED	P value‡
CC	34[a]	145[b]	335[c]	743[d]	28.9	0.001
n	23	24	24	23		
Chemical components						
ADF	311[a]	329[ab]	345[b]	356[b]	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[a]	130[a]	143[ab]	158[b]	9.3	0.001
Degradability						
$EDDM_{0.08}$§	470[ab]	501[b]	478[ab]	436[a]	21.7	0.032
$EDN_{0.08}$, g/kg N§	643[b]	682[b]	640[b]	531[a]	28.6	0.001
MDM_{FIM}¶	130	135	127	122	6.3	0.218
EDN_{FIM}, g/kg N¶	627	645	629	589	25.9	0.182
DOMD	647[b]	668[b]	631[b]	581[a]	18.5	0.001
EE	26.7[ab]	28.7[b]	27.4[ab]	23.2[a]	1.99	0.044
LA, g/kg	17.6[b]	16.2[b]	14.0[ab]	5.6[a]	3.42	0.003
pH	4.45[a]	4.41[a]	4.44[a]	5.23[b]	0.155	0.001
N, g/kg	7.8[a]	7.3[a]	7.9[a]	12.3[b]	0.93	0.001
NH_3-N, g/kg DM*100	48.3[a]	56.3[ab]	68.0[ab]	77.5[b]	9.71	0.018
TVC, g/kg ‖	28.4[b]	26.8[b]	24.0[ab]	14.3[a]	3.74	0.001
TVFA, g/kg ¥	23.3[b]	22.4[b]	20.8[ab]	11.6[a]	3.65	0.001
VCODM, g/kg	397[a]	350[a]	347[a]	498[b]	35.1	0.001
WSC	56.6	39.6	32.9	38.6	10.37	0.125

CC = clover concentration; EDDM = effective degradability of dry matter; EDN = effective degradable nitrogen; DOMD = digestible organic matter in total dry matter; EE = ether extract; LA = lactic acid; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; SED = standard error of the difference between means; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

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

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

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

¶ Degradability parameters determined by *in situ* incubation in the rumen, using the Feed Into Milk (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 proportion, k_{liq} = fractional outflow rate of the liquid pool (0.075/hr), b_D = degradable small particle proportion, b = degradable large particle proportion, c = fractional degradation rate of b, and k_f is the fractional outflow rate of the large particle pool (0.045/hr).

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

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

[a,b] Values within a row with different superscripts differ significantly at $P<0.05$.

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

Item	Measured mean	Predicted mean	Bias†	P value‡	r^2 §	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_{FIM}, 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_{FIM} ‖	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_3-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

DOMD = digestible organic matter in total dry matter; EDN = effective degradable nitrogen; EE = ether extract; LA = lactic acid; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; RMSEP = root mean standard error of prediction; RPD = ratio of standard deviation of the measured population to the standard error of prediction; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

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

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

§ Simple linear regression coefficient

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

‖ Degradability parameters determined by *in situ* incubation in the rumen, using the Feed Into Milk (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 proportion, k_{liq} = fractional outflow rate of the liquid pool (0.075/hr), b_D = degradable small particle proportion, b = degradable large particle proportion, c = fractional degradation rate of b, and k_f is the fractional outflow rate of the large particle pool (0.045/hr).

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

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

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

Item†	n‡	SEC	r^2 §	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
$EDDM_{0.08}$ ¥	174	2.15	0.88	5.28
$EDN_{0.08}$ ¥	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_3-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

EDDM = effective degradability of dry matter; EDN = effective degradable nitrogen; DOMD = digestible organic matter in total dry matter; EE = ether extract; LA = lactic acid; NH_3-N = ammonia nitrogen; SEC = standard error of calibration; SECV = standard error of cross-validation; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

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

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

§ Simple linear regression coefficient

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

‖ Alcohol is the sum of ethanol and propanol

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

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

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

Table 5 The results of a validation in which 10 grass-clover silages were used to test the prediction accuracy of new clover/grass-based NIRS equations generated from the spectra of 85 other grass-clover silages (in g/kg DM unless otherwise stated). Industry standardised data pre-treatment methods were used (1st derivative and 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
$EDDM_{0.08}$ ‖	452	438	14.7	0.68	16.3	1.58
$EDN_{0.08}$, 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_3-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

DOMD = digestible organic matter in total dry matter; EDN = effective degradable nitrogen; EE = ether extract; LA = lactic acid; NH_3-N = ammonia nitrogen; RMSEP = root mean standard error of prediction; RPD = ratio of standard deviation of the measured population to the standard error of prediction; SNVD, standard normal variate de-trending; TVC = total volatile content, TVFA = total volatile fatty acids; WSC = water soluble carbohydrates.

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

‡ Simple linear regression coefficient

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

¶ Alcohol is the sum of ethanol and propanol

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

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

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

Figure captions

Figure 1 The relationship between predicted and measured values where 94 grass-clover silages were utilised to assess prediction accuracy of grass-based near infra-red reflectance spectrometry (NIRS) equations for 15 chemical components when used on clover-containing samples. Graphs for each chemical component show a line of parity. VCODM = volatile corrected oven dry matter; TVFA = total volatile fatty acids; TVC = total volatile content; DOMD = digestible organic matter in total dry matter; ADF = acid detergent fibre; aNDF neutral detergent fibre; MDM = microbial dry matter yield; NH_3-N = ammonia nitrogen; EDN = effective degradable nitrogen; WSC = water soluble carbohydrate.

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